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Consequences of grazing on the vertical transmission of a fungal *Neotyphodium* symbiont in an annual grass population

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Abstract Symbiosis between cool-season grasses and vertically transmitted fungal endophytes are common and significantly impact on ecosystem function. This makes the understanding of the underlying mechanisms to symbiotic individuals frequency in local populations much more interesting. Most studies have been focused on the differential fitness between symbiotic and non-symbiotic counterparts (relative fitness), barely considering other mechanisms. We performed a microcosms experiment to evaluate whether grazing alters the dynamics of the endophyte *Neotyphodium occultans* in the annual grass *Lolium multiflorum* by simultaneously modifying the relative fitness and the endophyte efficiency to be transmitted from host plants to seeds. Grazing was simulated by means of clipping and trampling on symbiotic and non-symbiotic plants growing separately, in soils obtained from paddocks, differing in their agronomic management history (natural grassland *vs.* ryegrass promotion). Seed production showed a complex pattern as it depended on the symbiotic status of the plants, the level of grazing and the agro-ecological context. Grazed plants produced three times fewer seeds than ungrazed plants only in microcosms with endophyte-symbiotic plants in soils from ryegrass promotion. Endophyte benefits on seed production were exclusively observed in ungrazed plants in the same soil. Symbiotic plants produced symbiotic and non-symbiotic seeds in all the treatments. While the production of non-symbiotic seeds by these plants was not affected by grazing and the soil, grazing reduced the production of symbiotic seeds in both contexts. Grazing negative effect on the density of fully infected spikes determined a significant increment in the transmission failures which were not modified by agro-ecological contexts. Therefore, grazing can modulate symbiosis dynamics through reducing seed production and endophyte transmission efficiency. Transmission has been disregarded, but it is a context-dependent process that could lead to a gradual reduction in the symbiotic plants frequency in a population if the mutualism effectiveness does not outweigh transmission failures.

Key words: grass–endophyte symbiosis, *lolium multiflorum*, mutualism effectiveness, symbiosis dynamics, transmission efficiency.

INTRODUCTION

Species interactions are a widely spread phenomena in nature, affecting the ecology and evolution of participating partners and, thereby, altering the structure and functioning of the whole ecosystem (Omacini *et al.* 2005; Thompson 2005; Rudgers *et al.* 2009). Macro-organisms such as plants and animals interact not only between themselves but also with a myriad of symbiotic microorganisms that can have positive or negative effects on host fitness (Thompson 2005; Hartley & Gange 2009; Douglas 2010). Similar to every interaction between pairs of species, a third species may alter the outcome of plant-microorganism symbioses, from mutualism to parasitism, and the symbionts, in turn, may indirectly affect the performance of this species (Kareiva 1994; Thompson 2005; Omacini *et al.* 2006; Hartley & Gange 2009; Douglas 2010). A growing body of recent research has shown that mycorrhizal status may actually modify the host plant tolerance to herbivore (Koricheva *et al.* 2009; Piippo *et al.* 2011), while other symbionts of aerial tissues may alter the host colonization by mycorrhizal fungi and competitive ability (Omacini *et al.* 2006; Novas *et al.* 2009). Nonetheless, experimental studies usually involve no more than three species, focusing on macro-organisms

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rather than on the response of microorganism symbionts or on the symbiotic system as an integrated unit (Fitter *et al.* 2000; Gundel *et al.* 2011a).

