RYEGRASS AND TALL FESCUE RESPONSES TO INOCULATION WITH *Azospirillum amazonense* AND *Acaulospora scrobiculata*

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ABSTRACT: In the Southern Brazilian Plateau, farmers try to compensate the lack of forage from native grasslands cultivating gramineae such as ryegrass (*Lolium multiflorum* Lam.) and tall fescue (*Festuca arundinacea*). However, soil fertility often limits the productivity of these pastures. In this study, the authors investigated the effects of inoculating diazotrophic bacteria *Azospirillum amazonense* S22 and arbuscular mycorrhizal fungi (AMF) *Acaulospora scrobiculata* SCR855 on the growth and nutrient uptake of ryegrass and tall fescue. Both AMF and *Azospirilum* did not increase the dry weight and the nutrient concentration of ryegrass. However, *Azospirillum* inoculation promoted the growth of tall fescue. Mycorrhizal colonization was not affected by AMF inoculation, but it was increased by *Azospirillum* inoculation.
KEYWORDS: Southern Brazilian Plateau. Plant growth. Diazotrophic bacteria. Mycorrhizal colonization.

RESPOSTAS DE AZEVÉM E FESTUCA À INOCULAÇÃO COM *Azospirillum amazonense* E *Acaulospora scrobiculata*

RESUMO: No planalto do sul do Brasil, os produtores tentam compensar a falta de forragem das pastagens nativas cultivando gramíneas, como azevém (*Lolium multiflorum* Lam.) e festuca (*Festuca arundinacea*). Entretanto, a fertilidade do solo nessas áreas frequentemente limita a produtividade dessas pastagens. Neste estudo, foram investigados os efeitos da inoculação da bactéria diazotrófica *Azospirillum amazonense* S22 e do fungo micorrízico arbuscular (FMA) *Acaulospora scorbiculata* SCR855 no crescimento vegetal e absorção de nutrientes de azevém e festuca. Tanto FMA quanto *Azospirillum* não incrementaram a massa seca e as concentrações de nutrientes do azevém; porém, a inoculação de *Azospirillum* promoveu o crescimento da festuca. A colonização radicular micorrízica não foi afetada pela inoculação pelo AMF, mas foi aumentada pela inoculação com *Azospirillum*
PALAVRAS-CHAVE: Planalto sul brasileiro. Crescimento vegetal. Bactéria diazotrófica. Colonização micorrízica.

RESPUESTAS DE RAIGRÁS Y FESTUCA A INOCULACIÓN CON *Azospirillum Amazonense* Y *Acaulospora scrobiculata*

RESUMEN: En el altiplano del sur de Brasil, los productores intentan compensar la falta de forraje en pastajes nativas cultivando gramíneas, como raigrás (*Lolium multiflorum* Lam) y festuca (*Festuca arundinacea*). Sin embargo, la fertilidad del suelo en esas áreas a menudo limita la productividad de esas pastajes. En este estudio, se investigó los efectos de la inoculación de la bacteria diazotrófica *Azospirillum amazonense* S22 y del hongo micorrízico arbuscular (HMA), *Acaulospora scorbiculata* SCR855 en el crecimiento vegetal y absorción de nutrientes de raigrás y festuca. Tanto HMA como *Azospirillum* no incrementaron la masa seca y las concentraciones de nutrientes del raigrás; pero, la inoculación de *Azospirillum* promovió el crecimiento de la festuca. La colonización radicular micorrízica no se vio afectada por la inoculación por HMA, pero aumentó por inoculación con *Azospirillum*.
PALABRAS CLAVE: Altiplano Sur Brasileño. Crecimiento vegetal. Bacteria diazotrófica. Colonización micorrízica.

Introduction

The Southern Brazilian Plateau hosts large areas of native grasslands which are traditionally managed for cattle ranching. However, due to local edafoclimatic conditions, usually there is a shortage of forage during the winter. In order to avoid the lack of forage, farmers cultivate gramineae, particularly ryegrass (*Lolium multiflorum* Lam.) and tall fescue (*Festuca arundinacea* = *Lolium arundinaceum*). However, pasture productivity is limited by the high acid and poor fertility of the soils, once liming and phosphate fertilization are unfeasible. An alternative practice to improve forage productivity is the inoculation of pasture seeds by arbuscular mycorrhiza fungi (AMF) in association with diazotrophic

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bacteria (e.g. *Azospirillum* spp.). (BAUER et al., 2012; BAREA et al., 2013; WHITE et al., 2013).

Soil microorganisms are increasingly appreciated as important drivers of productivity and structure of plant communities. AMF and diazotrophic bacteria are well-known microbial functional groups that are key to plant nutritional needs for phosphorus and nitrogen, respectively (BAUER et al., 2012; BAREA et al., 2013).

