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Screening of biomass production of cultivated forage grasses in response to mycorrhizal symbiosis under nutritional deficit conditions

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Abstract

Arbuscular mycorrhizal fungi (AMF) colonize the root systems of most natural grassland species and usually increase plant growth by enhancing nutrients provision. This effect on growth responses of cultivated forage grasses is scarcely known, particularly under nutritional deficit conditions. We examined total biomass production, aboveground and belowground biomass and tillering of three temperate and three tropical cultivated forage grasses. Seedlings of each species were inoculated with a mixture of mycorrhizal fungi and later grown for 5 months under nutritional deficit conditions. The mycorrhizal symbiosis promoted aboveground and belowground biomass production in five out of six grass species. Grass species differed in their mycorrhizal responsiveness: tropical grasses (*Panicum coloratum* cv. Klein = *Brachiaria brizantha* cv. Marandú > *Paspalum dilatatum* cv. Primo) responded better than temperate (*Festuca arundinacea* cv. Royal > *Agropyron elongatum* cv. Hulk), while the temperate *Dactylis glomerata* cv. Porto did not respond to AMF inoculation. In four of the species, the changes observed in aboveground biomass were explained by the total number of tillers, while, in *P. dilatatum*, changes were accounted for by the individual weight of mature tillers. On the whole, the screening of cultivated forage grasses revealed that tropical grasses were highly responsive to mycorrhizae, in contrast to a lower effect on the growth of temperate grasses.

Introduction

Symbiosis with arbuscular mycorrhizal fungi (AMF) is a relevant relationship in most grassland plant species because: (i) they are generally colonized; (ii) soil nutrients availability is often limiting; and (iii) the mycorrhizal network is not recurrently disrupted as in annual crops. The main biological basis of this relationship is a bidirectional nutrient transfer: fungi provide minerals to roots in return for carbon substrates from the host plant (Smith and Read 2008). In this way, the association with AMF can improve the provision of poorly mobile nutrients, especially phosphorus (P), but also ammonium, copper, zinc and other micronutrients (Pacovsky 1986). Fungi seem to generate a smaller carbon cost per absorption area unit than roots, and they also allow a higher exploration of soil not accessed by roots (Koide 1991). Moreover, other potential benefits of mycorrhizal symbiosis have been mentioned, such as improved plant water relations and reduction of pathogenic infections (Newsham *et al.* 1995), promotion of soil aggregation (Wilson *et al.* 2009) as well as synergistic effects with other microorgan-

isms (Biró *et al.* 2000; Osorio and Habte 2013). However, the structures and propagules of AMF may be negatively affected by ploughing (Helgason *et al.* 1998), overgrazing (Gehring and Whitham 1994) and/or recurrent application of chemical products (Druille *et al.* 2013), thus disrupting the many beneficial effects of the symbiosis on plant community attributes and grassland ecosystem functioning (van der Heijden *et al.* 1998; Hartnett and Wilson 2002; Fitter *et al.* 2004).

The success of the symbiotic association is closely modulated by environmental conditions (Koide 1991; Janos 2007) in connection with the identity of the host plant and the fungus, as well as the interactions among different biological partners (Jones and Smith 1994). Environmental conditions are particularly favorable for mycorrhizal symbiosis in forage species due to the low availability of soil P limiting grassland productivity (Hartnett and Wilson 2002). Nevertheless, there are important differences in the response to mycorrhizae among major groups of forage species (i.e. legumes, temperate and tropical grasses). In forage legumes, most studies showed beneficial effects of AMF on plant growth and P uptake (Chalk *et al.* 2006), as well as interesting synergistic effects on plant co-inoculated with nitrogen-fixing bacteria (Biró *et al.* 2000; Kaschuk *et al.* 2009). For temperate and tropical grasses (C_3 and C_4, respectively), a recent meta-analysis reported responses that ranged from positive to negative, with a positive weighted average effect of AMF colonization on plant growth (Hoeksema *et al.* 2010). Interestingly, tropical grasses exhibited greater positive averaged responses to AMF symbiosis under field conditions than temperate grasses. This confirms the response pattern to the generation of mycorrhizae described for warm-season grasses, in comparison with cool-season grasses of the natural vegetation inhabiting the tallgrass prairie of the central Great Plains of North America (Hetrick *et al.* 1988; Wilson and Harnett 1998).

