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REGULAR ARTICLE

Can the defensive mutualism between grasses and fungal endophytes protect non-symbiotic neighbours from soil pathogens?

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Abstract
Background and aims It is proposed that *Epichloë* endophytes have a role protecting host grasses against pathogens. However, it is unclear whether this protection is extended to other non-symbiotic plants. Here we explored the effect of the asexual fungal symbiont, *Epichloë occultans*, on the interaction between *Lolium multiflorum* host plants and soil pathogens, and its potential positive side-effect on neighbouring plants.
Methods We conducted two microcosm experiments to assess the endophyte effect on seedling establishment of the host grass and other non-symbiotic grasses in the presence of soil pathogens. With an in-vitro experiment, we tested whether the endophyte inhibits, during seed germination, the growth of these pathogens.
Results Independently of pathogen identity, the endophyte improved host establishment (6 %). The endophyte also enhanced the establishment of the neighbouring grass *Bromus catharticus* (≈20 %) only in soil with *Rhizoctonia solani*. The endophyte in seed reduced the growth (≈20 %) of two out of four pathogens (*Fusarium acuminatum* and *R. solani*).
Conclusions We conclude that asexual endophytes could protect host grasses against pathogens but most importantly, that they can have protective effects beyond their hosts. Since effects depended on pathogen and plant identity, more experiments are needed in order understand the ecological meaning of these positive side-effects.

Keywords *Epichloë* · Asexual leaf endophytes · Pathogens · Grasses · Seedling establishment

Introduction

Symbioses with non-pathogenic microorganisms may produce a wide variety of effects on the interaction of host plants and other members of the community (Omacini et al. 2006, 2009; Rudgers and Clay 2007; Rudgers et al. 2005, 2007). These symbionts may impact on host plant interactions with competitors, herbivores and pathogens directly by enhancing host's defences and stress tolerance, and also indirectly through modifying their biotic and abiotic environment (Van Der Heijden et al. 2006a). For example, non-symbiotic neighbouring plants can take advantage of the soil nutrient enrichment as a side-effect of the symbiotic interaction of some plants with root-microorganisms such as mycorrhizal fungi and nitrogen-fixing bacteria (Pirhofer-Walzl et al. 2012; Van Der Heijden et al. 2006b). Although not so much studied, the symbioses

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with leaf fungal endophytes is suggested to produce multiple changes in the host rhizosphere that could modulate the performance of other non-symbiotic neighbouring plants (Kothamasi et al. 2009; Omacini et al. 2006; Rudgers et al. 2007, 2010).

Many cool season grasses are associated with asexual fungal endophytes of genus *Epichloë* which may produce significant changes in the physiology and ecology of host plants (Clay and Schardl 2002). These fungi colonize the intercellular spaces of meristems, leaf sheaths and culms and, during flowering, grow within developing-ovaries apparently without affecting seed development and its subsequent germination (Rodriguez et al. 2009; Schardl et al. 2004). Thus, the perpetuation of the symbiosis over time relies on the successful vertical transmission of the fungus, a process that can be disrupted by stressful conditions (García Parisi et al. 2012; Gundel et al. 2008, 2012b). The usually cited benefits of this symbiosis are an improved performance of host plants when facing different biotic and abiotic stress factors and in particular, an improved defence of plants to cope with natural enemies mainly herbivores. Less investigated however, an endophyte-mediated protection of plants against pathogens is usually suggested (Clay and Schardl 2002; Pérez et al. 2013; Rasmussen et al. 2008; Rúa et al. 2013). Those effects can impact positively on host fitness and are usually proposed to explain the high frequency of symbiotic individuals observed in grass populations (Clay and Schardl 2002; Gundel et al. 2011; Rudgers et al. 2010; Semmartin et al. 2015; Uchitel et al. 2011).

