*What happens next? Legacy effects induced by grazing and grass-endophyte symbiosis on thistle plants and their floral visitors*

**Cecilia Casas, Juan Pablo Torretta, Nina Exeler & Marina Omacini**

Springer

Springer

CrossMark

## REGULAR ARTICLE

# What happens next? Legacy effects induced by grazing and grass-endophyte symbiosis on thistle plants and their floral visitors

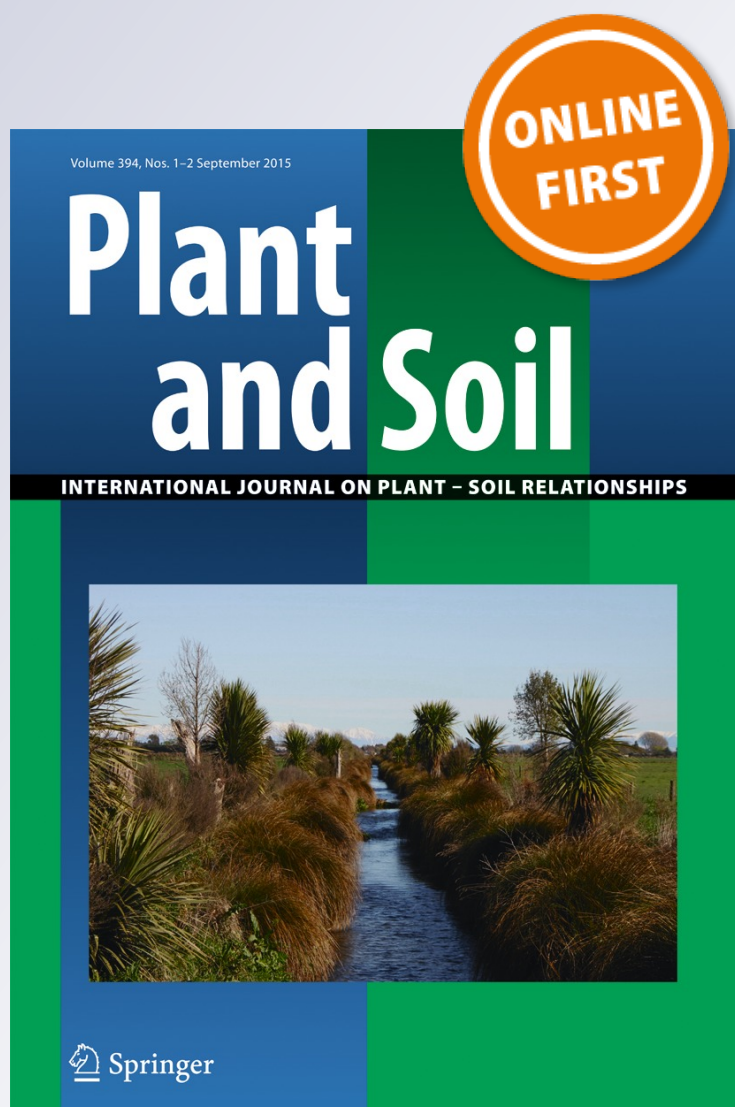**Cecilia Casas · Juan Pablo Torretta · Nina Exeler · Marina Omacini**

**Abstract**

*Background and aims* Complex webs of multiple interactions determine the final aboveground and belowground community structure. While the mechanisms are difficult to determine, soil conditioning may modify other plants performance and their interaction with other organisms. We aim to determine the extent to which aboveground *Epichloë* endophytes and consumers

C. Casas (✉)
Cátedra de Edafología, Universidad de Buenos Aires, Avenida San Martín 4453, Buenos Aires CPA 1417 DSE, Argentina
e-mail: ccasas@agro.uba.ar

J. P. Torretta
Cátedra de Botánica General, Universidad de Buenos Aires, Avenida San Martín 4453, Buenos Aires CPA 1417 DSE, Argentina

M. Omacini
IFEVA-CONICET, Cátedra de Ecología, Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, Buenos Aires CPA 1417 DSE, Argentina

N. Exeler
Department of Biology/Chemistry, Division of Ecology, University of Osnabrueck, Barbarastr. 13, D-49076 Osnabrueck, Germany

J. P. Torretta
CONICET, Buenos Aires, Argentina

induce legacy effects on subsequent plants and their interactions with floral visitors.
*Methods* We performed two mesocosm experiments in two phases. Firstly, annual ryegrass plants (*Lolium multiflorum*), in symbiosis or not with *Epichloë occultans* and subjected or not to grazing, were grown in mesocoms to generate four soil conditionings. Secondly, thistle plants (*Carduus acanthoides*) were grown in these conditioned soils. We assessed thistle plants aerial biomass, number of flower heads and their floral visitors.
*Results* The presence of one or both interactions (symbiosis and grazing) reduced total visits in subsequent thistle plants by 45 %. In particular, honeybees and other bees were reduced by 42 and 51 %, respectively. The flower head number or biomass of thistle plants may only partially mediate these effects.
*Conclusions* To better understand the rules structuring communities and ecological processes, it is critical to connect multiple interactions effects on soil conditions and their influence on trophic and non-trophic interactions during the subsequent generations.

**Abbreviations**

| | |
|---|---|
| E+ | Seeds of annual ryegrass (*Lolium multiflorum*) with high incidence of endophyte *Epichloë occultans* |
| E- | Seeds of annual ryegrass (*L. multiflorum*) with low incidence of endophyte *E. occultans* |

 Springer

| | |
|---|---|
| G+ | *L. multiflorum* population subject to simulated grazing |
| G- | *L. multiflorum* population do not subject to simulated grazing |
| Corg | Soil organic carbon |
| Nt | Soil total nitrogen |
| das | Days after seeding |
| ISTA | International seed testing association |

## Introduction

Plants live embedded in complex webs of positive and negative interactions which determine their direct or indirect effects on other plants of the same or different species (Callaway and Walker 1997; Ohgushi 2005; Scherber et al. 2010, and citations within). Either through their roots or dead tissues, which remain as detritus, they influence soil physico-chemical characteristics and its community structure (Moore et al. 2004; Bais et al. 2006; Eisenhauer and Reich 2012). While the mechanisms are difficult to determine, it has become clear that soil conditioned by plants may inhibit or favour other plants and modify their interaction with other organisms. Depending on the temporal and spatial dimensions of these plant–soil interactions, such effects can be termed as feedback, when they are detected in a neighbouring plant (Bever et al. 1997; De Deyn et al. 2003; Callaway et al. 2008; Kulmatiski et al. 2008; Reynolds et al. 2008), or as legacy effects, when they are detected in the subsequent generation (Gange and Smith 2005; Kardol et al. 2007; van der Putten et al. 2013). These legacy effects depend on the strength of plant–soil interactions and their time of residence (Kardol et al. 2007), also being capable of spreading to multiple interactions (Cahill et al. 2008). The importance of legacy effects mediated by multiple interactions, including herbivores and mutualists, has been poorly studied, although these kinds of effects may be critical for ecological processes like succession (Brown and Gange 1992) or invasion (Callaway and Ridenour 2004).

Asexual endophytes of the genus *Epichloë* (Clavicipitaceae) establish a highly specific interaction with many temperate grasses (Clay and Schardl 2002; Leuchtmann et al. 2014). They grow systemically within grass shoots and leaves, being vertically transmitted through host seeds (Clay and Schardl 2002; Cheplick and Faeth 2009). These obligate symbionts may deliver

a suite of fitness benefits to host plants under stressful conditions, while they obtain resources and means to multiply and disperse (Malinowski and Belesky 2000; Clay and Schardl 2002; Cheplick and Faeth 2009). This symbiosis was firstly known as responsible for toxic syndromes in livestock, associated to the production of secondary metabolites (i.e.: alkaloids), which protect host plants from herbivores and increase their fitness (Clay 1988; Clay and Schardl 2002). Due to fitness benefits, grass breeding programmes have developed forage grass-endophyte combinations which do not produce toxic alkaloids to livestock.

Besides, several symbioses which occur naturally produce a different profile of secondary metabolites which are not toxic to livestock and may be attractive in sustainable agriculture programmes worldwide (Rodríguez and Jacobo 2010; Iannone et al. 2012; Gundel et al. 2013). For instance, *Epichloë occultans* endophytes is an asexual hybrid with active loline biosynthetic pathways which produce N1-acetylloline, N1-acetylnorloline, N1-formylloline, N1-methylloline alkaloids and, it also has indole-diterpene genes (Schardl et al. 2012). Besides, various flavonoid and phenolic acids with systemic effect on *L. multiflorum* were found when this endophyte was present (Ponce et al. 2009). While in many cases the causal mechanisms and the compounds involved were not disentangled, this symbiosis has negative consequences on insect herbivores and their parasitoids (Omacini et al. 2001; Sugawara et al. 2006; Shiba et al. 2011).

Apart from aerial insect herbivory deterrence, *E. occultans* endophytes produce multiple changes in the host grass interactions with belowground organisms having consequences for fundamental ecological processes like decomposition (Omacini et al. 2004, 2006; Novas et al. 2008; Casas et al. 2011). Interestingly, grass-endophyte symbioses may generate legacy effects which persist even after the removal or death of the host. For instance, *Epichloë coenophial* symbiosis mediated negative legacy effects of *Schedonorus arundinaceus* (tall fescue) on other herbaceous and tree species (Clay and Holah 1999; Rudgers et al. 2007; Rudgers and Orr 2009). Besides, the presence of *E. occultans* endophyte modified legacy of *L. multiflorum* (annual ryegrass) through its litter, which promoted the establishment of fewer and larger plants regardless of symbiotic status of the annual ryegrass seeds (Omacini et al. 2009). These results suggest that litter produced by symbiotic plants may play an ecological role in community structure and dynamics.