The symbiosis between cool-season grasses (Poöideae) and epichloae fungi (Clavicipitaceae) has become an important study system in ecology and agronomy, though, much significantly less considered than the symbiosis between plants and mycorrhizal fungi or nitrogen-fixing bacteria. The interest was mainly triggered because *Neotyphodium* endophytes, the asexual form of epichloae fungi, synthesize bioactive alkaloids that have shown to be highly effective in deterring herbivores (Clay 1990; Clay & Schardl 2002; Schardl 2010). Thus, *Lolium arundinaceum* and *L. perenne* can be toxic to livestock because of the symbiosis with *N. coenophialum* and *N. lolii* respectively, but in the former, the toxicosis is mediated by ergot alkaloids while, in the latter, by lolitrem-B alkaloids (Clay & Schardl 2002; Schardl 2010). Alternatively, *L. multiflorum* is not toxic to livestock when it is naturally symbiotic with *N. occultans*, although it accumulates loline alkaloids that confer resistance to a wide spectrum of insects (TePaske *et al.* 1993; Moon *et al.* 2000; Omacini *et al.* 2009; Schardl 2010). In addition, endophyte-symbiotic plants usually show higher tolerance to different abiotic stresses, apparently as a consequence of a stimulated antioxidant system (Malinowski & Belesky 2000; Clay & Schardl 2002; White & Torres 2010). Plant stress tolerances as well as alkaloids profiles vary with host genotypes, fungal strains and environmental conditions (Cheplick 2007; Rasmussen *et al.* 2007; Cheplick & Faeth 2009). Therefore, the symbiosis outcome and prevalence in natural populations may depend on the ecological context explored (Müller & Krauss 2005; Cheplick & Faeth 2009; Rudgers *et al.* 2009).

During the last two decades, there has been an increasing interest in the possible underlying mechanisms determining the frequency of endophyte-symbiotic individuals in grass populations (Ravel *et al.* 1997; Saikkonen *et al.* 2002; Afkhami & Rudgers 2008; Gundel *et al.* 2008, 2009). Most studies has been centred on the differential fitness between symbiotic and non-symbiotic counterparts (i.e. relative fitness) underestimating the importance of other population processes (Saikkonen *et al.* 2002; Gundel *et al.* 2008; Rudgers *et al.* 2010). Correlations between frequency of endophyte-symbiotic individuals in grass populations and environmental gradients of any biotic or abiotic factor have provided insights about factors selecting for the endophyte-grass symbiosis and mutualism effectiveness (Lewis *et al.* 1997; Jensen & Roulund 2004; Novas *et al.* 2007; Kirkby *et al.* 2011b). Consequently, the frequency of symbiotic individuals in grass populations has been associated among others, to environmental climate variables, grazing pressure and level above sea (e.g. Lewis *et al.* 1997; Clay & Schardl 2002; Novas *et al.* 2007). Making inferences about the relative fitness based on a given frequency of symbiotic plants can be misleading as symbiotic individuals can produce symbiotic and non-symbiotic plants (Afkhami & Rudgers 2008; Gundel *et al.* 2008, 2009).

Understanding endophyte growth and transmission through a population approach is also essential to understand the dynamics of endophyte-grass symbioses in nature (Gundel *et al.* 2008). *Neotyphodium* fungus lives asymptomatically in the host plant, growing systemically by the apoplast in the aboveground tissues (Clay & Schardl 2002; Christensen *et al.* 2008; Schardl 2010). The endophyte is closely associated with plant meristems and, early in the organs differentiation, it reaches tillers, spikes (or panicles) and flowers (Philipson & Christey 1986). After ovary fertilization, the endophyte hyphae profusely grow into the developing embryos, and by reaching the seeds, the symbiont has found the way of multiplication and dispersion (Clay & Schardl 2002; Gundel *et al.* 2011a). It has recently been found that vertical transmission may not be perfect, with symbiotic plants producing a variable proportion of non-symbiotic seeds, and it has been suggested that failures in the transmission process can be characteristic of species, populations or certain environments (Afkhami & Rudgers 2008; Canals *et al.* 2008; Gundel *et al.* 2009). Whereas no significant effects have been found of drought and oxidative stress (herbicide) on endophyte transmission from the mother plants to the seeds (Davitt *et al.* 2011; Gundel *et al.* 2011a), there are still no evaluations of the impact of grazing on this process. However, grazing is considered one of the most important drivers of the endophyte symbiosis in grass population (Clay & Schardl 2002; Clay *et al.* 2005).