The presence of endophytic plants produces changes in the structure and function of recipient plant communities (Rudgers et al. 2005, 2007, 2010; Saikkonen et al. 2013). Among these changes, it can be mentioned a reduction of species richness of invaded communities, a switch in the diversity/productivity relationship, and a higher resistance to invasion in communities dominated by symbiotic grasses (Rudgers and Clay 2007; Rudgers et al. 2005, 2007; Saikkonen et al. 2013). All these effects are usually explained by a superior competitive ability of host plants with a consequent accumulation of dead biomass and interference of the emergence and growth of other species as well as by chemically mediated plant–plant interference, or allelopathy (Omacini et al. 1995, 2009; Vázquez-de-Aldana et al. 2013). Other endophyte-mediated effects that may indirectly account for the impact of the symbiosis on vegetation communities are through changes in the ecology of soil (Casas et al. 2011; Matthews and Clay 2001). Endophyte-mediated changes in host root exudates can change the soil environment and thus, potentially control the development of certain pathogens and to have feedback effects on other plants (Omacini et al. 2006; Rudgers and Orr 2009). For example, the negative indirect effect of endophyte on the development of plants of other species could be mediated by changes in the community of soil microorganisms affecting the success of non-symbiotic neighbours (Rudgers and Orr 2009).

There are numerous mechanisms proposed to explain the ways by which asexual endophytes may interact with soil microorganisms. A direct inhibitory effect over other microbes is supported by dual-culture experiments where the growth of pathogens is evaluated close to fungal endophytes (Pańka et al. 2013; Wäli et al. 2006). The effect of the symbiosis with endophytes on host plant mycorrhization has been puzzling although in general, inhibitory effects have been observed (Larimer et al. 2010; Omacini et al. 2012). All these effects mediated by the symbiosis with endophyte have been ascribed to the production of secondary compounds such as alkaloids (Bush et al. 1997) or other chemical compounds with antifungal activity such as volatile organic compounds (Steinebrunner et al. 2008). These changes may potentially have effects on the soil biota with ultimate impacts on the interaction of host plants with soil pathogens. More recently, Ma et al. (2014) showed that the endophyte enhanced the germination of perennial ryegrass (*Lolium perenne* L.) by providing protection against pathogenic fungi during early stages of establishment. This protection has been also observed in many other stages of plant development reinforcing the idea that the presence of endophytes may be key when pathogens are a threat (Clarke et al. 2006; Pańka et al. 2013; Pérez et al. 2013; Wäli et al. 2006). All these results together may indicate a potential for the endophyte to spread benefits of protection against pathogens to other non-host neighbours in the community.

In this paper, we focused on the effect of fungal endophytes on the interaction of the host plants with soil pathogens and the potential positive side-effect on other non-symbiotic neighbouring plants. We worked with the symbiosis between *Lolium multiflorum* and its fungal endophyte *Epichloë occultans*, in a recreated community context determined by the presence of pre-emergence pathogens and other grass species. We performed two microcosm experiments in order to understand the effect of the symbiosis on the establishment of the host grass and other non-symbiotic neighbouring species in the presence of soil pathogens. In parallel, we performed an in-vitro experiment to evaluate the effect of endophytes on the growth of the different pathogens during seed germination of the host. Our hypothesis is that the endophyte will have a positive effect on host seedling establishment protecting host plant in the presence of pathogens. We also hypothesize that this effect is delivered to other neighbouring plants species through an inhibitory effect of the endophyte on the pathogenic fungi. Under these hypotheses we predict that seedling establishment of *L. multiflorum* and other non-symbiotic species will be higher when *L. multiflorum* plants are symbiotic with endophytes than when they are endophyte-free.

Methods

Plant material

Lolium multiflorum Lam. (Italian ryegrass) is an annual grass with high constancy in grasslands of the Pampa region (Soriano 1992). Characterized as a competitive-ruderal species, its invasive behaviour in pampean grasslands has been associated with the presence of the endophytic fungus *Epichloë occultans* (formerly *Neotyphodium occultans*, see Leuchtmann et al. (2014); Moon et al. (2004)) (Omacini et al. 2009; Tognetti and Chaneton 2012). Surveys carried out in grasslands and old-fields of the Pampa region revealed very high frequency of symbiotic plants in naturalized populations (Gundel et al. 2009). Under field experimental conditions, symbiotic seeds have shown to produce more seedlings than endophyte-free plants (Uchitel et al. 2011). Furthermore, differences in seedling establishment has been favoured by a positive feedback of endophyte-mediated litter accumulation (Omacini et al. 2009).