The bias towards the study of isolated relationships has neglected issues such as if herbivory strengthens or weakens the effects of the grass-endophyte symbiosis on soil conditioning and how they mediate the legacy effects on the subsequent generation. In isolation, grazing modifies soil community structure and function via changes in root activity (i.e.: production of exudates), litter depositions (i.e.: quantity and quality) and trampling (Bardgett et al. 1998; Mikola et al. 2005; Sorensen et al. 2008; Schon et al. 2010; van der Putten et al. 2013). Importantly, grazing mediated legacy effects which control subsequent plants biomass allocation (Veen et al. 2014) and spread to multiple interactions like those established by these plants and their above or belowground consumers (Ohgushi 2005; Hartley and Gange 2009; Kostenko et al. 2012). Interestingly, Kostenko et al. (2012) showed that above and belowground insect herbivores, by affecting the soil fungal community, can alter the composition of secondary compounds in plants that later grow in the same soil, as well as the performance of the aboveground herbivores and parasitoids on those plants. These results remark that legacy effects of plants depend on the context they explore, which may be critical to understanding community dynamics. Even though very few studies have examined such interactive effects so far, the available evidence suggests that the simultaneous presence of foliar herbivores and fungal endophytes can, indeed, mediate legacy on other plant species (Matthews et al. 2001; Mikola et al. 2005; Sorensen et al. 2008).

In this study, we hypothesized that aboveground *Epichloë* symbionts and consumers induce legacy effects that will have an impact on the growth of subsequent plants and their interactions with floral visitors. To test our hypothesis, we performed two mesocosm experiments under natural environmental conditions, each consisting of two phases. In the first phase, annual ryegrass plants (*L. multiflorum*) in symbiosis or not with the endophyte *E. occultans* and, subjected or not to grazing, were grown in a full factorial design. This grass-endophyte symbiosis is very common in Pampean grasslands and pastures (De Battista 2005; Gundel et al. 2009), where it is promoted as winter forage (Rodríguez and Jacobo 2010). In the second phase, thistle plants (*Carduus acanthoides,* Asteraceae) were grown in conditioned soils to assess whether the legacy effects, induced by aboveground symbiosis and grazing, influence on plant growth and its reproductive performance. Then, we evaluated their floral visitors to determine the soil

legacy effects on aboveground non-thropic interactions. This experimental setting was performed in the same soil type subjected to two different agricultural practices: native and improved grasslands through annual ryegrass promotion. These different histories of use represented contrasting contexts where the induced legacy effects of grass-endophyte symbiosis and grazing might differ. For instance, soil communities of natural grassland, the least disturbed context, are considered more complex and resilient than those of improved grassland with higher degree of disturbance (Schipper et al. 2001; Wardle et al. 2003; Cragg and Bardgett 2010; Culman et al. 2010; Druille et al. 2013).

## Materials and methods

### Experimental setup: soil and seeds origins

Two controlled experiments were conducted to test the hypotheses. For each experiment, we used soils from neighbouring sites in the Flooding Pampa with the same taxonomy [typic Hapludoll, sandy loam series of Bolivar (Bv 9), US Soil Taxonomy] but, different history of use. One history represented an ungrazed humid mesophytic meadow, without other agricultural practices, dominated by native grasses (natural grassland site, hereafter; Fig. 1). The other history represented the same original natural grassland subjected to common practices to improve forage production during winter (improved grassland site, hereafter). This practice, known as promotion, consists of excluding the cattle during spring so that dominating species such as annual ryegrass produces the greatest possible quantity of seeds (Rodríguez and Jacobo 2010). In these grasslands, the establishment of annual ryegrass is usually favoured applying glyphosate herbicide before germination to reduce competition during its growing season (Rodríguez and Jacobo 2010). The result of this practice is an almost mono-specific annual ryegrass pasture which compensates the low forage productivity during winter. Particularly, in this improved grassland, promotion practices with glyphosate were performed for at least 4 years before transporting the soil for the experiment. The glyphosate applications were 4 $l.ha^{-1}$ in February (the dose was reduced to 2 $l.ha^{-1}$ in the next years as weed competition went down). In addition, at the beginning of April, herbicides 2–4 DB-ester (0.50 $l.ha^{-1}$) and Flumetsulan (0.20 $l.ha^{-1}$) were applied
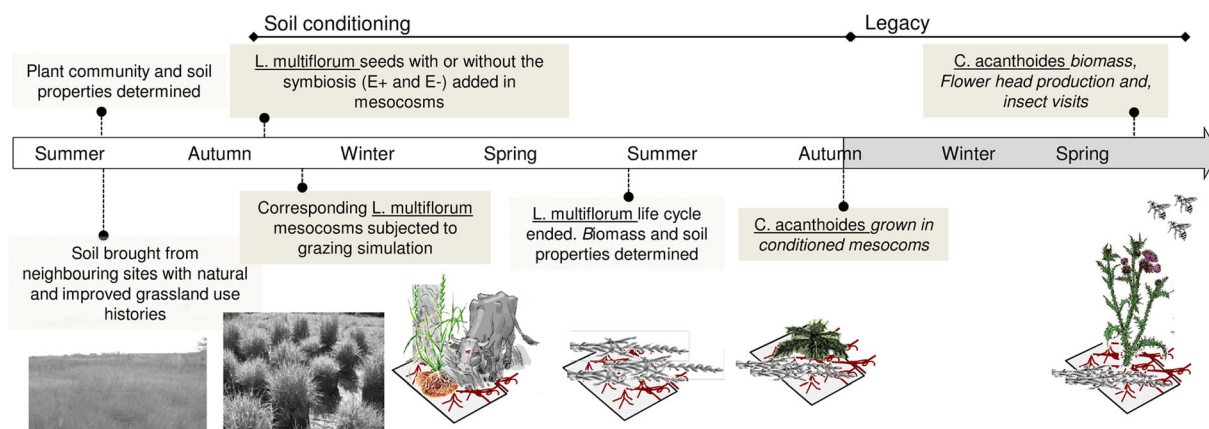
**Fig. 1** Timeline showing the experimental setting. Soil was brought from neighbouring sites (typical Hapludol, sandy loam) with natural and improved grasslands use histories. 24 mesocosms (0.25 m× 0.25 m×0.20 m depth) were filled with soil from each history of use to perform the conditioning treatments. The same amount of *L. multiflorum* seeds with or without the symbiosis (E+ and E-) were added in the mesocosms. Half of the mesocosms were subjected to grazing simulation including cutting and trampling. Five months after *L. multiflorum* life cycle had ended two legacy experiments were perform on the conditioned soils. The legacy to thistle plants (*C. acanthoides*) considered their biomass, number of flower heads and insect visits (see details in Materials and Methods)

to control dicotyledonous [i.e.: *Carduus acanthoides* (thistle), *Echium plantagineum* (purple flower), *Rumex crispus* (yellow dock)] (20–30 days residual power).

Both soils with different histories generated different environmental conditions for the growth of plants. Sites were characterized before the experiments. Three soil cores were taken from natural and improved grassland sites (2.5 cm diameter, 10 cm depth), homogenized, dried, and sieved (mesh #10) for chemical analyses during the end of April. Determinations included organic carbon (Corg; Walkley and Black 1934), total nitrogen (Nt; Kjeldahl), pH (1:2.5 distilled water), and water holding capacity (laboratory of the Department of Soil Science of the University of Buenos Aires). To avoid pseudoreplication or spurious analyses, we present the mean (± standard error) to describe the soil conditions at the beginning of the experiments (Table 1). To characterize the vegetation we used a simplified approach to the Braun-Blanquet method. Species cover was estimated as a percentage of the assessed vegetation. Those species that were observed only once were considered rare (Table 1).

Seeds of annual ryegrass with high incidence of *Epichloë* endophyte (E+ seeds>90 %) were collected from different mother plants in a grassland where symbiotic plants of annual ryegrass were dominant in December 2004 (deatails in Omacini et al. 2009). Distance from the seed collection to the soil collection site was less than 200 km. Half of these seeds were treated with the fungicide Triadimenol (5 mg.g$^{-1}$ seed) to obtain seeds with low incidence

level. Both, fungicide-treated and un-treated seeds were propagated in 1-m$^2$ plots under common environmental conditions in the experimental garden of the Faculty of Agronomy at the University of Buenos Aires. Plants were allowed to cross-pollinate freely, which contributed to homogenize the plant genetic background. The second generation from treated seeds (F2) with contrasting endophyte incidence were harvested in late spring (December 2005) and stored until the experiment. Microscopic examination determined the endophyte incidence level (E+: 90 vs. E-: 6 %, $n=30$ stained seeds per group). For this purpose, seeds were incubated in sodium hydroxide (2.5 %) for ~12 h, and then stained with Rose Bengal (ethanol 5 mL, Rose Bengal stain 0.5 g, distilled water 95 mL). This methodology has proven to be suitable for *Epichloë* endophyte determination (Koh et al. 2006). Germination tests according to ISTA (International Seed Testing Association) rules were carried out under laboratory conditions (15/25 °C, 8 h light, 5 days, $n=4$). Total germination did not differ between E+ and E- seeds [E+=98.7 % vs. E-=95 %. Welch *T*-test=1.56, $p=0.178$, $n=5$ (4.97)].

Thistle seeds were collected from a grassland in Inland Pampas during spring in 2003 (same field were annual ryegrass seeds were collected, see above) and stored at 4 °C until the experiments. Storage conditions and the period of storage (2003–2007) prior to the use ensured the loss of seed dormancy. Thistle seeds were placed on water agar

**Table 1** Characteristics of both sites used in the experiments

|  | Natural grassland | Improved grassland |
|---|---|---|
| Soil characteristics |  |  |
| Organic carbon (%) | 4.11 (±0.02) | 2.86 (±0.31) |
| Total nitrogen (%) | 0.42 (±0.07) | 0.3 (±0.03) |
| Mineral nitrogen ($\mu$g N.g soil$^{-1}$) | 21.41 (±2.92) | 19.11 (±2.34) |
| pH | 6.12 (±0.02) | 6.17 (±0.33) |
| Water holding capacity (%) | 36.6 (±5.60) | 25.65 (±1.23) |
| Plant cover (%) |  |  |
| Grasses |  |  |
| *Bromus mollis* | R | – |
| *B. unioloides* | 75 | R |
| *Cynodon dactylon* | 25 | ≤5 |
| *Lolium multiflorum* | – | 90* |
| *Paspalum dilatatum* | 5 | – |
| *Setaria* sp. | R | – |
| Dicots |  |  |
| *Ammi majus* | – | ≤3 |
| *Carduus acanthoides* | 1 | ≤5 |
| *Silybum marianum* | – | R |
| *Sonchus* sp. | R | – |

The determinations were performed on three soil samples at the beginning of the experiments we show the average (± standard error) with no statistical comparison. Species coverage was estimated as a percentage of vegetation per stand. Rare (R) species were observed only once in the vegetation stand

* *L. multiflorum* plants had high frequency of endophyte (greater than 95 %, $n=30$)

relations. In that concern, a dicot species has advantages over grasses as their flowers offers rewards (nectar and pollen) to insects which contribute with pollination (a non-trophic relation). Besides, thistle is similar to annual ryegrass in terms of origin and ecological behaviour. Both, species are exotic/ naturalized, considered as invasive species in Pampean temperate grasslands where usually prevailed in early and mid-successional stages (Tognetti et al. 2010). In particular, thistle is a large size species (from 30 cm to 2 m height), very persistent and has the ability to regenerate because of the longevity and large number of seeds that it produces. Besides, thistle invasion reduces productivity of pastures and grassland by suppressing growth of desirable vegetation and preventing livestock from eating plants growing in the vicinity of thistle stands (Desrochers et al. 1988).