Previous studies have shown that the inoculation of AMF has increased plant growth of several grasses under greenhouse conditions (e.g. BAREA; BONIS; OLIVARES, 1983; MUTHUKUMAR; UDAIYAN, 2006; BAREA et al., 2013), although there is evidence of negative responses of AMF inoculation on ryegrass (OMACINI et al., 2006). On the field, AMF inoculation resulted in the increase of total biomass production and seed yield of barley and wheat (JAKOBSEN, 1983; AL-KARAKI; MCMICHAEL; ZAK, 2004). Regarding the inoculation of diazotrophic bacteria on grasses, there are reports showing that sugarcane (BODDEY et al., 2003), elephant grass, *Pennsetum pureum* (REIS et al., 2001), maize and wheat (HUNGRIA et al., 2010) have been benefited through the inoculation of several *Azospirillum* spp strains. In fact, the rates of N_2 fixation of *Azospirillum* may be large enough to maintain plant biomass productivity without amendment with N fertilizers (BODDEY; DÖBEREINER, 1988). Andrews et al. (2011) reviewed the importance of studying diazotrophic bacteria and mycorrhizas for the development of inoculants to increase P availability and plant growth in pastures.

The objective of this study was to measure the responses of these pastures to inoculation with AMF *Acaulospora scorbiculata* and diazotrophic bacteria *Azospirillum amazonense*, with the hypothesis that inoculation with AMF and *Azospirillum amazonense* on ryegrass and tall fescue increases plant growth and nutrient concentration.

Material and Methods

Experimental conditions

Twenty-five pre-germinated seedlings of ryegrass and tall fescue pot^{-1} were cultivated in pots containing 2.5 kg soil, under greenhouse conditions during three months. The plants received the same amount of distilled water whenever necessary. Unsterilized soil (Control) was used to test whether the inoculation is suitable for local conditions, and also because, in previous trials, soil sterilization caused plant toxicity due to Mn solubilization. The soil, classified as Dystric Cambisol (FAO, 1988), was sampled from natural grassland, sieved and treated with dolomite lime enough to mitigate the toxic effects of exchangeable Al. After liming, the soil presented the following characteristics: pH in water, 5.4; clay content, 79%; organic matter, 3.5%; P, 1.7 mg kg^{-1}; K, 61 mg kg^{-1}; exchangeable Al, 36 mg kg^{-1}; Ca, 1003 mg kg^{-1}; and Mg, 535 mg kg^{-1}.

Inoculation

A. scrobiculata SCR855 (Provided by Federal University of Lavras) was inoculated on ryegrass and tall fescue applying 35 g (approximately 100 AMF spores) of sorghum-rhizosphere soil pot^{-1} in 2 cm depth, forming the surface. Non-AMF treatments received the same amount of steamed sterile soil inoculant. The species *A. amazonense* S22 (provided by Federal University of Lavras) was grown in 15-mL potato-dextrose liquid-based (200 g potato added with 20 g dextrose L^{-1}) for 6 days at 27 °C. The bacterial suspension was centrifuged (10,000 rpm, 4 °C, 10 min) and the pellet was rinsed twice with saline solution (0.55% NaCl). Neubauer-counting estimated 1.35 x 10^8 bacterial cells mL^{-1}. Then, 1 mL of this inoculant was used to soak 25 per pot pre-germinated seeds during 3 hours. Before germination, seeds of ryegrass and tall fescue were disinfested in a 70% alcohol solution for 5 min, hydrogen peroxide (5%) for 5 min, rinsed with sterile water for 2 min, and then incubated at 32 °C until the radicles became visible.

Measurements

Shoots were harvested and dried at 60 °C until dry mass was constant. Fresh roots (0.5 g) were sampled for mycorrhizal evaluations, and the remaining roots were dried at 60 °C until constant mass.

Shoot and root N, K, Ca and Mg concentrations were measured after a sulfuric acid/hydrogen peroxide digestion (TEDESCO et al., 1995). Ca and Mg concentrations were determined by inductively coupled plasma spectrometer, and K concentration was estimated by flame photometer. Digested samples were distillated in NaOH; the diffused N was trapped in a boric acid-indicator solution and determined by titration with diluted H_2SO_4.

Mycorrhizal colonization was assessed by clearing and staining roots (BRUNDRETT et al., 1996). The percentage of vesicles, arbuscules and hyphae in roots were estimated as described by McGonigle et al. (1990). The total colonization (Col_t) was transformed through the equation: $Col._t = \left(ArcSin\sqrt{Col.(\%)/100}\right).(180/\pi)$.

Statistical analysis

The experiment consisted in a factorial design of three inoculation levels (with AMF and/or *Azospirillum* and its combination), and a treatment control without inoculation. In greenhouse, all treatments were completely randomized, with 4 replications each. All data were submitted to one-way analysis of variance - ANOVA and Duncan Test SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA) in order to compare averages.