For this study, mature seeds of *L. multiflorum* were collected from a population occurring in an older than ten years old-field grassland in Pampa region, Argentina (35°55′14.70″S 61° 9′29.24″W). A preliminary evaluation established that the harvested seed lot (≈200 g, ≈100,000 seeds) presented a 95 % of endophyte symbiotic individuals (based on microscopic observation of 100 seeds stained with Rose Bengal dye) (Bacon and White 1994). As counterpart, a non-symbiotic population was generated by treating half of these seeds with the systemic fungicide triadimenol (150 g ai kg −1, dose: 5 mg per gram of seed). Treated and untreated seeds (from now on E- and E+, respectively) were sown in monocultures in adjacent plots of 1 m^2 in the experimental field of the Faculty of Agronomy, University of Buenos Aires. During flowering, free pollination was allowed in order to keep wide the same genetic background (Gundel et al. 2012a). Here, we worked with the seeds produced by these plants (F1). A hundred of seeds from each subpopulation were checked to confirm the effectiveness of the fungicide treatment. The frequency of endophyte-symbiotic seeds was 90 % (90/100) and 10 % (10/100) for E+ and E- subpopulations.

Soil origin

Soil was brought from the same old-field where *L. multiflorum* seeds were collected. Soils in this location are well-drained, sandy-loam Typic Hapludols (or Haplic Phaeozem, FAO system) developed from loess materials, with a deep and well-drained upper horizon with 2.5 % organic matter.

Neighbouring species

Three native grass species whose persistence within the pampean grassland could be threatened by the invasion of *L. multiflorum* were selected: *Bromus catharticus, Briza subaristata,* and *Stipa neesiana*. Within these three species, *B. catharticus* is a biannual ruderal species with similar germination dynamics to that of *L. multiflorum*, and is often found sharing the same successional grassland. Meanwhile the perennials *B. subaristata* and *S. neesiana* are typical of the pristine grasslands with later and slower germination than *L. multiflorum*, being rarely found over 20 years of old-field succession (Tognetti and Chaneton 2012; Tognetti et al. 2010). Seeds of these three species were collected from a remnant of pristine grassland distanced 6 km from the old fields (35°55′14.70″S 61° 9′29.24″W). These unplowed grasslands are dominated by *Paspalum quadrifarium* Lam., a perennial native C4 species that accumulates about 90 % of the standing biomass of the community (Perelman et al. 2003). The remaining biomass is generally contributed by less dominant native species such as *Bromus catharticus*, *Briza subaristata*, and *Stipa neesiana*.

Selection of pathogens and methods of inoculation

For our experiments, we selected a set of generalist soil pathogens which, in particular, have been described as pathogens of the aforementioned grass species and *L. multiflorum* (Farr and Rossman 2014). Four pathogens were selected through pathogenicity pre-essays from a total of 15 pathogens provided by the Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires and the Agricultural and environmental Biosciences Research Institute (INBA-CONICET), Faculty de Agronomy University of Buenos Aires. Preessays were carried out in 30×20 cm (6 cm depth) plastic trays (hereafter, 'microcosms') filled with sterile sand. In each microcosm, a mix of 40 E+ and E- *L. multiflorum* seeds were sown and covered with a thin layer of sterile sand and inoculated with a solutions for the different species prepared following the protocol proposed by Lichtenzveig et al. (2006). We observed differences in the emergence of *L. multiflorum* for the following pathogens: *Fusarium acuminatum* (F.a. BAFCcult:1057); *Fusarium graminearum* (F.g. BAFCcult: 122); *Fusarium oxysporum* (F.o. BAFCcult:126); *Rhizoctonia solani* (R.s. BAFCcult:759). The sand was sifted and the recovered not-emerged seeds were plated in Potato Dextrose Agar medium in order to isolate and establish causation. From the selected pathogens, fungi from genus Fusarium and Rhizoctonia are generally soilborne, necrotrophic, plant pathogens causing primarily vascular wilts and rots of seeds roots and stem (Agrios 1997). The inoculation solutions for the different species were prepared following the protocol by Lichtenzveig et al. (2006). A sample of 1 ml of each solution was subjected to successive solutions in sterile distilled water and 1 ml of each was plated in potato Dextrose Agar medium. Colony forming units (cfu) were quantified. The solution concentrations were 2.35×106 cfu for *Fusarium acuminatum*; 6.7×10^5 cfu for *Fusarium graminearum*; 2.67×10^6 cfu for *Fusarium oxysporum*; and 1.23×10^5 cfu for *Rhizoctonia solani* per milliliter. Sterile Potato Dextrose medium was used as control. Each solution was diluted 1: 4 with distilled water.