*Phase 1: soil conditioning*

The soils from each site were used to fill 24 mesocosms (plastic containers of 0.25 m×0.25 m× 0.20 m depth; considering both soil origins, 48 in total). Before filling the mesocosms, the soils from both sites, natural and improved grasslands, were homogenised by crumbling and cleaning from roots. To generate the different conditioning treatments, we seeded annual ryegrass with high or low level of fungal endophyte incidence (E+ or E-, respectively) and simulated or not cattle grazing (G+ or G-, respectively) in a factorial arrangement. We use the same experimental design in each experiment with randomized complete blocks (six blocks each). Annual ryegrass with or without the symbiosis (E+ or E-) were manually seeded at a density of 3000 plants.m$^{-2}$ (equivalent to 200 plants per mesocosm) in early May 2006. Grazing was simulated during the growing season of annual ryegrass according to cut off frequency in rotational grazing systems typical in Pampean region [40 and 130 das (days after seeding)] (Giordani 1973). The annual ryegrass biomass was cut to 6 cm height using scissors. The G+ treatment also included a simulation of trampling with an artificial hoof (adapted from Striker et al. 2006) (Fig. 1). The plants were grown under natural environmental conditions of light and temperature and, watered to maintain soil moisture in experimental garden of the Faculty of Agronomy at the University of Buenos Aires. Environmental conditions explored during the experiments were

plates under controlled conditions (growth chamber at 22 °C) to induce germination. Consequently, the effect of treatments on the germination was not considered. This species is considered a weed in crop and livestock systems, and it is usually found in early successional stages in Pampean grasslands and pastures where annual ryegrass is a common species (Omacini et al. 2005). Success as an invasive species has been attributed to the prolific seed production, seed longevity, wind dispersal, competitive ability, the absence of natural enemies (Feldman and Refi 2006; Mandák et al. 2009). The flower heads are solitary or in small clusters and are visited mainly by *Bombus* spp. and *Apis mellifera* and several species of other bees and wasps (Desrochers et al. 1988).

Thistle plants and their visitors is an interesting model to evaluate legacy effects on trophic and non-trophic

similar to local historical average and according to temperate climatic conditions (Fig. 2).

We estimated annual ryegrass aerial (including leaves, tillers and spikes) and root biomass at the end of its life cycle (mid December 2006). Plant biomass helped us to identify possible mechanisms for legacy effects: whether legacy effects occur indirectly through plant size effects or potential soil changes. Estimations were made on three sub-samples taken at random with a core (6 cm diameter and 5 cm depth). Annual ryegrass aerial biomass was estimated from the material above the core and, root biomass from the roots in the core after removing the soil. The biomass was oven dried (60 °C, 48 h) and weighed (accuracy of±0.01 g). Both biomass values are expressed as kg.m$^{-2}$. Total biomass included aboveground material plus the cut material during grazing simulation which was oven dried and weight. Note that E- seed production did not depend on grazing and was similar in natural and improved grassland (García Parisi et al. 2012). In contrast, grazing reduced E+ seed production in about 70 and 40 % in improved and natural grassland, respectively. Finally, grazing reduced the endophyte transmission to seeds in both type of grasslands (García Parisi et al. 2012). Soil separated from roots was collected, dried and sieved (mesh #10) at the end of ryegrass life cycle. We determined soil mineral N (μg N.g soil$^{-1}$), pH and water holding capacity (%) (Laboratory of the Department of Soil Science of the University of Buenos Aires). Soil was considered rhizospheric as root density was very high throughout the volume of the sample.

*Phase 2: legacy effects on thistle plants and their floral visitors*

We set a legacy experiment in soils where annual ryegrass plants had grown under the combination of the symbiosis (E+ or E-) and grazing (G+ or G-) interactions. Thistle (*C. acanthoides*) plants were grown in the mesocosms used during phase 1. Annual ryegrass biomass varied with grazing and so did the amount of litter produced by these plants. Therefore, the amount of litter deposited in each pot was homogenized harvesting all the material produced and placing 12.5 g of litter on the surface [equivalent to 2000 kg ha$^{-1}$ reasonable in temperate pasture (Sosa et al. 2006)] straight after the end of annual ryegrass life cycle. Accordingly, litter only varied in relation to the level of endophyte infection (E+ or E- but not in quantity). We controlled the quantity of litter to generate similar conditions in terms of soil temperature, light and water dynamics. This procedure avoided mixed effects and allowed evaluating differences due to litter quality.

Five months after the end of *L. multiflorum* life cycle, three thistle seedlings were transplanted in each mesocosm (June 2007. See Figs. 1 and 2 for weather conditions details). The most vigorous thistle plant, within each mesocosm was selected to continue the experiment a week after transplanting. To determine the frequency of visits we performed observation censuses on each thistle during the flowering period (07th December, 2007 - 31th January, 2008). Each census lasted 15 min between 8:00 and 14:00 h (when insect activity is the highest) and on sunny days when the wind speed was low. Only insects that were collected on an
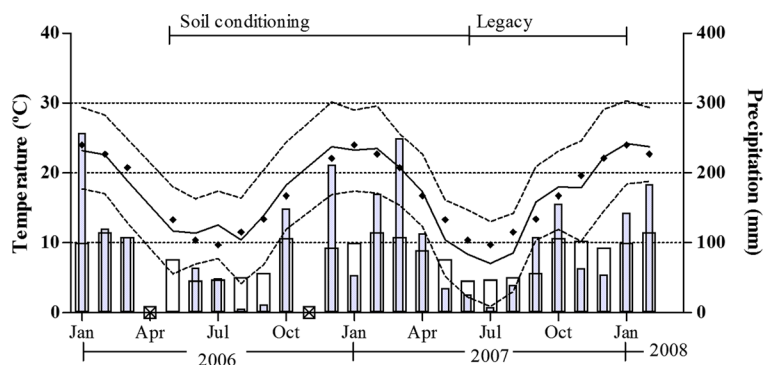


**Fig. 2** Monthly average of mean temperature (*full line*), minimum temperature (*lower dotted line*) and maximum temperature (*upper dotted line*). All expressed as °C), and precipitation (*gray bars*. Expressed as mm) during phases of soil conditioning and legacy of the experiments which were recorded at the nearest weather station. Historical mean temperature (*diamonds*) and historical mean precipitation (*white bars*) help to compare with average weather condition of the region. Non-available data are indicated with crosses at the *bottom line* (Source: http://ds.data.jma.go.jp/gmd/tcc/tcc/index.html. Accessed: 3rd May, 2015)

open flower head were considered as visitors. On each observation census, 4 to 5 thistle plants were randomly selected. Considering that the census of the eight conditions (i.e.: four treatments and two soil histories) was impossible in 1 day, randomization of thistle plants including the eight conditions minimized spurious differences due to the date of sampling along the census period. We observed and identified a total of 2197 insects belonging to 16 morphotypes. Wasps (one species of tribe Bembecini, in family Crabronidae: Hymenoptera) were excluded from the analyses as only three individuals were observed along the experiments. Morphotypes were classified according to honeybees, other bees (this group included bumble bees, native bees, and/or solitary bees), butterflies (different Lepidoptera species), beetles (two species of Coleoptera), and flies (including Diptera species, see Table 3). Honeybees were the most abundant species (10:1 to the other insect morphotypes) and, they are important visitors of thistle. The frequency of floral visitors is expressed as the average of visits per hour in relation to the number of censuses done on the thistle plant. A total of 36 thistle flowered plants were harvested to estimate biomass when the flowering period was over in February 2008. At this moment, almost all the flower heads had seed already ripe. Some of the thistle plants remained as rosettes along the experiment, and were not taken into account in the results as no association between the rosette stage plants and treatments was found. In total, we used 19 flowering plants in the natural grassland (n: E-G-=4, E-G+=5, E+G-=4, E+G+=6) and 17 in the improved grassland (n: E-G-=4, E-G+=5, E+G-=4, E+G+=4). The harvested material (stems, leaves and flower heads) was dried to constant weight (for at least 72 h at 60 °C) and weighed (with an accuracy of 0.001 g). Flower heads were counted to estimated individual flower head biomass. Aerial biomass and flower head values were expressed as g.plant$^{-1}$ and g.flower head$^{-1}$, respectively. Given the variation in flowering onset, duration of flowering phenology and number of flower heads between the plants in each census, we recorded the days to onset flower since first observation. In addition, we counted the open flower heads from all thistle plants. Days to onset, maximum number of open flower heads and number of open flowers at flowering end represented the duration and magnitude of the visual and olfactory attraction to insects. Note that flower biomass per plant includes the total amount of flowers (with different attractiveness depending on if they were close, open or already senescent) at the end and does not represent the attraction along the flowering period.