Cultivated forage grasses constitute a very important group of perennial crops because their elevated biomass production sustains cattle grazing in many regions of the world. However, a comparative screening of mycorrhizal responsiveness (MR) of cultivated forage grasses under homogenous controlled conditions is still lacking. Our aim is to evaluate the response to AMF symbiosis of cultivated forage grasses under nutritional deficit conditions. A manipulative experiment was designed involving three temperate grasses: *Dactylis glomerata* L., *Agropyron elongatum* (Host) P. Beauv. and *Festuca arundinacea* Schreb., as well as three tropical grasses: *Paspalum dilatatum* Poir., *Panicum coloratum* L. and *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf. The screening of AMF responses of cultivated forage grasses has particular interest regarding the key role of mycorrhizal symbiosis in grassland ecosystems.

Materials and methods

Plant material and experimental design

Six cultivated grasses with high forage quality were selected. The three temperate grasses, (i) *D. glomerata* cv. Porto, (ii) *A. elongatum* cv. Hulk and (iii) *F. arundinacea* cv. Royal, and the three tropical grasses, (i) *P. dilatatum* cv. Primo, (ii) *P. coloratum* var. *coloratum* (cv. Klein) and (iii) *B. brizantha* cv. Marandú, were used as host plants. All species are tussock grasses worldwide used as cultivated pastures for direct grazing and/or hay and silage production. Two of the tropical species, *P. dilatatum* and *P. coloratum*, could also be cultivated at temperate zones, while *B. brizantha* only at lower latitudes. Seeds were washed for 20 min in NaOCl (6% active chlorine) for surface sterilization and germinated in polystyrene boxes containing absorbent white paper saturated with distilled water in an incubator (20–30°C). After 4–5 days, two to three seedlings were individually transplanted into pots (11 cm diameter, 26 cm height) and placed in a glasshouse at the Faculty of Agronomy at University of Buenos Aires. The substrate for growth was sterilized sand with a small amount of phosphate rock, as a P source of low availability (171 mg P_2O_5 kg^{-1} sand, Grimoldi *et al.* 2005). Simultaneously, a source of inocula (7 g per pot) was added to the substrate of half of the pots (a mixture of three species of the genus *Glomus: G. mosseae* BEG 12, *G. intraradices* BAFC 3108 and *G. hoi* BEG 104) after propagating them on *Zea mays* L., *Lolium perenne* L. and *Trifolium repens* L. as hosts under greenhouse conditions for 3 months. Three different species of AMF were used, in order to increase the possibility of colonization of the experimental plants. The inocula of the three species were separately checked a few days before the beginning of the experiment: spore isolations were performed by wet sieving and decanting (Gerdemann and Nicolson 1963), being the supernatant centrifuged in a sucrose gradient (Walker *et al.* 1982). The viability of the spores was checked by tetrazolium bromide vital stain (An and Hendrix 1988). The inocula, consisting of colonized roots, external mycelium and spores (approximately 100 viable spores per 100 g of dry inoculum), were thoroughly mixed with quartz sand used as substrate to ensure contact between roots of the grass plants and AMF. Pots with and without AMF inocula were placed in separate plastic boxes to prevent contamination of the noninoculated plants. Plants within species were arranged in a randomized design with five replicates per species and treatment. All pots and boxes were rotated every 7 days during the experiment.