In this article, we performed a microcosms experiment to study the impact of grazing on the symbiosis dynamics between *N. occultans* and *L. multiflorum* (annual ryegrass) in different agro-ecological contexts. Given that *N. occultans* is not an effective deterrent to livestock, intense grazing is likely to occur in *L. multiflorum* populations and it may be considered an important stress factor. Grazing was simulated by clipping and trampling symbiotic or non-symbiotic plants growing in soils obtained from paddocks with two different agronomic management histories. We propose that grazing by domestic herbivores influences the dynamics of the grass–endophyte symbiosis through two mechanisms. Grazing reduces the difference in seed production between endophyte symbiotic and non-symbiotic plants (e.g. relative fitness, Gundel *et al.* 2008). Moreover, the reduction in biomass increases the relative cost of harbouring the endophyte, thus, grazing negatively affects the fungus transmission efficiency to the seeds. By integrating the response of these two not completely independent

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processes, we also predict that livestock grazing may modulate the proportion of symbiotic and non-symbiotic seeds of an annual host grass that enters soil seed bank. Considering that outcomes of the grass–endophyte symbiosis are context-dependent, the effects of grazing on both processes (relative fitness and transmission efficiency) were evaluated in plants growing in soils with different types of human management that may change soil and plant biodiversity.

METHODS

System of study

Lolium multiflorum, ordinarily known as Italian or annual ryegrass, is a cool-season, C3 annual grass species extensively naturalized in temperate regions worldwide (Soriano 1991). In temperate grasslands of South America, it is an excellent alternative to promote high quality forage in winter (Rodriguez & Jacobo 2010). This species establishes a symbiosis with *N. occultans* endophyte fungus (Moon *et al.* 2000). Naturalized and promoted populations of *L. multiflorum* have shown high proportion of endophyte-symbiotic individuals in Río de la Plata Pampean grasslands and pastures (de Battista 2005; Gundel *et al.* 2009). The symbiosis has been found to increase host ability to withstand abiotic and biotic stresses, improving seedling recruitment and plant biomass production (Omacini *et al.* 2001, 2009; Miranda *et al.* 2011; Uchitel *et al.* 2011). Variations in the endophyte transmission efficiency from plant to offspring have been found to naturally occur associated to vegetation communities (Gundel *et al.* 2009).

Experimental design

Between March and December 2006 we conducted an outdoor experiment in microcosms (square pots, 0.25 × 0.25 m side, 0.20 m depth) in the experimental field at the Agronomy Faculty, Buenos Aires University, Argentina (34°35′S, 58°35′W). The experiment consisted in a full factorial randomized block design with six replicates and three factors: symbiotic status (S+, S–), grazing (G+, G–) and agro-ecological context (Natural grassland, Ryegrass promotion). The 48 pots were organized into a grid of 6 by 8 in an area of 25 m^2 protected against vertebrate herbivores. A meta-aldehyde based product was used in the area to exclude snails and slugs from the experimental area. When invertebrate herbivores were observed, they were removed manually. Microcosms were watered as required to keep soil moisture at field capacity.

Pots were filled with sieved soil brought from two adjacent paddocks at the Inland Pampa, Argentina (35°22′S, 59°26′W). Both soils are typic Hapludolls, series Bolivar (INTA) that differ in their agronomic management history and, in consequence, in the structure of current plant communities (Rodriguez & Jacobo 2010). The history determines two different agro-ecological contexts: the 'natural grassland', characterized by dominance of summer native grasses and a very low cover of *L. multiflorum*, while 'ryegrass promotion', characterized by the dominance of the symbiosis *L. multiflorum–Neotyphodium occultans* and a very low cover of specially summer native grasses (pers. obs.). This latter pattern results from the annual application of glyphosate herbicide [N-(phosphonomethyl) glicine] in order control summer species and to promote winter forage productivity (i.e. *L. multiflorum*; Rodriguez & Jacobo 2010). *L. multiflorum* plants growing in the ryegrass promotion paddock showed a symbiotic status of 100% (30 seedlings were evaluated by the stained method; Bacon & White 1994). As a consequence of management, soils from both paddocks now also showed differences in organic carbon and nitrogen levels (natural grassland: %C = 4.11 ± 0.46, %N = 0.42 ± 0.04; and ryegrass promotion: %C: = 2.58 ± 0.18, %N = 0.26 ± 0.02). We acknowledge that soils with a certain management history were sampled from only one paddock (~30 ha), but other paddocks with natural grasslands or ryegrass promotions would involve an unwieldy heterogeneity considering the different management practices (i.e. tillage, hebicides, fertilizers) developed to increase forage production in pampean grasslands (Rodriguez & Jacobo 2010).