Experiment 1

To test our first hypothesis, a greenhouse experiment was carried out in 30×20 cm (6 cm depth) plastic trays (hereafter, 'microcosms') filled with the soil mentioned above. In each microcosm, 40 E+ or E- *L. multiflorum* seeds were sown and covered with a thin layer of soil. At the same time, each microcosm was inoculated with 40 ml solution of each pathogen selected (i.e. *F. acuminatum, F. graminearum, F. oxysporum*, and *R. solani*). The solutions were gently and uniformly poured on the soil surface. A set of 5 microcosms were used as controls as they were inoculated with sterile culture medium. The emergency was daily recorded until no further seedlings appeared. The response variable was the percentage of established seedlings out of 40 sown seeds. Each treatment was replicated 5 times.

Experiment 2

To explore the potential inhibitory effect of the endophyte on the pathogens, an experiment was carried out in Petri dishes filled with 5 ml of potato-dextrose-agar (PDA) medium where each pathogen from our first experiment was individually plated along with an E+ or E- *L. multiflorum* seed. In each Petri dish, a 5 mm plug of one of the pathogens was placed 30 mm away from a symbiotic or non-symbiotic *L. multiflorum* seed. Since our E+ and E- subpopulations may present low but certain level of contamination (e.g. some E+ seeds in the E- subpopulation), the symbiotic status of each *L. multiflorum* seed was *a posteriori* confirmed. To estimate the pathogens' performance under each endophyte treatment (E+ or E-), the colony growth was followed by scanning each Petri dish using the WinRHIZO software (version 2003b, Regent Instrument, Quebec, Canada). Each treatment had 10 replicates.

Experiment 3

To test our second hypothesis, a second greenhouse experiment was carried out in which seeds of other three grasses were sown together with E+ or E- *L. multiflorum* seeds in presence or absence of the pathogens. The experimental units were microcosms as those described before and filled with the same soil. Each one was inoculated with 40 ml of culture of the following pathogens: *F. graminearum* and *R. solani*. Control microcosms were inoculated with sterile culture medium. In each one, 20 seeds of each of the native grass species (*Bromus catharticus, Stipa neesiana and Briza subaristata*) were randomly sown along with 20 E+ or

E- *Lolium multiflorum* seeds. The establishment of each sown species (including *L. multiflorum*) was counted daily until no further appearances were observed. For each experimental unit, the response variable was the percentage of established seedlings out of 20 sown seeds per species. Each treatment was replicated 10 times.

Statistical analyses

Establishment results obtained in our first microcosm experiment were analyzed with analysis of variance (ANOVA) of two factors with symbiotic status and pathogen inoculation as factors (Experiment 1). The results from the Petri dish experiment were analyzed by repeated measures ANOVA with symbiotic status as a factor. Analyses were performed separately for each pathogen (Experiment 2). Finally, in our third experiment, species establishment in each microcosm was analyzed using a Multivariate Analysis of Variance (MANOVA) with symbiotic status and pathogen inoculation as factors. Protected ANOVAs were then performed for each grass species individually (Experiment 3).

Results

Experiment 1

Seedling establishment of *L. multiflorum* was significantly affected by its symbiotic status and the pathogen inoculation treatment. Endophyte presence within the seeds enhanced the establishment of the host irrespective of soil inoculation with pathogens (Endophyte: $F_{1.40}$=5.20, p=0.028, Fig. 1). On the other side, the inoculation with pathogens reduced equally *L. multiflorum* establishment from symbiotic and non-symbiotic seedlings (Pathogens: $F_{4.40}$=2.99, p=0.029, Fig. 1, Endophyte × Pathogens : $F_{4.40}$=1.74, p=0.1605).

Experiment 2

Endophyte presence in *L. multiflorum* seeds reduced significantly the growth of two of the four pathogens tested in dual-culture experiments. From the three *Fusarium* species, only *F. acuminatum* showed a near to 5 % reduction in its growth that became significant at the third day and so on ($F_{4.72}$=5.11, p=0.001) (Fig. 2).