In order to create a nutritional deficit condition, the original formulation of the full nutrient Hoagland's solution was modified. Relative to the full original solution,

our solution had 90% less nitrogen, potassium and calcium, and 70% less iron and sodium. The macroelement composition (mmol L^{-1}) was: 0.3 KNO_3, 0.4 Ca $(NO_3)_2$, 0.1 $MgSO_4$ and 0.1 KCl, and the microelement composition (μmol L^{-1}) was: 0.04 Fe-EDTA, 2.5 H_3BO_3, 0.2 $MnCl_2$, 0.2 $ZnCl_2$, 0.05 $CuCl_2$ and 0.05 MoO_3. Additionally, to facilitate the generation of mycorrhiza, the solution was soluble P-free and supplied with ammonium nitrate (0.11 mmol L^{-1}). In this way, the only source of P was phosphate rock. All plants were watered to field capacity with the modified Hoagland's solution (approximately 50 mL) every day during the whole experiment. To avoid the accumulation of nutrients in the substrate, once a week all pots were flushed with 125 mL tap water and as the substrate was inert, they were once more watered with the Hoagland′s solution. Seedlings were thinned to one plant per pot and grown in a glasshouse for 5 months at 18–25°C and midday photosynthetic photon flux density (PPFD) of 1650 $\pm$ 125 μmol m^{-2} s^{-1}.

Sampling procedure

At the end of the experiment, each plant was harvested and roots were freed from the substrate by washing them with running water. Each plant was then dissected into shoots and roots. A sample of the fresh root material was weighed and used for determining AMF colonization. Shoots were separated into reproductive tillers, vegetative mature tillers (at least one fully developed leaf) and daughter tillers (no fully expanded leaf), which were counted. The total number of tillers was the sum of these three types of tillers. Vegetative mature tillers were dissected into blade and sheath. Additionally, mature tiller weight was estimated from the sum of blade and sheath biomasses divided by the number of vegetative mature tillers. All biomass samples were weighed after oven drying for 72 h at 80°C.

AMF colonization and blade phosphorus concentration

Mycorrhizal colonization was determined by histological detection of mycorrhizal structures (hyphae, arbuscules and vesicles) after root staining (Phillips and Hayman 1970). Briefly, a sample of the fresh root material was cleared with KOH (10% w/v, 10 min, 90°C), acidified in HCl (1% v/v, 5 min) and stained with trypan blue (0.05% w/v, 10 min, 90°C) in acid glycerol. The percentage of root length with AMF colonization was assessed by microscopic examination at 200 × magnification for 100 random intersections of the root for each plant (McGonigle *et al.* 1990).

P concentration was determined on samples of 200 mg of blades of the vegetative mature tillers which were ashed in a muffle furnace. The resulting ash was digested in HNO_3/HCl and quantities of P were measured by phosphovanado-molybdate colorimetry (Hanson 1950). Reference material of grass leaves was included with every 15 samples to check digestion and analytical procedures.

Calculations and statistical analyses

In order to discuss the relative importance of AMF inoculation across species, MR was calculated for each plant species as follows: percentage MR = ([total biomass [+AMF] plant – total biomass [−AMF] plant]/total biomass [−AMF] plant) × 100 (Baon *et al.* 1993; reviewed by Janos 2007). Standard errors of this parameter were obtained accounting for propagation of independent samples. Variables were analyzed by one-way analysis of variance (ANOVA) per species with AMF inoculation as main factor (Steel and Torrie 1980). The normality and homoscedasticity of data were previously checked. Biomass data were transformed (log x) to comply with the ANOVA assumptions. Statistical analyses were performed using Statistica package for Windows (StatSoft, Tulsa, OK, USA). All results are presented as means $\pm$ SE (n = 5).

Results

All mycorrhizal plants averaged up to 50% root length colonized by AMF (Table 1). Overall, the AMF colonization tended to be relatively higher for the tropical grasses (*P. dilatatum*, *P. coloratum* and *B. brizantha,* 63–80%), than for the temperate grasses (*D. glomerata*, *A. elongatum* and *F. arundinacea*, 54–69%). None of the noninoculated plants were colonized by mycorrhizal fungi. Blade P concentration was low (approximately 0.7 mg P g^{-1} dry weight) for all species and inoculation treatments (as a reference 1.5–3 mg P g^{-1} dry weight is the P for optimal plant growth; Table 1). Only the tropical grass *B. brizantha* showed a slight increment ($P < 0.05$) on blade P concentration in response to AMF inoculation. The set-up conditions, high mycorrhizal colonization and low P status, were confirmed for all studied grass species at the end of the experimental period (Table 1).