Microcosms were sown with 0.4 g of *L. multiflorum* seeds (approx. 200 seeds) with high (S+: >90%) or low (S–: approx. 6%) proportion of symbiotic individuals. To obtain these seeds with contrasting proportions of symbiotic individuals, 1 year before the experiment, we collected seeds that were naturally endophyte-symbiotic (approx. 95%) from an old-field grassland at Inland Pampa dominated by *L. multiflorum* (Carlos Casares, Argentina 34°06′S, 60°25′W). Half of them were treated with triadimenol fungicide (0.5 g p.a./100g seeds) to remove the endophyte (see details in Omacini *et al.* 2004, 2009). Fungicide treated and non-treated seeds were cultivated in adjacent 1 m^2 plots in the experimental field Agronomy Faculty, Buenos Aires University (34°35′S, 58°35′W). After a growing cycle during 2005, the harvested seeds showed a contrasting proportion of endophyte-symbiotic individuals (indicated above) and were used in the experiment.

Half of microcosms were subjected to simulated livestock grazing (G+ and G–) by applying defoliation and trampling. Livestock grazing was simulated by clipping the *L. multiflorum* sward twice in growing cycle (e.g. at tillering and at stem elongation). Clipping was 6 cm tall carried out with scissors and trampling with an artificial hoof weighing 3.8 kg. The hoof was dropped inside a pipe five times per microcosm, from a height of 50 cm above ground level (adapted from the methodology used by Striker *et al.* 2006).

Sampling and determinations

At the end of the growing cycle (from November 2006 to January 2007), we harvested all mature spikes falling within a sampling area (ring of 15.9 cm^2 diameter) in each microcosm. Harvesting was synchronic to spikes maturity. In each microcosm, we estimated spike density by recording the number of spikes in the sampling area, seed production after threshing every spike and weighting seeds, and endophyte transmission efficiency by estimating the proportion of symbiotic seeds per spike (all the seeds were evaluated). Endophyte transmission was only evaluated in all the spikes

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in S+ microcosms. Endophyte presence was determined by microscopic observation in stained seeds (Bacon & White 1994). According to the proportion of symbiotic seeds detected in each spike, we established three different categories. When all the seeds were free of endophyte, the spike was considered negative (E^-); when all the seeds were found endophyte-symbiotic, the spike was perfect positive (E^+) and; when at least one but not all the seeds were endophyte-symbiotic, the spike was imperfect positive ($E^{+/-}$). The endophyte transmission efficiency was calculated on a spike basis as the proportion of endophyte-symbiotic seeds over the total seed evaluated. Then, the endophyte transmission efficiency (T) was calculated on a microcosm basis as the weighted average of the spike transmission efficiency (t) from the corresponding sampling area. For convenience, we estimated the endophyte transmission failures as the complement of transmission efficiency ($1 - T$). The production of endophyte-symbiotic seeds was calculated as the sum of the product of seed production and transmission efficiency of each spike (1). Conversely, the production of non-symbiotic seeds was calculated as the sum of the product of seed production and transmission failures (2):

$$\text{Endophyte-symbiotic seeds}\left(\text{m}^{-2}\right) = \sum\left[t_i \times \text{seed production}\left(\text{m}^{-2}\right)_i\right] \qquad (1)$$

$$\text{Non-symbiotic seeds}\left(\text{m}^{-2}\right) = \sum\left[1 - t_i \times \text{seed production}\left(\text{m}^{-2}\right)_i\right] \qquad (2)$$

where t_i is the transmission efficiency of the i^{th} spike, and seed production$_i$ is the number of seed produced (m^{-2}) by the i^{th} spike.

Statistical analysis

Data were analysed using generalized least squares models (GLS) with the package nlme (Pinheiro & Bates 2009) using R statistical software (R Development Core Team). The model used to compare spike density and total seed production per microcosm included symbiotic status, grazing and agro-ecological context as fixed factors. After checking the infection level of non-symbiotic microcosms, 11 of them, whose infection level was higher than 30%, were discarded.