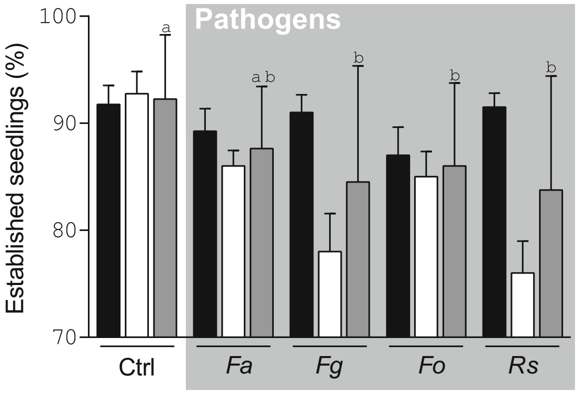

Fig. 1 Establishment of symbiotic (*black bars*), non-symbiotic (*white bars*) and overall (*grey bars*) *L. multiflorum* seedlings sown in non-inoculated soils (*control: Ctrl*) or inoculated with *Fusarium acuminatum* (Fa)*; Fusarium graminearum* (Fg)*; Fusarium oxysporum* (Fo)*; Rhizoctonia solani* (Rs) (*grey box*). Values are means and SE (n=5)

In the case of *R. solani*, the growth reduction became significant from day 3 and continued up to day 5 ($F_{3.54}$=5.11, p=0.004) (Fig 2). The excessive growth of the mycelia of this fungus did not allow considering results from day 5 onwards.

Experiment 3

In terms of the establishment of neighbouring plant species, an interaction among pathogens inoculation and symbiotic status was observed (Table 1). In absence of pathogens, *L. multiflorum* association with endophytes reduced the establishment of the native species *B. catharticus* (protected ANOVA: $F_{2.54}$=7.29, p=0.0016). However, this negative effect of the endophyte symbiosis on non-symbiotic neighbouring plants was not observed in presence of pathogens, being significant only in the presence of *R. solani* where a promotion in the establishment of *B. catharticus* seedlings was observed (Fig. 3). The establishment of *Stipa neesiana* and *Briza subaristata* seedlings was not affected either the symbiotic status of *L. multiflorum* seeds or by pathogen inoculation treatments.

Discussion

In this work, we found that grass-endophyte symbiosis has a positive effect not only on the host grass but also on other non-symbiotic neighbouring plants. Our results showed that the association of *L. multiflorum* with the