The mycorrhizal symbiosis significantly stimulated total biomass production in five out of the six cultivated grass species ($P < 0.001$; Figure 1). Dry weight of mycorrhizal plants was higher than that of nonmycorrhizal ones by 202 and 206% for the tropical species *B. brizantha* and *P. coloratum,* respectively, by 98% for *P. dilatatum,* and by 69 and 54% for the temperate *F. arundinacea* and *A. elongatum,* respectively (Figure 1). Thus, tropical

Table 1 Arbuscular mycorrhizal fungi (AMF) colonization and blade phosphorus (P) concentration of nonmycorrhizal (−AMF) and mycorrhizal (+AMF) plants of six cultivated forage grasses grown under nutritional deficit conditions. Note that none of the noninoculated plants (−AMF) were colonized by mycorrhizal fungi

Plant species	AMF colonization (%)	Blade P concentration (mg P g^{-1})	
	+AMF	−AMF	+AMF
Dactylis glomerata	54.8 ± 2.4	0.63 ± 0.02	0.70 ± 0.03
Agropyron elongatum	68.8 ± 4.7	0.71 ± 0.12	0.73 ± 0.06
Festuca arundinacea	59.2 ± 9.5	0.61 ± 0.11	0.64 ± 0.02
Paspalum dilatatum	80.4 ± 2.7	0.70 ± 0.05	0.71 ± 0.06
Panicum coloratum	80.2 ± 4.6	0.74 ± 0.01	0.77 ± 0.04
Brachiaria brizantha	63.4 ± 3.5	0.38 ± 0.01	0.44 ± 0.01*

Values are means ± SE (n = 5). Asterisk indicates significant differences of blade P concentration (*P < 0.05) between AMF treatments within one species.

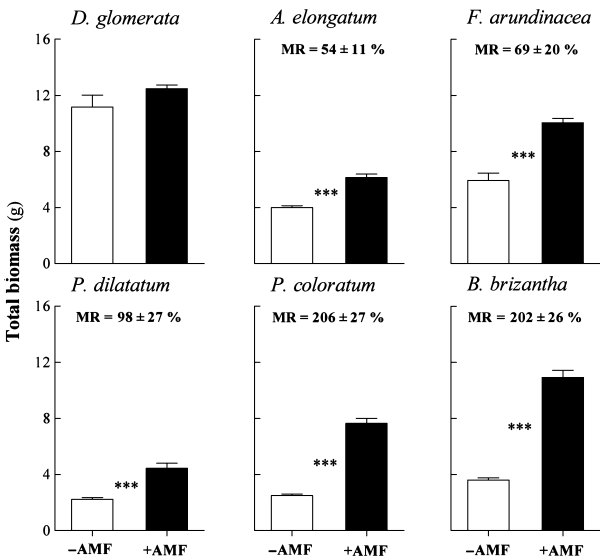

Figure 1 Total biomass of nonmycorrhizal (−AMF, open bars) and mycorrhizal (+AMF, filled bars) plants, and mycorrhizal responsiveness (MR) ± SE, indicated by bold text on top of each figure. The six cultivated forage grasses grown under nutritional deficit conditions were *Dactylis glomerata, Agropyron elongatum, Festuca arundinacea, Paspalum dilatatum, Panicum coloratum* and *Brachiaria brizantha*. Values are means ± SE (n = 5). Asterisks indicate significant differences (***P < 0.001) between treatments for each species. MR was calculated as ([total biomass [+AMF] plant − total biomass [−AMF] plant]/total biomass [−AMF] plant) × 100.

species (*P. coloratum* = *B. brizantha* > *P. dilatatum*) were more responsive to mycorrhizae than temperate species. In contrast, the temperate grass *D. glomerata* was the only species that did not respond significantly (P = 0.22) to mycorrhizal symbiosis.