The density of spikes E^+, E^- and $E^{+/-}$, the seed production per each type of spike, the transmission failures and the symbiotic and non-symbiotic seed production per microcosms were only evaluated in S+ treatments. Thus, the models which compared these variables included the grazing and the agro-ecological context as fixed factors. Blocks were considered as a factor in all the models. Normal distribution of data was evaluated using shapiro.test function with the residuals (basic package). For each analysis the homogeneity of variance was evaluated using leveneTest function within the treatments (car package, Fox 2009). When necessary, varFunc = varIdent function was used to stratify the variances within the levels of a factor (Pinheiro & Bates 2009). The varIdent function was applied for grazing factor when analysing transmission failures. The estimates produced by GLS allowed modelling the variances and the correlation of errors within each group (a combination of factors levels) for the fixed effects models (Pinheiro & Bates 2009). Akaike Information Criterion (AIC) was used to compare the models for each response variable and to choose the best adjusted models (Zuur *et al.* 2009). ANOVA results of the selected models are only shown.

RESULTS

All microcosms

Seed production per microcosm presented a complex pattern as it depended on the symbiotic status of the plants, the level of grazing and the agro-ecological context (Table 1). Grazing reduced the number of seeds produced, but the difference between G– and G+ was only significant in microcosms with endophyte-symbiotic plants, growing in soils from ryegrass promotion (Fig. 1). In this latter condition, ungrazed plants showed a threefold seed production

Table 1. All microcosms results of least squares models for the effect of grazing, agro-ecological context and symbiotic status on seed production per microcosm and spike density

		Seed production per microcosm		Spike density	
	d.f.	*F*-value	*P*-value	*F*-value	*P*-value
Block	5	0.57	0.720	0.88	0.509
Grazing (G)	1	42.19	**<0.0001**	18.94	**0.0002**
Context (C)	1	0.06	0.813	3.95	0.059
Symbiosis (S)	1	6.73	**0.016**	0.07	0.793
G × C	1	6.61	**0.017**	0.02	0.897
G × S	1	3.22	0.086	2.37	0.136
C × S	1	6.32	**0.019**	1.55	0.225
G × C × S	1	2.95	0.099	0.01	0.923
Error	24				

The table shows the degrees of freedom (d.f.), the statistical values of *F*- and *P*-values. Bold letters indicate significant *P*-values ($P < 0.05$)

over grazed plants and twice as many as ungrazed plants growing in S– microcosms with ryegrass promotion soils (Fig. 1, Table 1). In contrast, grazing reduced the spike density ($\#/\text{m}^2$) in about 54%, a pattern consistent in symbiotic and non-symbiotic *L. multiflorum* plants in both contexts (Table 1).

Symbiotic microcosms

Symbiotic plants produced non-symbiotic and symbiotic seeds in all the treatments (Fig. 1). Neither grazing nor agro-ecological contexts modified non-symbiotic seed production (Fig. 1, Table 2), but grazing reduced symbiotic seed production, being more effective in a ryegrass promotion context than in a natural grassland context (Fig. 1, Table 2). This detrimental effect was driven mainly through a reduction on E^+ spikes density, because E^- or $E^{+/-}$ spikes density did not vary between grazing treatments (Table 3, Fig. 2). There was no effect caused by the agro-ecological context on the density of any type of spike (Table 3). Furthermore, grazing significantly reduced seed production per E^+ spike in the ryegrass promotion microcosms (G+: 68.93 ± 3.69 *vs.* G–: 27.25 ± 4.80, mean ± SE), while it did not have any effect on natural grassland microcosms (G+: 38.50 ± 8.15 *vs.* G–: 43.71 ± 7.20) (Table 3). Neither grazing nor agro-ecological context altered number of seeds produced by $E^{+/-}$ and E^- spikes.

Grazing negative effect on the density of E^+ spikes determined a significant increment in the transmission failures per microcosms (Fig. 3, Table 2). As the agro-ecological context did not modify grazing effects (Table 2), the per cent of non-symbiotic seeds increased from 5% to 18–25% under grazing treatments in S+ microcosms with soils from both natural grassland and ryegrass promotion (Fig. 3).