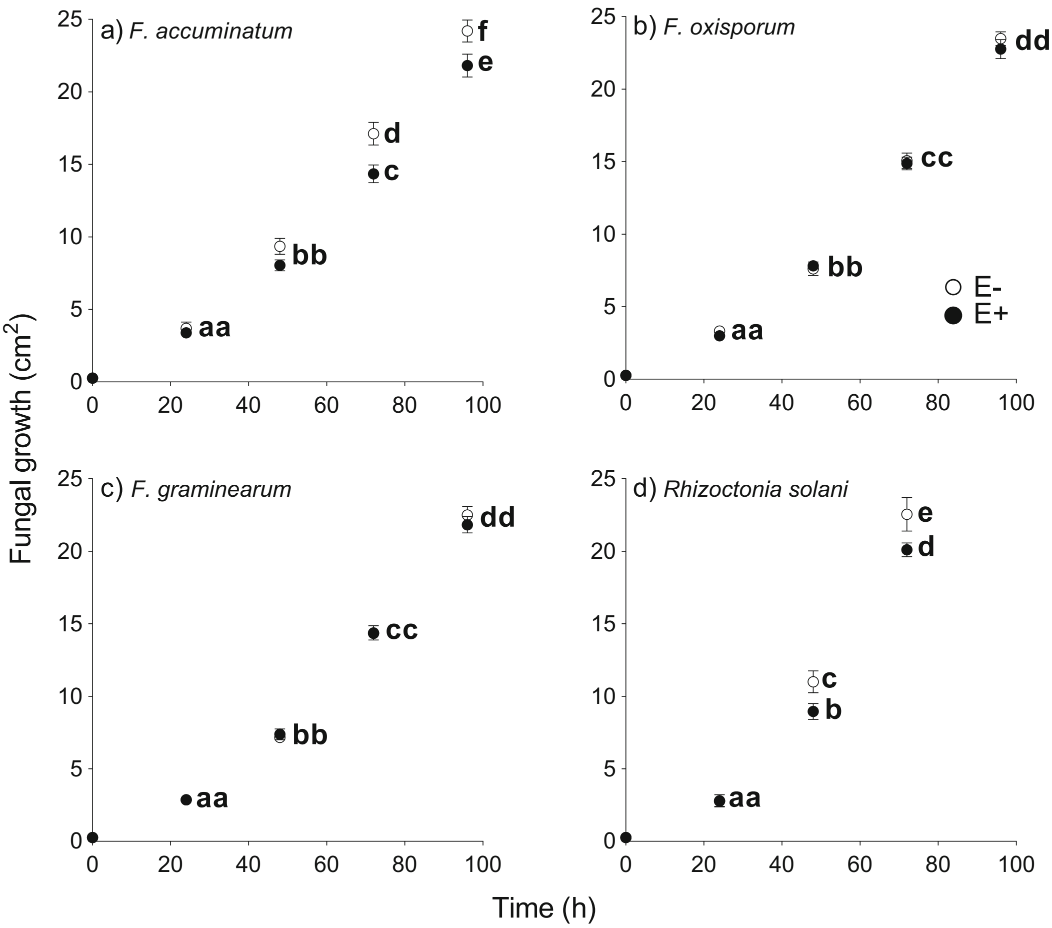

Fig. 2 Fungal growth of pathogenic fungi *Fusarium acuminatum, Fusarium graminearum, Fusarium oxysporum* and *Rhizoctonia solani* plated in PDA medium along with a symbiotic (*filled circles*) or non-symbiotic (*empty circles*) seed of *L. multiflorum.* Values are means and SE (n=10). *Letters* indicates significant differences between treatments for each variable for each date (Tukey test, p<0.05)

fungal endophyte *Epichloë occultans* altered the growth of certain generalist soil pathogens, which may be linked to the differential success of host plants versus endophyte-free counterparts and the extended benefits to other plant species in community. Even though changes in the host rhizosphere modifying the interaction among host plants and microorganisms were detected in previous experiments (Casas et al. 2011), it is not possible to establish whether this effect is produced by the endophyte itself or if it turns out from the holobiont during seed germination (Ma et al. 2014). Long-term consequences of positive side-effects mediated by the symbiotic interaction of grasses with asexual *Epichloë* endophytes on other non-symbiotic plants merit further research.

Table 1 Results of multivariate analysis of variance (MANOVA) for the effects of symbiotic status and pathogen inoculation on grasses' establishment

Effect	Pillai's trace	d.f.	F	*p*-level
Symbiotic status (S)	0.07	4.51	0.94	0.45
Pathogen inoculation (P)	0.26	8104	1.91	0.06
S × P	0.51	4104	4.47	>0.01

We detected an effect of endophytes promoting the establishment of host grass under different environmental conditions as it was observed in previous experiments (Omacini et al. 2009; Uchitel et al. 2011). Present

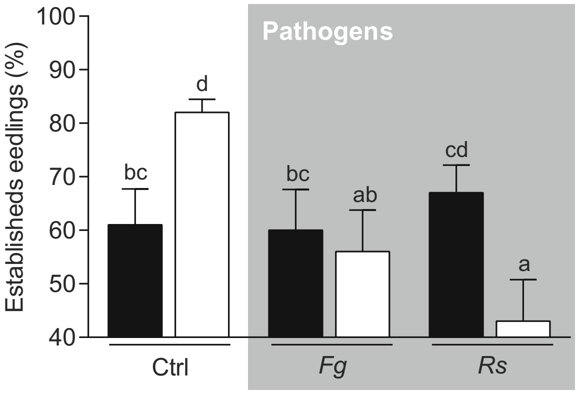

Fig. 3 Seedling establishment of *Bromus catharticus* in soil seeded along with symbiotic (*black bars*) and non-symbiotic (*white bars*) *L. multiflorum* seeds and inoculated with sterile culture medium (*control*) or with pathogens [*Fusarium graminearum* (Fg) or *Rhizoctonia solani* (Rs)] (*grey box*). Values are means and SE ($n=10$). *Different letters* indicates significant differences between treatments for each variable (Tukey test, $p<0.05$)

pathogens in the rhizosphere did not modify the benefits conferred by the endophyte to the host plant, and contrary to expectation they did not reduce significantly the establishment of non-symbiotic *L. multiflorum* seedlings. This could be associated to the identity of the pathogens and their evolution history along with host grass. In previous studies we proposed that pre-existent co-evolutionary processes could shape this triple interaction between plants, endophytes and pathogens (Pérez et al. 2013). So, we would expect that the effect of endophyte would become significant when pathogens come from the same communities from which the seed used in these tests were obtained. Our hypothesis is that the co-evolution of plants associated with endophytes and pathogens will increase the magnitude of the observed results. The mechanisms behind a reduction in the infection due to the symbioses with endophytes may be the result of complex interaction selected over time (Burdon and Thrall 2009; Rúa et al. 2013). Further experiments should consider this factor by including pathogens isolated from the same communities where all these grasses co-exist.