Aboveground (shoots) and belowground (roots) biomasses were higher for mycorrhizal plants than for nonmycorrhizal plants in most of the studied species (P < 0.01; Table 2). This means that both parameters explained the increments observed at total biomass production (Table 2). The temperate grass *D. glomerata* was the only species showing no differences between mycorrhizal treatments (P > 0.05). Overall, the total number of tillers per plant was the main factor explaining the changes observed on aboveground biomass (Table 2). For this variable, the tropical grass *B. brizantha* showed the most remarkable promotion by AMF (100%; Table 2). In *P. dilatatum*, the number of tillers per plant was not affected by the presence of mycorrhizae, while the individual weight of mature tillers was significantly increased by AMF colonization (−AMF: 0.17 ± 0.02 vs. +AMF: 0.25 ± 0.02 g $tiller^{-1}$; P = 0.01). In the other species, individual weight of mature tillers was similar between mycorrhizal treatments (P > 0.05; data not shown). Remarkably, mycorrhizal plants of the tropical grass *P. coloratum* were the only ones showing the presence of reproductive tillers (15% of the total number of tillers).

Discussion

Under nutritional deficit conditions, mycorrhizal symbiosis was beneficial for growth of most cultivated forage grass species assessed in our experiment. However, in the literature, there is evidence of a high degree of variation in the functional effect of symbiosis in relation to the identity of partners involved in the relationship (Munkvold *et al.* 2004; Smith *et al.* 2004; Reinhart *et al.* 2012). Accordingly, besides the general positive response, we observed that studied grass species notably differ in their MR (Figure 1). The tropical species were highly responsive to mycorrhizae (*P. coloratum* = *B. brizantha* > *P. dilatatum*), in contrast to the lower effect of AMF on the growth of the temperate species (*A. elongatum* and *F. arundinacea*; Table 1; Figure 1). Besides, *D. glomerata* did not respond significantly to mycorrhizal symbiosis. In this sense, our results coincided with those obtained for natural grassland species (i.e. Hetrick *et al.* 1988; Wilson and Harnett 1998; Hoeksema *et al.* 2010), where warm-season grasses exhibited greater positive responses to AMF symbiosis in relation to cool-season grasses. In particular, it was found that the tropical grasses *Panicum virgatum* L. (Wilson and Harnett 1998) and *B. brizantha* (Kanno *et al.* 2006) were more responsive to symbiosis, in comparison to most of the cool-season grasses evaluated (Hetrick *et al.* 1988; Wilson and Harnett 1998). For *P. dilatatum*, it was previously reported that the species is highly colonized under natural conditions in the Flooding Pampa Grasslands (Grigera and Oesterheld 2004). But, to

Table 2 Aboveground and belowground biomass and total number of tillers of nonmycorrhizal (−AMF) and mycorrhizal (+AMF) plants of six cultivated forage grasses grown under nutritional deficit conditions

	Aboveground biomass (g)		Belowground biomass (g)		Total number of tillers (Tillers per plant)	
Plant species	−AMF	+AMF	−AMF	+AMF	−AMF	+AMF
Dactylis glomerata	4.7 ± 0.3	3.4 ± 0.6	6.5 ± 0.9	9.1 ± 0.8	22.3 ± 0.9	20.0 ± 2.1
Agropyron elongatum	1.7 ± 0.2	2.5 ± 0.1**	2.3 ± 0.2	3.7 ± 0.3**	13.0 ± 0.7	17.7 ± 1.5*
Festuca arundinacea	2.9 ± 0.1	3.8 ± 0.1**	3.0 ± 0.6	6.3 ± 0.3**	10.8 ± 1.1	15.3 ± 0.7*
Paspalum dilatatum	1.1 ± 0.1	1.9 ± 0.2**	1.2 ± 0.1	2.5 ± 0.2***	6.2 ± 0.5	7.0 ± 0.5
Panicum coloratum	1.6 ± 0.1	4.2 ± 0.3***	1.0 ± 0.1	3.5 ± 0.2***	11.0 ± 0.4	15.5 ± 2.9*
Brachiaria brizantha	1.9 ± 0.1	4.8 ± 0.4***	1.7 ± 0.1	6.1 ± 0.3***	3.8 ± 0.5	7.4 ± 0.2***