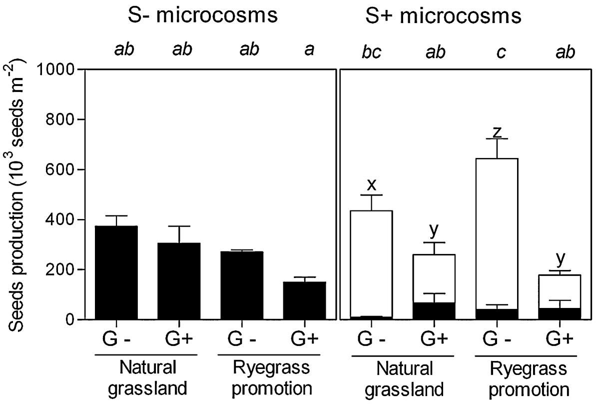

Fig. 1. Endophyte-symbiotic and non-symbiotic seeds (white and black bars, respectively) from nonsymbiotic and symbiotic *Lolium multiflorum* plants (S– microcosms and S+ microcosms, respectively) growing in soils from natural grassland or ryegrass promotion agro-ecological contexts and that were subjected or not to simulated grazing (G+ or G–, respectively). Bars are mean ± standard error of six replicates. Different letters in italic above graph-box (a, b and c) indicate significant differences ($P < 0.05$, Tukey test) among treatments for total seed production. Different letters inside graph-box (x, y and z) indicate significant differences ($P < 0.05$, Tukey test) among treatments within S+ microcosms, for endophyte-symbiotic seed production. For non-symbiotic seed production in S+ microcosms no significant differences among treatments ($P > 0.05$, Tukey test) were detected and, thus, no letters were included above these bars. Analyses were performed separately for endophyte-symbiotic and non-symbiotic seeds.

DISCUSSION

Our results show that grazing can simultaneously reduce seed production of the annual grass *L. multiflorum* plants bearing the fungus *N. occultans*, as well as the vertical transmission rate of this endophyte, by reducing the proportion of symbiotic spikes at the end of the growing cycle. The relative fitness between symbiotic and non-symbiotic plants, and the transmission efficiency can contribute to the local dynamics of the grass–endophyte symbiosis; although both processes are not independent but can respond differently to the environmental context (Gundel *et al.* 2008, 2011b). Recently, an experimental study has

Table 2. Symbiotic microcosms results of least squares models for the effect of grazing and agro-ecological context on non-symbiotic and symbiotic seed production of S+ *L. multiflorum* plants and transmission failures per microcosms

		Production of non-symbiotic seed		Production of symbiotic seed		Transmission failures	
	d.f.	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Block	5	1.21	0.351	1.26	0.329	1.25	0.337
Grazing	1	1.31	0.270	40.87	**<0.0001**	4.64	**0.048**
Context	1	0.02	0.885	0.02	0.877	0.15	0.702
G × C	1	1.07	0.317	4.62	**0.048**	0.86	0.369
Error	15						

The table shows the degrees of freedom (d.f.), the statistical values of *F*- and *P*-values. Bold letters indicate significant *P*-values ($P < 0.05$).

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Table 3. Symbiotic microcosms results of least squares models for the effect of grazing and agro-ecological context on each spike category produced by S+ *Lolium multiflorum* plants

	E^{+} spikes density			Seed per spike in E^{+}		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Block	5	0.94	0.484	5	0.40	0.838
Grazing	1	19.52	**0.001**	1	13.63	**0.002**
Context	1	0.16	0.694	1	1.62	0.225
G × C	1	0.74	0.403	1	7.32	**0.017**
Error	15			14		

	E$^{+/-}$ spikes density			Seed per spike in E$^{+/-}$		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Block	5	0.69	0.640	5	1.45	0.277
Grazing	1	0.14	0.715	1	1.96	0.186
Context	1	0.75	0.399	1	2.98	0.110
G × C	1	0.75	0.399	1	0.02	0.893
Error	15			12		

	E^{-} spikes density			Seed per spike in E^{-}		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Block	5	1.01	0.447	1	1.84	0.268
Grazing	1	1.42	0.253	1	0.86	0.423
Context	1	0.06	0.815	1	0.08	0.799
G × C	1	0.00	1.000	1	1.06	0.379
Error	15			3		

The table shows the degrees of freedom (d.f.), the statistical values of *F*- and *P*-values. Bold letters indicate significant *P*-values ($P < 0.05$). E^{+}, perfect positive spikes; E$^{+/-}$, imperfect positive spikes; E^{-}, negative spikes.