Our results obtained from the in-vitro experiment showed a negative effect of the seed-borne endophyte on the growth of two of the four pathogens tested. Previous studies showed that the concentration of alkaloids in endophytic seeds is high and that a 20 % of them are leached to the medium during the imbibition phase of the germination process (Justus et al. 1997). It has been suggested that these alkaloids could have a positive effect by preventing host plants from being infected by pathogenic fungi but at the same time, it could also be negative if it inhibits mutualistic symbionts such as mycorrhiza (Mack and Rudgers 2008; Novas et al. 2011; Omacini et al. 2006). In the case of the symbiosis between *L. multiflorum* and *E. occultans*, the amount and diversity of alkaloids is particularly low when compared to other symbioses (TePaske et al. 1993). This suggests that special attention should be paid to other secondary compounds produced by the endophyte fungus that may affect the host interaction with other organisms (Hamilton et al. 2012; Rasmussen et al. 2007). Thus together with allelopathic compounds (Vázquez-De-Aldana et al. 2011), asexual endophytes could mediate the exudation of compounds from the host root with antimicrobial properties (e.g., phenolics, phytoalexin) and thus affect the rhizosphere not only of host grasses but also of other neighbour grasses. It has been suggested that some of these changes could be responsibleof the resistance to root pathogen in symbiotic grasses (Malinowski et al. 1998). The higher tolerance of endophyte-symbiotic seedlings of *Lolium perenne* to pathogens was found to be related to improved antioxidant machinery (Ma et al. 2014), although it is unknown whether this effect can be found in the shared space in a way that other plants may benefit from the inhibition of fungal pathogens.

The among-grasses variation in the effect of endophyte symbiont on the establishment of neighbouring plants can be associated with the differences in life-history traits of the grass species studied. Although the four species included in this study are cool-season grasses with an autumn-winter-spring growing cycle, they differentiate in other traits. For example, while *L. multiflorum* is annual and *B. catharticus* biannual, the other two species (*B. subaristata* and *S. neesiana*) are perennials (Burkart et al. 2011). On the one hand, emergence dynamics of *B. catharticus* seedling may be comparable to that of *L. multiflorum*, while *B. subaristata* and *S. neesiana* show slow germination with a significant lag phase (Bourdôt and Hurrell 1992; Ferrari and Lopez 2000). Therefore, we propose that the higher synchrony in seed germination and seedling emergence between *L. multiflorum* and *B. catharticus* may explain at least a portion of the observed effect. According to this hypothesis, neighbouring species with similar emergence rate to that of host grasses could benefit from the reduced pathogen growth window generated by the presence of the endophyte. Certainly, the potential for a nurse effect to occur would be dependent on the population density of *L. multiflorum*, the frequency of endophyte-symbiotic individuals and the proximity of non-symbiotic plants to symbiotic seeds. In successional grasslands of Pampa region, *Bromus catharticus* is one of the few ruderal native species that subsist even after the invasion of the endophyte-symbiotic *Lolium multiflorum* (Tognetti and Chaneton 2012). These results support the recently proposed idea that the protective effect of endophyte may not be only for its host, but also for other community members (García Parisi et al. 2014).

In conclusion, our study adds to the notion that the symbiosis with fungal endophytes may act at different levels of organization regulating the establishment of the host plant and other non-symbiotic grasses within plant community. While the effect of the endophyte on host interaction with other fungi (Bacon et al. 1997; Chu-Chou et al. 1992; Pańka et al. 2013) and their consequences on community dynamics (Rudgers and Orr 2009) have received certain attention, here we provide evidence on the possible mechanisms involved. Through different approaches we demonstrated that asexual endophytes can modulate, on one side the establishment of the host grass and on the other the recruitment of other non-symbiotic native species by reducing the growth of soil pathogens. Our results show a high consistence along the approaches suggesting that the mechanisms observed *in vitro* could be responsible of the patterns observed in experiments performed in soil (Kaur et al. 2009). Even when we took a step further of the classical dual culture experiments and those focused on host grass only, further experiments should be oriented on seeking greater realism in experimental conditions. This may be achieved through the experimentation in natural communities, and through long-term studies using pathogens isolated from the same sites. This will help us understand how the changes observed in this paper will finally impact on the structure and functioning of plant community.

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