Results and Discussion

AMF inoculation in ryegrass and tall fescue did not increase plant growth when compared to the control treatment (Table 1). However, *Azospirillum* inoculation showed a significant increase in ryegrass and tall fescue dry biomass production when compared to the control treatment (Table 1). In the review by Bashan, Holguin e De-Bashan (2004), it is concluded that *Azospirillum* inoculation is a very good growth promoter of grass plants. This explains the findings in the present study.

Table 1: Plant dry biomass (g pot^{-1}) of ryegrass and tall fescue uninoculated and inoculated with AMF *Acaulospora scrobiculata* SCR855 and/or *Azospirillum amazonense* S22 and its combination.

	AMF	***Azospirillum***	***AMF + Azospirillum***	**Non-inoculated (Unsterilized soil)**
Ryegrass				
Shoot	1.82 ± 0.17 b	2.28 ± 0.33 a	2.07 ± 0.16 ab	2.34 ± 0.33 a
Root	1.19 ± 0.15^{ns}	1.55 ± 0.42	1.26 ± 0.11	1.54 ± 0.26
Total	3.01 ± 0.25 b	3.84 ± 0.75 a	3.33 ± 0.25 ab	3.88 ± 0.59 a
Shoot : Root	1.55 ± 0.21^{ns}	1.51 ± 0.17	1.64 ± 0.11	1.53 ± 0.05
Tall Fescue				
Shoot	1.91 ± 0.10 b	2.83 ± 0.38 a	1.87 ± 0.25 b	1.98 ± 0.25 b
Root	1.38 ± 0.16 b	1.88 ± 0.06 a	1.25 ± 0.21 b	1.42 ± 0.35 b
Total	3.28 ± 0.20 b	4.71 ± 0.38 a	3.12 ± 0.43 b	3.40 ± 0.60 b
Shoot : Root	1.40 ± 0.18^{ns}	1.50 ± 0.22	1.52 ± 0.18	1.43 ± 0.18

Mean values ± Standard Deviation (SD) are shown; ns=not significant. Means within row followed by different letters are significantly different with the Duncan test $p \leq 0.05$.

There were no significant differences between shoot and root nutrient concentrations, particularly in ryegrass (Table 2). One probable explanation is that ryegrass and tall fescue do not sufficiently respond to mycorrhizal colonization (OMACINI et al., 2006) and these plants are not as dependent as other plant species. Ryegrass could have benefits from AMF in others way, such as supporting more abiotic stress and having less attack from plant pathogens, but these aspects were not tested in our study.

Table 2: Shoot and root nutrient concentration (mg g^{-1}) of ryegrass and tall fescue uninoculated and inoculated with AMF *Acaulospora scrobiculata* SCR855 and/or *Azospirillum amazonense* S22 and its combination.

	Shoot				**Root**			
	AMF*	AZ	AMF+AZ	Cont	AMF	AZ	AMF+AZ	Cont
Ryegrass								
N	10.9 ± 0.7^{ns}	9.4 ± 1.1	11.9 ± 1.5	9.4 ± 2.5	6.0 ± 0.2 ab	5.5 ± 1.1 b	6.8 ± 0.4 a	5.5 ± 0.4 b
K	22.9 ± 1.5^{ns}	18.1 ± 3.0	19.7 ± 4.3	19.4 ± 2.4	4.5 ± 0.4^{ns}	4.8 ± 0.6	4.9 ± 0.7	4.9 ± 0.3
Ca	6.3 ± 0.7^{ns}	6.5 ± 0.7	6.5 ± 0.4	6.6 ± 0.6	4.2 ± 0.1 ab	4.6 ± 1.0 a	4.5 ± 0.2 a	3.7 ± 0.2 b
Mg	5.5 ± 0.7^{ns}	5.7 ± 0.3	5.7 ± 0.4	5.9 ± 0.3	1.8 ± 0.1 bc	2.0 ± 0.1 b	2.3 ± 0.2 a	1.6 ± 0.4 c
Tall Fescue								
N	9.5 ± 1.2 a	6.0 ± 1.7 b	10.4 ± 1.2 a	9.3 ± 1.3 a	5.4 ± 0.4^{ns}	6.5 ± 1.6	6.2 ± 0.5	6.9 ± 0.8
K	19.8 ± 2.8 a	13.5 ± 6.2 b	22.3 ± 1.3 a	18.0 ± 2.6 ab	4.7 ± 0.2^{ns}	4.9 ± 0.0	4.7 ± 0.4	4.8 ± 0.3
Ca	5.7 ± 0.3 a	3.7 ± 0.8 b	5.7 ± 0.5 a	5.0 ± 0.2 a	3.9 ± 0.3 b	3.4 ± 0.2 c	3.6 ± 0.2 bc	4.4 ± 0.4 a
Mg	6.2 ± 0.5 a	4.8 ± 1.1 b	6.7 ± 0.3 a	6.4 ± 0.7 a	1.6 ± 0.2^{ns}	1,6 ± 0.2	1.6 ± 0.2	1.5 ± 0.3