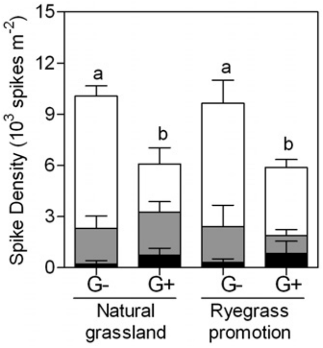

Fig. 2. Density of negative (E^{-}, black), imperfect positive (E$^{+/-}$, grey) and perfect positive (E^{+} white) *Lolium multiflorum* spikes of endophyte-symbiotic *Lolium multiflorum* plants growing in soils from natural grassland or ryegrass promotion agro-ecological contexts and that were subjected or not to simulated grazing (G+ or G–, respectively). Bars are mean ± standard error of six replicates. Different letters above columns indicate significant differences ($P < 0.05$, Tukey test) between treatments for E^{+} spike density. As neither E$^{+/-}$ nor E^{-} spike density were different among treatments ($P > 0.05$, Tukey test), no letters are shown above these bars. Analyses were performed separately for each spike category.

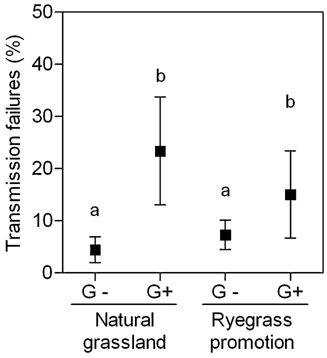

Fig. 3. Failures in the endophyte transmission of endophyte-symbiotic *Lolium multiflorum* plants growing in soils from natural grassland or ryegrass promotion agro-ecological contexts and that were subjected or not to simulated grazing (G+ or G–, respectively). Bars are mean ± standard error of six replicates. Different letters above graph-box indicate significant differences ($P < 0.05$, Tukey test) among treatments.

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shown that the level of the imposed drought increased the relative fitness of *Agrostis hyemalis* symbiotic plants but it did not affect the vertical transmission of the endophyte *Epichloë amarillans* (Davitt *et al.* 2011). However, Gundel *et al.* (2011a) found a positive relationship between the transmission efficiency and *L. multiflorum* biomass, and suggested that the costs of maintaining the endophyte (sl., Cheplick 2007) can be transferred into the transmission process in at least, this annual host grass. Therefore, vertical transmission of the endophyte seems to be clearly dependent on the environmental factor considered, being likely to generate spatial and temporal variability in the number and proportion of symbiotic seeds per plant as it has recently been observed for native and introduced grasses (Afkhami & Rudgers 2008; Gundel *et al.* 2009).

It has been argued that symbioses involving strictly vertically transmitted microbes must be mutualistic to persist in grass populations (Clay 1993; Clay *et al.* 2005; Selosse & Schardl 2007), though the benefits conferred to the plant can be very small, as long as the transmission efficiency is larger enough (Gundel *et al.* 2008). In our experimental conditions, endophyte presence was associated, in general, with a tiny insignificant increment of seed production, a consistent result with other works on the *L. multiflorum-N. occultans* association (Omacini *et al.* 2001; Miranda *et al.* 2011; but see Omacini *et al.* 2006, 2009). In fact, endophyte-positive effects were observed only in ungrazed plants growing in microcosms with soils from the most intensified agricultural management (e.g. ryegrass promotion). Thus, considering the detected failures during transmission, the symbiosis would be lost if this pattern persists under grazing and if there are not any other benefits which compensate the differences in seed number (Saikkonen *et al.* 2002; Gundel *et al.* 2008; Davitt *et al.* 2011). *Neotyphodium occultans* does not generate toxic effects on cattle and it has not been detected in livestock preference for endophyte-free *L. multiflorum* plants (de Battista 2005). However, previous studies suggest that positive endophyte effects can be expected in processes such as seed germination or seedling establishment, and resistance to invertebrate herbivores (Omacini *et al.* 2004, 2009; Uchitel *et al.* 2011), which can increase the frequency of symbiotic individuals in naturalized populations of *L. multiflorum* in grazed Pampean grasslands (de Battista 2005; Gundel *et al.* 2009). Nevertheless, it is important to consider that, during the experiment, grazing was simulated by both defoliation and trampling and that indirect effects of grazers are complex and variable (Wardle *et al.* 2004; Bardgett *et al.* 2005), also having complex still unconsidered effects on transmission and/or on mutualism effectiveness.