**Table 2** Means (± standard error) for the aerial and root biomass of *Lolium multiflorum* and, the values of soil pH, water holding capacity and mineral nitrogen

| | G- | | G+ | |
| --- | --- | --- | --- | --- |
| | E- | E+ | E- | E+ |
| **Natural grassland** | | | | |
| Lm aerial biomass (kg.m$^{-2}$) | 7.15 (±0.82) [a] | 6.87 (±1.38) [a] | 1.76 (±0.20) [b] | 1.55 (±0.36) [b] |
| Lm root biomass (kg.m$^{-2}$) | 1.24 (±0.08) [a] | 0.97 (±0.07) [ab] | 0.76 (±0.09) [b] | 0.96 (±0.16) [ab] |
| Soil mineral N (µgN.g soil$^{-1}$) | 50.45 (±5.78) [a] | 52.41 (±7.57) [a] | 39.48 (±3.70) [b] | 40.89 (±4.13) [b] |
| Soil pH | 6.95 (±0.18) [a] | 6.97 (±0.11) [a] | 6.42 (±0.13) [b] | 6.31 (±0.17) [b] |
| Water holding capacity (%) | 36.49 (±1.85) a | 36.13 (±0.97) a | 28.75 (±1.31) b | 28.69 (±2.21) b |
| **Improved grassland** | | | | |
| Lm aerial biomass (kg.m$^{-2}$) | 3.65 (±0.67) [a] | 5.21 (±0.97) [a] | 1.30 (±0.26) [b] | 1.13 (±0.21) [b] |
| Lm root biomass (kg.m$^{-2}$) | 0.81 (±0.13) [a] | 1.29 (±0.23) [a] | 0.70 (±0.11) [b] | 0.68 (±0.08) [b] |
| Soil mineral N (µgN.g soil$^{-1}$) | 30.66 (±3.51) [a] | 27.22 (±3.32) [a] | 24.76 (±3.52) [b] | 20.89 (±2.01) [b] |
| Soil pH | 7.27 (±0.09) [a] | 7.45 (±0.15) [a] | 6.92 (±0.14) [b] | 7.09 (±0.12) [b] |
| Water holding capacity (%) | 29.00 (±1.59) [a] | 28.17 (±1.90) [a] | 17.12 (±0.87) [b] | 15.77 (±2.08) [b] |

Different letters within the cells indicate significant differences for comparisons per row (*P*-value <0.05). Lm=*L. multiflorum*

Environments generated by history of use (natural and improved grasslands) were analyzed independently. The letters G and E in the column headings indicate grazing (G- and G+) and symbiosis (E- and E+) treatments

**Table 3** Observed morphotypes of insects. Morphotypes were classified according to honey bees, other bees, butterflies, flies and beetles. Columns show the group and the identified visitors included

| Morphotype group (Order) | Species/morphotype |
| --- | --- |
| Honey bees (Hymenoptera) | *Apis mellifera* |
| Other bees (Hymenoptera) | *Augochlora* sp. |
| | *Bombus atratus* |
| | *Lasioglossum* sp. |
| | *Megachile gomphrenae* |
| | Meliponini sp. |
| | *Mellissodes tintinnans* |
| | *Xylocopa augusti* |
| Butterflies (Lepidoptera) | Unclassified |
| Beetles (Coleoptera) | *Chauliognathus scriptus* |
| | Chrysomelidae sp. |
| Flies (Diptera) | *Allograpta exotica* |
| | *Eristalix tenax* |
| | Muscidae sp. |
| | *Toxomerus* sp. |

Statistical analysis

Analyses were performed separately for each experiment consisting in soils with different history of use (i.e. natural and improved grassland soils) to avoid pseudoreplication in history of use and not suitable conclusions (note that soil histories do not have genuine repetitions as soils were brought from two neighbouring sites in the Flooding Pampa with the same taxonomy. Comparisons were performed using linear mixed models. The values of annual ryegrass aerial and root biomass and, soil pH, moisture and mineral nitrogen, thistle aerial biomass, flower head biomass per plant and per individual flower head and, days to onset flower since first observation, maximum number of open flower heads and, the number of open flowers at flowering end were compared using the lme function [nlme package (Pinheiro and Bates 2000; Pinheiro et al. 2009)] with normal distribution of error and, considering Symbiosis and Grazing as fixed factors; and block as a random factor. When was necessary variance functions were tried including weights=varIdent (form=~1|factor) for Symbiosis or Grazing. Nested models were compared with AICtab function from bbmle package (Bolker and Team 2014). The normality assumption was evaluated by shapiro.test function (the basic package) with residues of the models [shapiro.test (residuals(model))]. The assumption of homogeneity of variances was evaluated by leveneTest function [car package (Fox and Weisberg 2011)] for treatments (combination of levels of factors). When the error distributions did not fit the normal distribution assumptions, we fitted models for Poisson error distribution family or negative binomial as explained below for frequency of visits,

The frequency of total visits and groups described above: total visits, honeybees, bees other than honeybees, butterflies, flies and beetles were compared using generalized mixed effect models. We used the glmer function [lme4 package (Bates et al. 2014)] for Poisson error distribution family and, the glmmadmb function [glmmADMB package (Bolker et al. 2013)] for negative binomial distribution (Table 5). Both models considered Symbiosis and Grazing as fixed factors; and block as a random factor. Dispersion was evaluated with the overdisp_fun proposed by Bolker et al. (2009).

To examine the relationship between thistle traits and frequency of visits, a second analysis consisted in running three different mechanistic models. These mechanistic models were run for the visits groups with influenced by the symbiosis, the grazing or the interaction between both factors (See Table 5). The mechanistic factors in the models were flower head biomass (g.thistle plant$^{-1}$), maximum number of open flower heads and open flower heads at the end of the experiment. Each mechanistic model included the factor and the interactions with Symbiosis and Grazing as predictors as predictor in order to test their importance as a possible mechanism through which the effects may spread. Note that the analysis of the visits expressed by each of the trait as a response variable would not allow analysis of these interactions. Inferential analyses were done with Anova function [car package (Fox and Weisberg 2011)] which performed an analysis of deviance (Type II tests with degrees of freedom associated Chisq$_{1,10}$ for natural grassland and Chisq$_{1,8}$ for improved grassland). The lack of development of some *C. acanthoides* plants determined unbalanced data (natural grassland, $n=19$; improved grassland, $n=17$). All analyzes were performed with the R-cran program, version 2.10 (R Development Core Team 2007). Figures in the results section show the averages of the replicates±standard error.

Plant Soil

## Results

### Soil conditioning

At the end of the soil conditioning phase, the symbiosis did not influence either the aerial and root biomass of annual ryegrass or soil measured parameters. Instead, G+ treatment reduced the aerial biomass of annual ryegrass (76.4 %), soil pH (8.5 %), moisture (20.9 %) and mineral nitrogen (21.9 %) in the natural grassland soil experiment. The interaction between symbiosis and grazing influenced root biomass of annual ryegrass. Grazing (G+ level) reduced root biomass by 38 % in the absence of symbiosis (E- treatment) and had no effect when symbiosis was present (E+ treatment, Table 2).

Likewise, at the end of the soil conditioning phase, symbiosis did not affect either annual ryegrass biomass (aerial and root) or soil measured parameters, but grazing reduced the aerial and root biomass of annual ryegrass (73 and 52 %, respectively), soil pH (4.8 %), moisture (42.5 %) and mineral nitrogen (21.1 %) in the improved grassland soil experiment (Table 2).

### Legacy effects on thistle plants

Symbiosis and grazing on annual ryegrass do not modify flower head biomass per thistle plant but, the symbiosis reduced individual flower head biomass in the natural grassland soil experiment. Individual flower head biomass was reduced in average by 20 % in the E+ compared with E- treatments (Table 4 and Fig. 3a and c). In contrast, grazing reduced in average by 17 % flower heads biomass per thistle plant in the improved grassland soil experiment. Symbiosis and grazing on annual ryegrass do not modify individual flower head biomass in the improved grassland soil experiment (Table 4 and Fig. 4b and d). Grazing anticipated flowering by 7 days in average in the natural grassland soil experiment (Table 4 and Fig. 4a). Symbiosis and grazing on annual ryegrass did not influence the maximum number of open flower heads per thistle plant nor the number of open flower at flowering end, which were 13 and 10, respectively on average in the natural grassland soil experiment (Table 4 and Fig. 4c and e). Meanwhile, the days to onset depended on the interaction between symbiosis and grazing in the improved grassland soil experiment (Table 4 and Fig. 4b). This interaction showed that E-G- treatment anticipated flowering by 22 and 18 days compared with E+G- and E-G+, respectively. Besides, both interactions reduced the maximum number of open flowers and, the number of open flowers at flowering end of thistle plants growing in the improved grassland soil experiment (Table 4 and Fig. 4d). Maximum number of open flowers was reduced by 22.5 % under grazing and by 45 % under symbiosis conditions and the number of open flowers at flowering end was reduced by 30 % under grazing and by 60 % under symbiosis conditions. Neither symbiosis nor grazing on annual ryegrass changed the aerial biomass of thistle in both natural or improved grassland soil experiments (Table 4).

### Legacy effects on thistle floral visitors

Neither symbiosis nor grazing on annual ryegrass affected the frequency of visits in the natural grassland soil experiment (Table 5 and Fig. 5). In contrast, both interactions (symbiosis and grazing) had negative effects on the frequency of visits to thistle plants in the improved grassland soil experiment (Table 5 and Fig. 6). The presence of one or both interactions reduced the total visits by 45 % compared with E-G- treatment. In particular, a similar pattern was observed for the honeybees and other bees where the presence of one or both interactions reduced the frequency of visits by 42 and 51 % compared with E-G- treatment, respectively. In the case of butterflies, the symbiosis reduced the frequency of visits to thistle plants by 48 %. We did not detect changes due to symbiosis or grazing in flies and beetles visits, the least abundant ones. The three models, which included the flower head biomass, the maximum number of open flower heads or the number of open flower heads at the end of flowering as predictors, indicated that these variables are involved in the effects observed interacting with symbiosis and grazing and thus, partially explained the effects observed on total visits and honeybees (Table 6). The maximum number of open flower heads and the number of open flower heads at the end of flowering were involved in the effect on other bees and any of the three predictors were not involved in the effect of the symbiosis on butterflies (Table 6).