Values are means ± SE (n = 5). Asterisks indicate significant differences (*P < 0.05, **P < 0.01, ***P < 0.001) between treatments for each species and biomass component.

our knowledge, there is no information about biomass responses to AMF symbiosis for this species, or for other species of the same gender. Therefore, this is the first evidence about its MR. Overall, it was suggested that root system morphology (i.e. a poorly branched root system) characteristic of tropical grasses seem to be associated with higher MR (Baylis 1972; Hetrick *et al.* 1991; Newsham *et al.* 1995), presumably in relation to a promotion in uptake of poorly mobile nutrients. Based in our observations, the three tropical grasses used in our experiment presented deep root systems with very few branches. Unfortunately, we did not find in the literature specific mention of the root branching pattern for this species. But remarkably, in our study the presence of mycorrhizae still played a significant positive role in the growth of temperate grasses (i.e. *A. elongatum* and *F. arundinacea*). This fact was already reported in some cereal crops (Smith and Smith 2011) and different genders of cultivated temperate grass species (Powell and Daniel 1978; Hall *et al.* 1984; Grimoldi *et al.* 2005; Covacevich and Echeverría 2008) at different soil stressful conditions.

It has been reported that many of the grassland species that form symbiosis with AMF show positive effects on plant biomass components (Hartnett and Wilson 2002; Hoeksema *et al.* 2010). Generally, the effect of increased uptake of P on the ecological success of the host plant is considered to be the most important benefit of mycorrhizal symbiosis (Koide 1991; Smith and Read 2008). In fact, it has been demonstrated that shoots of mycorrhizal plants could contain higher internal P concentrations (Stribley *et al.* 1980; in our screening the tropical grass *B. brizantha*), and/or increased biomass production and consequently higher P content at the whole plant level (all species of our screening except the temperate grass *D. glomerata*). For tussock forage grasses, the increase of biomass production of the plants due to nutritional changes is mainly explained by variations in bud initiation and thus the number of mature tillers per plant (Chapman and Lemaire 1993). This relationship is particularly strong for cool-season grasses, but it may differ in warm-season grasses (Williamson *et al.* 2012). For most species in our study, the rise in aboveground biomass of mycorrhizal plants was explained by increases in the number of tillers (Table 2), whereas for *P. dilatatum* the increase in dry weight of mature tillers accounted for the response. Additionally, mycorrhizal *P. coloratum* plants, the species with the highest MR, reached a size adequate to generate reproductive tillers. In contrast, in the temperate grass *D. glomerata*, the association with AMF did not show any enhancement of aboveground biomass components or P uptake of plants. We speculate that the occurrence of a high degree of colonization in this species may be explained by other beneficial effects on plant performance (Newsham *et al.* 1995), or that the optimum cost-benefit relationship would be evident at relatively higher soluble P availabilities (Janos 2007). Additionally, we could not discount the possibility that the AMF get more benefits (e.g. spore propagation and proportion of external hyphae) than host plants. Consequently, to elucidate the role of mycorrhizal symbiosis in this latter species, we need further investigation work under different stressful conditions and/or higher doses of fertilization, quantifying effects on the different fungus parameters (internal structures, external hyphae and spores).

To summarize, our results show that species responded differently to mycorrhizal symbiosis: the tropical species (*P. coloratum* = *B. brizantha* > *P. dilatatum*) showed greater responsiveness than temperate species (*F. arundinacea, A. elongatum* and *D. glomerata*). We supported the idea that the greater MR in tropical forage grasses can be a possible mechanism that explains their abilities for growth in nutrient deficient soils. In conclusion, this work is particularly relevant because it provides novel

and useful information for the selection of model species, with different MR, in order to study the symbiosis. Additionally, these findings could optimize grass forage production in grassland and cultivated pastures under nutritional deficit conditions.

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