Grazing increased transmission failures by reducing mainly the endophyte ability to reach the host spikes completely (e.g. E^+ spikes) and, in consequence, diminishing the proportion of symbiotic seeds in patches previously dominated by symbiotic *L. multiflorum* plants. Considering the close connection between grass and fungus throughout their life cycles, transmission inefficiencies may occur because the endophyte failed to reach seedlings, tillers, spikes or panicles, spikelets and ovaries during flowering (Philipson & Christey 1986; Ravel *et al.* 1997), processes that are crucial in annual host species (Gundel *et al.* 2008). Our experimental approach allowed recognizing that the environmental context can triplicate the failures from the host plants to their seeds, without affecting the number of non-symbiotic seeds produced. In soils from ryegrass promotion, grazing also reduced the number of seeds produced by these E^+ spikes. There are no evaluations considering the transmission process during the whole plant cycle, though there are several studies that evaluated how storage conditions affect endophyte viability within the seed (Siegel *et al.* 1984; Kirkby *et al.* 2011a), or how environmental conditions affect endophyte growth within the plant (Ju *et al.* 2006; Rasmussen *et al.* 2007; Mack & Rudgers 2008). A few recent studies have documented imperfect transmission, detecting variability among species in the same site, and populations of the same species in different environmental conditions (Afkhami & Rudgers 2008; Canals *et al.* 2008; Gundel *et al.* 2009). For *L. multiflorum*, it was found that the transmission varied between ecotypes of the grass, and was not affected by the herbicide dose while it was 10% increased by the level of resources, a difference smaller to the one detected in our study (Gundel *et al.* 2011a).

Physiological mechanisms behind the variability observed in transmission efficiency remain unclear (Gundel *et al.* 2011b). It is possible that the level of resources determined these effects, considering that transmission efficiency can be high and invariable in plants or spikes with high seed production, while intermediate and lower in low seed production plants and spikes (Gundel *et al.* 2011a). Evolutionary theory of symbiosis proposes that, when the symbiont became parasitic, the host could apply sanctions, restricting the amount of resources allocated to the symbiont (West *et al.* 2002; Kiers *et al.* 2003). The way in which annual host plants sanction vertically transmitted symbionts could be by controlling the transmission process. In this way, mutualism effectiveness (or relative fitness) would be closely related to transmission efficiency (Gundel *et al.* 2011a). Our findings partially support these ideas, where grazing reduced mutualism benefits, host sanctioned the endophyte by restricting its growth and reducing its vertical transmission rate. Even when relative fitness decay was not as high as in ryegrass promotion context, transmission efficiency in plants growing in a natural grassland context was markedly reduced by grazing, suggesting that the relationship between transmission and relative fitness is also context dependent.

In conclusion, our results exhibited that grazing can modulate symbiosis dynamic through its effects on the vertical transmission of the endophyte and the relative fitness of host plants, two processes apparently synergistic that can determine the persistence of the symbioses. Mutualism effectiveness was only detected in ungrazed populations of ryegrass promotion context, while we found that vertical transmission was reduced in all situations. This could lead us to hypothesize that endophyte-symbiotic populations could exist in specific contexts without high grazing pressures. However, it is necessary to check out if this pattern is maintained for other soils on which ryegrass has been cultivated intensively. Moreover, it must be considered that other compensation mechanisms could exist under field conditions and be absent in our experimental approach. Now, it is necessary to evaluate if these effects are repeated in a larger scale for developing management strategies in order to promote the usual benefits that endophytes provide to the host.

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