## Discussion

This study demonstrated that aboveground interactions such as symbiosis with fungal endophytes and grazing

**Table 4** Results of the models for the legacy effects of symbiosis (E+ vs. E-) and grazing (G+ vs. G-) on the biomass of flower heads (flowers head biomass. thistle plant$^{-1}$), individual flower biomass, days to onset flowering since first observation (Days to onset flowering), the maximum number of open flower heads, open flowers at flowering end and, the aerial biomass of *C. acanthoides*

| | Natural grassland | | | Improved grassland | | |
|---|---|---|---|---|---|---|
| | Symbiosis (E) | Grazing (G) | E × G | Symbiosis (E) | Grazing (G) | E × G |
| Flower head biomass | 0.30 (0.58) | 2.31 (0.12) | 0.64 (0.42) | 3.19 (0.07) | **4.33 (0.03)** | 0.71 (0.39) |
| Individual flower head biomass | **7.64 (0.005)** | 0.97 (0.32) | 0.45 (0.49) | 0.05 (0.81) | 1.69 (0.19) | 0.23 (0.63) |
| Days to onset flowering | 0.01 (0.92) | **9.94 (0.001)** | 0.61 (0.43) | 3.19 (0.07) | 1.37 (0.24) | **15.37 (0.000)** |
| Maximum open flower heads | 0.03 (0.85) | 0.08 (0.77) | 1.17 (0.27) | **22.7 (0.000)** | **10.18 (0.001)** | 2.13 (0.14) |
| Open flowers at flowering end | 0.32 (0.57) | 2.39 (0.12) | 0.16 (0.68) | **22.79 (0.000)** | **4.93 (0.02)** | 0.38 (0.53) |
| Aerial biomass | 0.36 (0.54) | 0.15 (0.70) | 0.14 (0.70) | 1.60 (0.21) | 1.57 (0.21) | 0.78 (0.37) |

The conditions created by history of use (natural and improved grasslands) were analyzed independently. The table shows the values of the statistic with degrees of freedom associated ($F_{1,15}$ and $F_{1,13}$ for natural and improved grasslands, respectively) and the probability value (*p*-value). Bold numbers correspond to significant effects (*p*-values< 0.05)

on the same annual grass induced soil legacy effects, mainly by delaying flowering period and by reducing the number of open flower heads available for floral visitors in the subsequent generation of thistle plants. Studies on aboveground-belowground interactions have mainly evaluated the effects considering interactions on
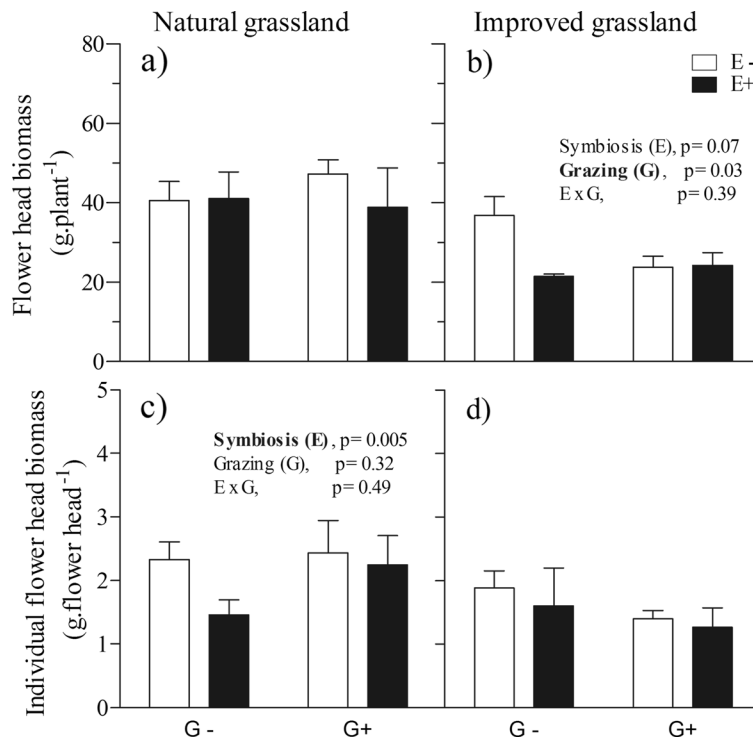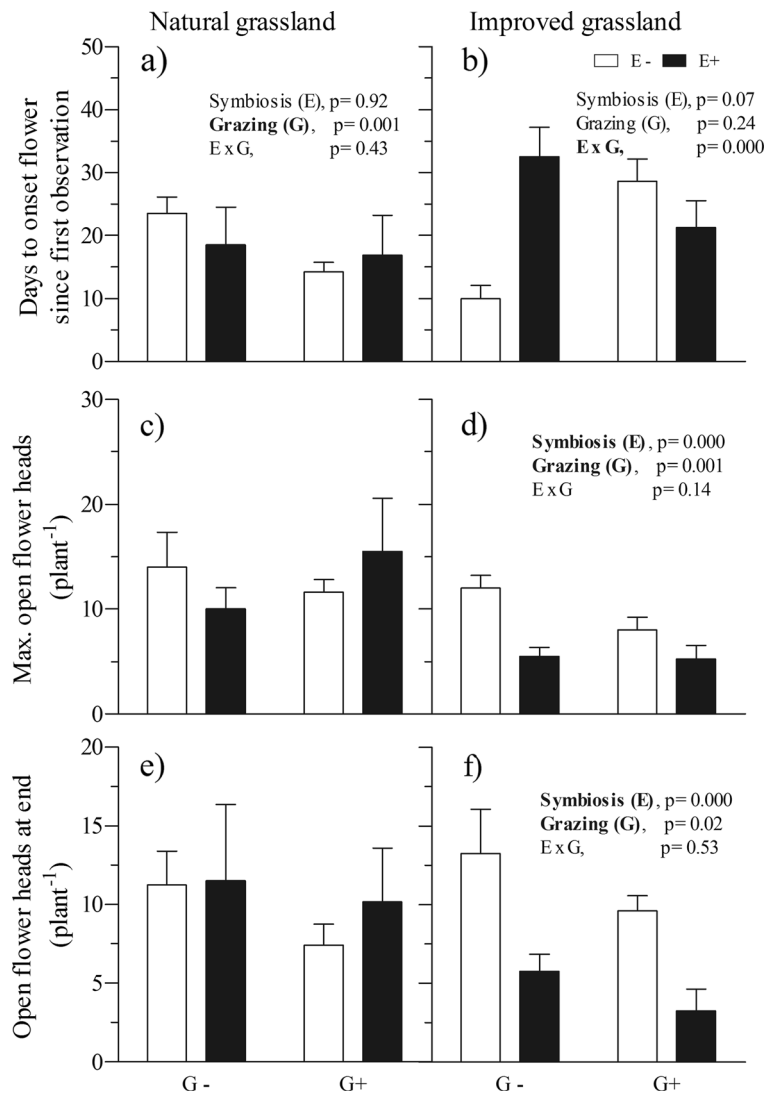


**Fig. 3** Flower head biomass per plant (**a**, **b**), individual flower head biomass (**c**, **d**) of *C. acanthoides*. Plants were grown in soil from natural grassland (*right*) or improved grassland (*left*) and were subject to legacy effects associated with the presence of *L. multiflorum* plants with or without the symbiosis (E+ vs. E-) and the with or without simulated grazing (G+ vs. G-) the year before. The combination of symbiosis and grazing generated four growth condition for *C. acanthoides*. The *bars* show average (± standard error). Probability value (p=) are indicated beside the factors when there was significant effects in the analysis. Only flowered thistle plants were considered in the analyses. In total, we used 19 flowering plants in the natural grassland (n: E-G-= 4, E-G+= 5, E+G-= 4, E+G+= 6) and 17 in the improved grassland (n: E-G-= 4, E-G+= 5, E+G-= 4, E+G+= 4)

**Fig. 4** Days to onset flower since first observation (**a**, **b**), maximum number of open flower heads during flowering period (**c**, **d**) and, number of open flowers at flowering end (**e**, **f**) of *C. acanthoides*. Plants were grown in soil from natural grassland (*right*) or improved grassland (*left*) and were subject to legacy effects associated with the presence of *L. multiflorum* plants with or without the symbiosis (E+ vs. E-) and the with or without simulated grazing (G+ vs. G-) the year before. The combination of symbiosis and grazing generated four growth condition for *C. acanthoides*. The bars show average (± standard error). Probability value (p=) are indicated beside the factors when there was significant effects in the analysis. Only flowered thistle plants were considered in the analyses. In total, we used 19 flowering plants in the natural grassland (n: E-G-= 4, E-G+= 5, E+G-= 4, E+G+= 6) and 17 in the improved grassland (n: E-G-= 4, E-G+= 5, E+G-= 4, E+G+= 4)



the same plant such as the effects of arbuscular mycorrhizal fungi on leaf-mining or pollinating insects (Gange et al. 2003; Gange and Smith 2005). Besides, legacy effects have been mostly studied considering one kind of interactions such as the effects of soil microbial community composition (Kardol et al. 2007) as well as, foliar- and root-feeding insects (Brown and Gange 1992; Kostenko et al. 2012) on succeeding plants. The results presented here contribute with novel insights into plant legacy research for several reasons: i) they remark the importance of interactive effects of two aboveground relations of different nature (grass-endophyte symbiosis and grazing), ii) both relations drove legacy effects on a different plant species with potential consequences on its reproductive success, iii) both relations, through effects

on soil, had also consequences on aboveground interactions, as they reduced the frequency of floral visitors to those plants. It is important to consider that these effects were only evident in the soil from improved grassland under intensive management practice (i.e.: herbicide applications) and with greater cattle disturbance.