Mean values ± SD are shown; ns=not significant. Means within row, within ‘shoot’ or ‘root’, followed by different letters are significantly different with the Duncan test $p \leq 0.05$.
*AMF = Arbuscular mycorrhizal fungus; AZ = Azospirillum amazonense; Cont = Non-inoculated (Unsterilized soil)

Overall, there were no differences in the total mycorrhizal colonization of ryegrass and tall fescue, but there was a significantly higher percentage of colonization and arbuscules when both AMF and *Azospirillum* were simultaneously inoculated in ryegrass (Table 3). However, it is important to mention that colonization values of non-inoculated plants could have been overestimated due to measuring errors.

Table 3: Intra-radical root AMF colonization of ryegrass and tall fescue uninoculated and inoculated with AMF *Acaulospora scrobiculata* SCR855 and/or *Azospirillum amazonense* S22 and its combination.

	AMF	***Azospirillum***	**AMF + *Azospirillum***	**Uninoculated (Unsterilized soil)**
Ryegrass				
Total	38.3 ± 6.4 ab	31.5 ± 3.0 b	40.2 ± 5.7 a	30.7 ± 2.8 b
Vesicles	6.8 ± 1.9^{ns}	4.9 ± 2.0	5.7 ± 5.1	9.7 ± 4.6
Arbuscules	14.8 ± 6.1 b	16.3 ± 5.1 ab	23.6 ± 4.7 a	10.4 ± 3.4 b
Tall fescue				

Total	34.9 ± 4.9^{ns}	34.4 ± 18.5	42.0 ± 5.4	39.6 ± 8.3
Vesicles	8.9 ± 5.6^{ns}	10.1 ± 9.8	6.9 ± 5.6	12.5 ± 5.7
Arbuscules	12.0 ± 4.1^{ns}	14.4 ± 8.0	18.5 ± 3.7	14.4 ± 3.5

Mean values ± SD are shown; ns=not significant. Means within row followed by different letters are significantly different with the Duncan test $p \leq 0.05$.

The role of inoculation is to promote a more efficient contact of roots with the beneficial microbial (KASCHUK et al., 2010; BAUER et al., 2012; MALUSÁ; SAS-PASZT; CLESIELSKA, 2012; WHITE et al., 2013). The fact that inoculation did not increase root colonization suggests that the chosen strains in this study are not competitive against background native strains. Local soils are rich in organic matter and host high microbial diversity. A pioneer study about mycorrhizal diversity in local soils showed that, together with other genus, *Acaulaspora* is very abundant and diverse in soils in the Southern Brazilian Plateau (PURIN; KLAUBERG-FILHO; STÜRMER, 2006).

On the other hand, tall fescue was benefited from the inoculation with *Azospirillum* (Table 1), probably due to the increase in the number of beneficial microbes in the rhizosphere. It is expected that inoculation of pure cultures of *Azospirillum* increases the colony-forming unities in the rhizosphere by 20 to 44 % (BIRÓ et al., 2000; MUTHUKUMAR; UDAIYAN, 2006). The benefits of *Azospirillum* are attributed to N_2 fixation, but also to changes in the plant hormone balance, resulting in larger root system with improved water and nutrient uptake (REIS-JUNIOR et al., 2004).

Inoculation with *Azospirillum* seems to be a promising technology for increasing grass productivity (BAREA et al., 2013; WHITE et al., 2013). Recently, Brazil has developed a commercial inoculant with *A.brasiliense* for wheat and maize (HUNGRIA et al., 2010). One of the great challenges in developing inoculants from beneficial microbes is the screening program for strains which are simultaneously efficient and competitive against native soil strains. As a matter of fact, a high diversity of *Azospirillum* spp. is expected, since this genera is widespread in tropical and temperate climates (REIS-JUNIOR et al., 2004). Therefore, as well as evaluating the plant growth responses, other studies should consider the evaluation of diazotrophic diversity associated with native and cultivated pastures in the region (BAREA et al., 2013; WHITE et al., 2013).

Conclusions

A.amazonense S22 increased tall fescue growth. However, it is possible that some native strains are able to bring similar or even greater benefits, and therefore, there is the need for isolating and screening for more effective native *Azospirillum* spp.

A. scrobiculata SCR855 is not a prospective candidate for inoculation on ryegrass and tall fescue in the soil conditions in the Southern Brazilian native grasslands.

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