Remarkably, changes induced by symbiosis and grazing levels in this annual grass, generated unexpected and non-additive legacy effects on thistle, which were evident by the delay in the flowering period, reduction of flowers head biomass and, the number of open flower heads. Considering interactions simultaneously allowed us to observe interesting patterns such as non-additive legacy effects mediated by grass interaction with its symbiont and grazers. Importantly, our

**Table 5** Results of the models for the legacy effects of symbiosis (E+ vs. E-) and grazing (G+ vs. G-) on total visitors and morphotypes classified according to honey bees (*A. mellifera*), other bees, butterflies, flies and beetles visiting *C. acanthoides* flower heads
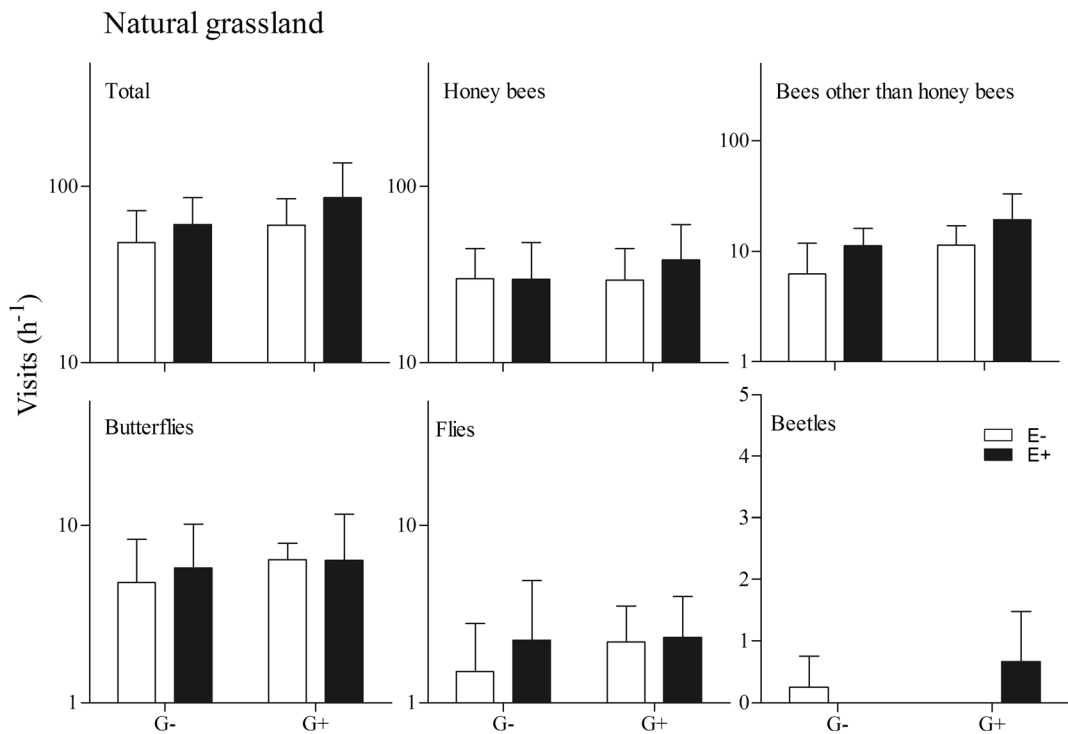
|  | Total visits | Honey bees | Other bees | Butterflies | Flies | Beetles |
|---|---|---|---|---|---|---|
| **Natural grassland** | | | | | | |
| Symbiosis (E) | 0.54 (0.46) | 0.00 (0.98) | 1.78 (0.18) | 0.16 (0.68) | 0.30 (0.58) | 0.00 (0.99) |
| Grazing (G) | 0.57 (0.44) | 0.00(0.95) | 2.05 (0.15) | 0.46 (0.49) | 0.31 (0.58) | 0.00 (0.99) |
| E × G | 0.09 (0.78) | 0.33 (0.56) | 0.01 (0.91) | 0.11 (0.73) | 0.28 (0.59) | 0.00 (0.99) |
| Model | Neg. Binom | Neg. Binom | Neg. Binom | Neg. Binom | Poisson | Poisson |
| Disp. parameter | 5.6 (±1.92) | 4.3 (±1.56) | 3.8 (±1.64) | 4.3 (±2.62) | 1.7(p 0.04) | 0.6 (p0.84) |
| **Improved grassland** | | | | | | |
| Symbiosis (E) | 38.87 (0.000) | **20.66 (0.000)** | **4.31 (0.03)** | **5.49 (0.02)** | 0.55 (0.46) | 0.43 (0.51) |
| Grazing (G) | 34.79 (0.000) | **14.39 (0.000)** | **5.09 (0.02)** | 3.63 (0.05) | 0.17 (0.68) | 0.01 (0.92) |
| E × G | **4.63 (0.03)** | 2.88 (0.08) | 1.57 (0.21) | 0.18 (0.67) | 2.56 (0.10) | 0.00 (0.99) |
| Model | Poisson | Poisson | Neg. Binom | Poisson | Poisson | Poisson |
| Disp. parameter | 2.4 (p 0.004) | 0.8 (p 0.58) | 6.4 (±4.06) | 1.9 (p 0.02) | 1.2(p 0.24) | 0.85(p0.60) |

Wasps were excluded from the analyses as they have a different trophic behaviour and only three individuals were observed along the experiment. The conditions created by history of use (natural and improved grasslands) were analyzed independently. The table shows the Chi square statistic from the analysis of deviance (Type II tests with degrees of freedom associated $Chisq_{1,10}$ for natural grassland and $Chisq_{1,8}$ improved grassland) and the probability value (*p*-value). Below the factors the table shows the family or error distribution (Poisson, negative binomial or quasi-poisson) and the corresponding dispersion parameter. Bold numbers correspond to significant effects (*p*-values< 0.05)

results showed for the first time that induced legacy of both symbiosis and grazing mediated by the annual ryegrass had a similar impact (effect size) on thistle plants during the subsequent generation (i.e.: number of open flower heads at the end of the experiment). Meanwhile, we did not find any evidence of change in the aboveground biomass allocation of thistle as expected from previous works (Veen et al. 2014). Recent works support the idea that grass-endophyte symbiosis mediates legacy effects controlling the structure of the plant community during the following growing season (Rudgers et al. 2007; Rudgers and Orr 2009; Omacini et al. 2009) through mechanisms still difficult to elucidate.

Concerning feasible mechanisms through the soil, previous studies shown that this particular symbiosis can influence rhizosphere chemistry (Ponce et al. 2009; Vignale et al. 2014), reduce litter decomposition rates (Omacini et al. 2004) and affect the activity of soil organisms (Omacini et al. 2006; Novas et al. 2008; Casas et al. 2011). In particular, exudation of allelopathic compounds from *L. multiflorum* infected plants are likely to explain, at least in part, the effects on soil communities and functioning (Vignale et al. 2014). However, very low values of loline alkaloids reported in *L. multiflorum* infected plants (less than 100 $\mu g.g^{-1}$)

(see Omacini et al. 2009; Siegrist et al. 2010) makes this group of compounds unlikely to generate legacy effects. Meanwhile, there are several evidences that grazing can influence soil environment (Hamilton and Frank 2001; Mikola et al. 2001; Kourtev et al. 2002; Pietikäinen et al. 2009; Eisenhauer and Reich 2012). For instance, grazers promoted root exudation of carbon, which was quickly assimilated by microbial community in the rhizosphere (Hamilton and Frank 2001). Moreover, their effects on microbial community increased soil mineral nitrogen, plant nitrogen uptake and leaf nitrogen content. Interestingly, Bultman and collaborators (2004) detected that clipping enhanced alkaloids production in endophytic tall fescue (*S. arundinaceus*) plants which altered host plant relationship with aphids. Thus, the effects of simulated grazing on endophytic annual ryegrass plants may also increase loline compounds levels or other alkaloids production and trigger changes in host exudates and soil chemistry. Furthermore, higher soil nitrogen levels under simulated grazing makes this possibility even more feasible since it may stimulate alkaloid production (Arechavaleta et al. 1992). It will also be essential to know the dynamics of these products in the soil and their ability to be transferred to other plants (see Lehtonen et al. 2005) to assign them a role in the legacy effects.
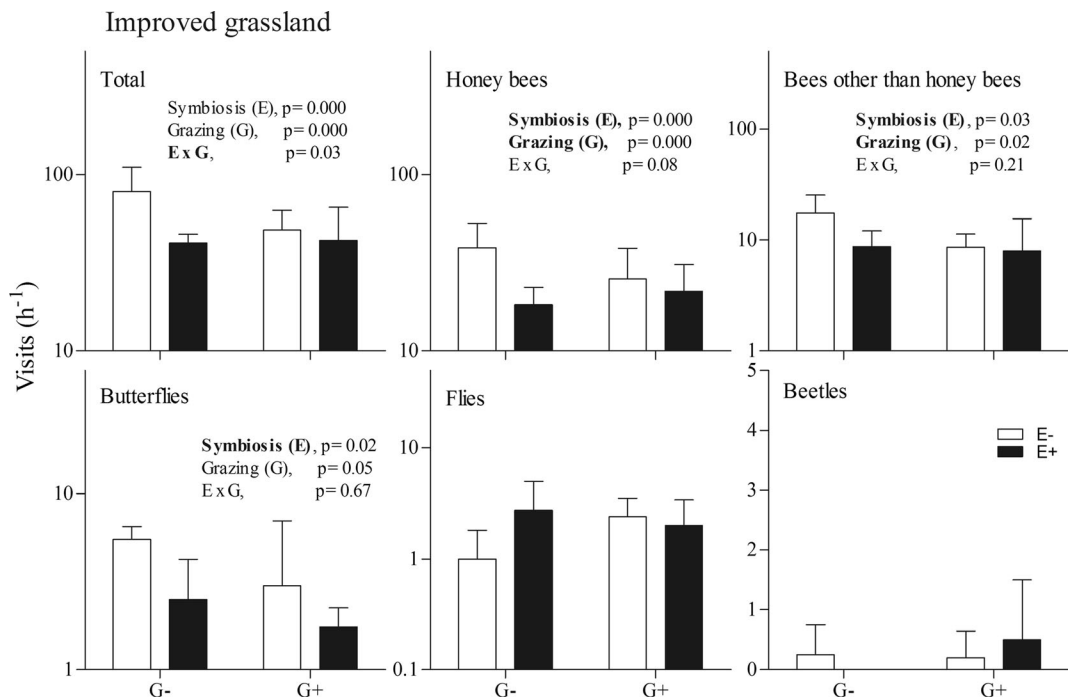
## Natural grassland



**Fig. 5** Frequency of total visitors, and morphotypes classified according to honey bees (*A. mellifera*), other bees, butterflies, beetles, and flies visiting *C. acanthoides* flower heads. Wasps were excluded from the analyses as they have a different trophic behavior and only three individuals were observed along the experiment. *C. acanthoides* plants grown in soil from natural grassland subjected to legacy effects of *L. multiflorum* plants with or without the symbiosis (E+ vs. E-) and the with or without simulated grazing (G+ vs. G-) the year before. The combination of symbiosis and grazing generated four growth conditions for *C. acanthoides*. The bars show the average (± standard error). Probability value (p=) are indicated beside the factors when there was significant effects in the analysis. We included 19 flowered thistle plants in the analyses (n: E-G-= 4, E-G+= 5, E+ G-= 4, E+G+= 6)

Our experimental approach allowed us to address the potential effects of the symbioses and grazing through the litter quality on soil environment. We controlled the quantity of litter to generate similar conditions in soil temperature, light and water dynamics with the purpose to isolate endophyte and grazing legacy effects due to litter quality. Further experiments should address whether the effects observed here would have persisted in the presence of natural differences in litter quantity generated by grazing. Concerning to feasible mechanisms, Siegrist et al. (2010) demonstrated that ergot alkaloids (ergovaline and ergovalinine) do not persist in litter from *S. arundinaceus* - *E. coenophialum* symbiosis. In consequence, they suggested that, at least, these alkaloids were not involved in effect of endophytes on decomposition rates. We cannot completely discard loline alkaloids produced by *L. multiflorum* - *E. occultans* symbiosis and contained in the litter may also mediate legacy effects . Although secondary compounds have not been measured in litter from endophyte-

infected *L. multiflorum* plants, for instance, we detected that it altered the establishment and herbivory of seedlings, underlying a hidden ecological role of this symbiosis after the host life (Omacini et al. 2009).

The significant interaction among symbiosis, grazing and number of open flower heads suggested that other not elucidated mechanisms were involved in the specific soil legacy which reduced the attraction for visitors (Wyatt 1982) and potentially, reproductive performance of thistle plants. We propose that changes in plant nutritional quality as well as in production or quality of secondary compounds in thistle plants might cause the observed effects (eg.: Joosten et al. 2009; Hol et al. 2010). Joosten et al. (2009) showed that soil-borne microorganisms as well as the type of soil influenced on the composition and quantity of secondary metabolites produced as defences in aerial and root tissues. Meanwhile, Hol et al. (2010) found that reduction of rare soil microbes increased both plant biomass and plant nutritional quality and in turn, the susceptibility to insect

Fig. 6 Frequency of total visitors, and morphotypes classified according to honey bees (*A. mellifera*), other bees, butterflies, beetles, and flies visiting *C. acanthoides* flower heads. Wasps were excluded from the analyses as they have a different trophic behavior and only three individuals were observed along the experiment. *C. acanthoides* plants grown in soil from improved grassland subjected to legacy effects associated with the presence of *L. multiflorum* plants with or without the symbiosis (E+ vs. E-) and the with or without simulated grazing (G+ vs. G-) the year before. The combination of symbiosis and grazing generated four growth conditions for *C. acanthoides*. The *bars* show the average (± standard error). Probability value (p=) are indicated beside the factors when there was significant effects in the analysis. We included 17 flowered thistle plants in the analyses (n: E-G-= 4, E-G+= 5, E+G-= 4, E+G+= 4)

herbivores. Note that in these works (Joosten et al. 2009; Hol et al. 2010), interactions were studied on the same plant and not on plants growing during the subsequent generation (i.e.: considering legacy effects).

In addition to the attractiveness generated by the number of open flower heads, floral visitors, such as honeybees, possibly respond to changes in the distinctive nectar smell or the presence of other volatile compounds at short distances (Giufa and Núñez 1993). The results are consistent with previous works suggesting that visits of honeybees to thistle flower heads are not random but, they are guided by the nectar reward (Shafir et al. 1999) or a repellent generated by them once the flowers were visited and depleted (Giufa and Núñez 1992, 1993; Goulson 1999). For instance, Kostenko et al. (2012) demonstrated that insect herbivory (above and belowground) in ragwort (*Jacobaea vulgaris*, Asteraceae) greatly influenced the secondary compounds, biomass and aboveground multitrophic interactions of succeeding ragwort plants under controlled

experimental conditions. Although we could not identify the compounds responsible, changes in the rewards as well as in the odorous signals are possible mechanisms through which induced legacy could drive trophic and non-trophic interactions (i.e.: the reduction in floral visits unexplained by the number of open flower heads, the flower head biomass, the or the number of open flower heads at the end of flowering). Besides, other feasible mechanisms like the presence of attractive compound with toxic effects to the insect visits or, the presence of harmful compounds which were not detected by them can not be rule out. In consequence, honeybees as well as other bees may visit other species and eventually influence the ecological success of neighbouring species. However, the consequences of these effects on the structure and dynamics of the community are more difficult to predict (Ohgushi 2005).

In recent years, it has become clear that not only antagonistic (Mills and Bever 1998; Callaway and Ridenour 2004), but also mutualistic interactions (Bais et al. 2008; Casanova-Katny et al. 2011) between exotic

**Table 6** Results of the three mechanistic models for the legacy effects of symbiosis (E+ vs. E-) and grazing (G+ vs. G-) on total visitors and morphotypes classified according to honey bees (*A. mellifera*), other bees, butterflies in the conditions created by the improved grassland

|  | Total visits | Honey bees | Other bees | Butterflies |
|---|---|---|---|---|
| Symbiosis (E) | 25.21 (0.000) | **12.73 (0.000)** | 0.00 (0.96) | 0.27 (0.60) |
| Grazing (G) | 21.16 (0.000) | 8.44 (0.003) | 0.21 (0.63) | 0.18 (0.67) |
| Flower head Biomass (B) | 0.09 (0.75) | 0.02 (0.87) | 0.80 (0.36) | 1.23 (0.26) |
| E × G | **6.01 (0.01)** | 3.89 (0.05) | 0.42 (0.51) | 0.54 (0.45) |
| E × B | 0.41 (0.52) | 0.32 (0.56) | 0.19 (0.66) | 0.71 (0.39) |
| G × B | 1.82 (0.17) | **6.00 (0.01)** | 0.73 (0.39) | 0.93 (0.33) |
| E × G × B | 2.07 (0.14) | 0.01 (0.89) | 0.92 (0.33) | 0.88 (0.34) |
| Model | Poisson | Poisson | quasi-Poisson | Neg. Binom |
| Disp. parameter | 3.0 (p 0.002) | 0.51 (p 0.84) | 2.66 (±1.02) | 1.36 (±0.44) |
| Symbiosis (E) | 0.01 (0.93) | 0.04 (0.84) | 1.83 (0.17) | 2.85 (0.09) |
| Grazing (G) | 1.50 (0.22) | 0.08 (0.77) | 1.13 (0.28) | 2.21 (0.13) |
| Open flower (F) | 15.21 (0.000) | 13.31 (0.000) | 7.39 (0.006) | 0.40 (0.52) |
| E × G | 1.92 (0.16) | **3.94 (0.04)** | 2.08 (0.14) | 0.00 (0.95) |
| E × F | 0.46 (0.49) | 1.51 (0.22) | 1.23 (0.26) | 0.00 (0.99) |
| G × F | 1.27 (0.26) | **5.17 (0.02)** | 1.81 (0.17) | 0.27 (0.60) |
| E × G × F | **11.14 (0.000)** | 2.54 (0.11) | **4.47 (0.03)** | 2.52 (0.11) |
| Model | Poisson | Poisson | quasi-Poisson | Poisson |
| Disp. parameter | 1.2 (p 0.26) | 1.2 (p 0.27) | 1.3 (±0.53) | 2.1 (p 0.03) |
| Symbiosis (E) | 8.85 (0.002) | 6.98 (0.008) | 2.07 (0.14) | 1.91 (0.16) |
| Grazing (G) | 20.93 (0.000) | 9.77 (0.001) | 5.17 (0.02) | 2.23 (0.13) |
| Open flower head at end (O) | 0.17 (0.67) | 0.00 (0.96) | 0.30 (0.58) | 0.82 (0.36) |
| E × G | 20.10 (0.000) | **12.23 (0.000)** | 9.33 (0.002) | 1.40 (0.23) |
| E × O | 1.03 (0.31) | 0.24 (0.62) | 1.69 (0.19) | 0.28 (0.59) |
| G × O | 10.05 (0.001) | **7.39 (0.006)** | 2.32 (0.12) | 1.86 (0.17) |
| E × G × O | **4.91 (0.02)** | 0.53 (0.46) | **10.84 (0.000)** | 1.43 (0.23) |
| Model | Poisson | Poisson | Poisson | Neg. Binom |
| Disp. parameter | 0.69(p 0.69) | 0.63 (p 0.74) | 1.44 (p 0.17) | 1.0 (±0.0007) |

This mechanistic models were run for the visits groups with influenced by the symbiosis, the grazing or the interaction between both factors (See Table 5). The mechanistic factors in the models were flower head biomass (g.thistle plant$^{-1}$), maximum number of open flower heads and open flower heads at the end of the experiment. Each mechanistic model included the factor and the interactions as predictor in order to test their importance as a possible mechanism through which the effects may spread (see Fig. 3 y 4). The table shows the Chi square statistic from the analysis of deviance (Type II tests with degrees of freedom associated Chisq$_{1,10}$ for natural grassland and Chisq$_{1,8}$ improved grassland) and the probability value (*p*-value). Below the factors the table shows the family or error distribution (Poisson, negative binomial or quasi-poisson) and the corresponding dispersion parameter. Bold numbers correspond to significant effects (*p*-values< 0.05)

plants and soil community in the new range can drive the invasion process (but see, Suding et al. 2013). Currently, annual ryegrass as well as thistle used in our experimental system, may be considered exotic species naturalized in Pampean grassland. Although we did not focus on ecological processes like invasion, our results suggest that the impact of these invasive species on soil and aerial communities structures depended on the traits of the new range that they explored. This was evident as the impact of the legacy differed critically between the natural grassland soil with no evidence of legacy effects and the improved grassland soil with important legacy on thistle and its floral visitors. These results supported the idea that systems under intensive management and disturbance are less fertile, more fragile and susceptible to changes which spread along generations and multiple interactions. From these results, we feel encouraged to go deeper in this field in order to better understand how plant invasion indirectly affects other communities and the ecological meaning of this impact.

We conclude that legacy effects induced by grass-endophyte symbiosis and grazing can mediate trophic and non-trophic interaction in other subsequent plant species depending on the history of soil use. This study shows that specific aboveground interactions between annual plants, fungal endophyte symbionts and grazers can even extend beyond a single growth period, emphasizing the complexity of ways by which plants interact in the present and the past and, their consequences. In particular, annual ryegrass is naturally associated with *E. occultans* endophyte and with long history of cattle in European grasslands and pastures. It is likely that these simultaneous interactions have a major influence in shaping these grassland and pastures communities (Bardgett et al. 1998). Undoubtedly, the observed soil legacies induced by grazing and symbiosis can play a role in ecological processes such as succession and invasion, but tests under more complex conditions are required. Besides, studies to determine specific compounds or soil organisms involved in the legacies observed are needed. The contribution of this study arises from connecting simultaneous interactions effects on soil conditions and their influence on trophic and non-trophic interactions during the subsequent generations. Finally, we consider that research projects with a broader and more complex framework, instead of single interactions, are an actual opportunity to better understand the rules that structure communities and ecological processes.

# References

Arechavaleta M, Bacon CW, Plattner RD et al (1992) Accumulation of ergopeptide alkaloids in symbiotic tall fescue grown under deficits of soil water and nitrogen fertilizer. Appl Environ Microbiol 58:857–861

Bais HP, Weir TL, Perry LG et al (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266. doi:10.1146/annurev.arplant.57.032905.105159

Bais HP, Broeckling CD, Vivanco JM (2008) Root exudates modulate plant—microbe interactions in the rhizosphere.

Second. Metab. soil Ecol. Springer Berlin Heidelber, pp 241–252

Bardgett RD, Wardle DA, Yeates GW (1998) Linking aboveground and below-ground interactions: how plant responses to foliar herbivory influence soil organisms. Soil Biol Biochem 30:1867–1878. doi:10.1016/S0038-0717(98)00069-8

Bates D, Maechler M, Bolker B et al (2014) lme4: Linear mixed-effects models using Eigen and S4 Maintainer. 1:1–7

Bever JD, Westover KM, Antonovics J (1997) Incorporating the soil community into plant population dynamics: the utility of the feedback approach. J Ecol 85:561–573

Bolker B, Team RDC (2014) bbmle: tools for general maximum likelihood estimation. R Packag. version 1.0.17

Bolker BM, Brooks ME, Clark CJ et al (2009) Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol Evol (Personal Ed 24:127–35. doi: 10.1016/j.tree.2008.10.008

Bolker B, Skaug H, Magnusson A, Nielsen A (2013) Getting started with the glmmADMB package. 1–17

Brown VK, Gange AC (1992) Secondary plant succession: how is it modified by insect herbivory? Vegetatio 101:3–13

Bultman TL, Bell G, Martin WD (2004) A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. Ecology 85:679–685

Cahill JF, Elle E, Smith GR, Shore BH (2008) Disruption of a belowground mutualism alers interactions between palnts and their floral visitors. Ecology 89:1791–1801

Callaway RM, Ridenour WM (2004) Novel weapons : invasive success and the evolution of increased competitive ability. Front Ecol Environ 2:436–443

Callaway RM, Walker LR (1997) Competition and facilitation: a synthetic approach to interactions in plant communities. Ecology 78:1958–1965

Callaway RM, Cipollini D, Barto K et al (2008) Novel weapons: invasive plant suppresses fungal mutualists in America but not in its native Eupore. Ecology 89:1043–1055

Casanova-Katny MA, Torres-Mellado GA, Palfner G, Cavieres LA (2011) The best for the guest: high Andean nurse cushions of *Azorella madreporica* enhance arbuscular mycorrhizal status in associated plant species. Mycorrhiza 21:613–622. doi:10.1007/s00572-011-0367-1

Casas C, Omacini M, Montecchia MS, Correa OS (2011) Soil microbial community responses to the fungal endophyte Neotyphodium in Italian ryegrass. Plant Soil 340:347–355. doi:10.1007/s11104-010-0607-8

Cheplick GP, Faeth SH (2009) Ecology and evolution of the grass-endophyte symbiosis. Oxford University ress, New York

Clay K (1988) Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology 69:10–16. doi:10.2307/1943155

Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Science 285(5434):1742–1744

Clay K, Schardl CL (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat 160:99–127

Cragg RG, Bardgett RD (2010) How changes in soil faunal diversity and composition within a trophic group influence decomposition processes. Soil Biol 33:2073–2081

Culman SW, DuPont ST, Glover JD et al (2010) Agriculture, ecosystems and environment long-term impacts of high-

input annual cropping and unfertilized perennial grass production on soil properties and belowground food webs in Kansas, USA. Agric Ecosyst Environ 137:13–24. doi:10.1016/j.agee.2009.11.008

De Battista J (2005) Neotyphodium research and aplication in South America. In: Roberts CA, West CP, Spiers DE (eds) Neotyphodium cool. Grasses, 1st edn. Blackwell Publishing Ldt, Oxford, pp 65–71

De Deyn GB, Raaijmakers CE, Zoomer HR et al (2003) Soil invertebrate fauna enhances grassland succession and diversity. Nature 422:711–713

Desrochers AM, Bain JF, Warwick SI (1988) The biology of Canadian weeds: *Carduus nutans* L. and *Carduus acanhoides* L. Can J Plant Sci 68:1053–1068

R Development Core Team (2007) R: a language and environment for statistical computing. Vienna, Austria

Druille M, Omacini M, Golluscio RA, Cabello MN (2013) Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. Appl Soil Ecol 72:143–149. doi:10.1016/j.apsoil.2013.06.011

Eisenhauer N, Reich PB (2012) Above- and below-ground plant inputs both fuel soil food webs. Soil Biol Biochem 45:156–160. doi:10.1016/j.soilbio.2011.10.019

Feldman SR, Refi RO (2006) Cambios en la composición florística de un pastizal pampeano bajo diferentes prácticas de manejo. Cienc Investig Agrar 33:109–116

Fox J, Weisberg S (2011) An {R} companion to applied regression

Gange AC, Smith AK (2005) Arbuscular mycorrhizal fungi influence visitation rates of pollinating insects. Ecol Entomol 30:600–606

Gange AC, Brown VK, Apllin DM (2003) Multitrophic links between arbuscular mycorrhizal fungi and insect parasitoids. Ecol Lett 6:1051–1055. doi:10.1046/j.1461-0248.2003.00540.x

García Parisi PA, Casas C, Gundel PE, Omacini M (2012) Consequences of grazing on the vertical transmission of a fungal Neotyphodium symbiont in an annual grass population. Austral Ecol 37:620–628. doi:10.1111/j.1442-9993.2011.02325.x

Giordani CA (1973) Método para aprovechamiento de pasturas. Revista CREA 8:1–19

Giufa M, Núñez JA (1992) Foraging by honeybees on *Carduus acanthoides*: pattern and efficiency. Ecol Entomol 17:326–330

Giufa M, Núñez JA (1993) Efficient floret inspection by honeybees in capitula of *Carduus acanthoides*. Ecol Entomol 18:116–122

Goulson D (1999) Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. Perspect Plant Ecol Evol Syst 2:185–209

Gundel PE, Martínez-Ghersa MA, Garibaldi LA, Ghersa CM (2009) Viability of Neotyphodium endophytic fungus and endophyte-infected and noninfected *Lolium multiflorum* seeds. Botany 87:88–96. doi:10.1139/B08-119

Gundel PE, Pérez LI, Helander M, Saikkonen K (2013) Symbiotically modified organisms: nontoxic fungal endophytes in grasses. Trends Plant Sci 18:420–427. doi:10.1016/j.tplants.2013.03.003

Hamilton WE III, Frank DA (2001) Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecology 82:2397–2402

Hartley SE, Gange AC (2009) Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. Annu Rev Entomol 54:323–342. doi:10.1146/annurev.ento.54.110807.090614

Hol WHG, de Boer W, Termorshuizen AJ et al (2010) Reduction of rare soil microbes modifies plant- herbivore interactions. Ecol Lett 13:292–301. doi:10.1111/j.1461-0248.2009.01424.x

Iannone LJ, Novas V, Young CA et al (2012) Endophytes of native grasses from South America: biodiversity and ecology. Fungal Ecol 5:357–363. doi:10.1016/j.funeco.2011.05.007

Joosten L, Mulder PPJ, Klinkhamer PGL, van Veen JA (2009) Soil-borne microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. Plant Soil 325:133–143. doi:10.1007/s11104-009-9963-7

Kardol P, Cornips NJ, van Kempen MML et al (2007) Microbe-mediated plant–soil feedback causes historical contingency effects in plant community assembly. Ecol Monogr 77:147–162. doi:10.1890/06-0502

Koh S, Vicari M, Ball JP et al (2006) Rapid detection of fungal endophytes in grasses for large-scale studies. Funct Ecol 20:736–742. doi:10.1111/j.1365-2435.2006.01150.x

Kostenko O, van der Voorde TFJ, Mulder PPJ et al (2012) Legacy effects of aboveground-belowground interactions. Ecology 15:813–821. doi:10.1111/j.1461-0248.2012.01801.x

Kourtev PS, Ehrenfeld JG, Häggblom M (2002) Exotic plant species alter the microbial community structure and function in the soil. Ecology 83:3152–3166

Kulmatiski A, Beard KH, Stevens JR, Cobbold S (2008) Plant – soil feedbacks: a meta-analytical review. Ecol Lett 11:980–992. doi:10.1111/j.1461-0248.2008.01209.x

Lehtonen P, Helander M, Wink M et al (2005) Transfer of endophyte-origin defensive alkaloids from a grass to a hemiparasitic plant. Ecol Lett 8:1256–1263. doi:10.1111/j.1461-0248.2005.00834.x

Leuchtmann A, Bacon CW, Schardl CL et al (2014) Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106:202–215. doi:10.3852/13-251

Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci 40:923–940

Mandák B, Zákravský P, Kor D et al (2009) Low population differentiation and high genetic diversity in the invasive species Carduus acanthoides L. (Asteraceae) within its native range in the Czech Republic. Biol J Linn Soc 98:596–607

Matthews JW, Clay K, Feb N (2001) Influence of fungal endophyte infection on plant-soil feedback and community interactions. Ecology 82:500–509

Mikola J, Yeates GW, WARDLE DA et al (2001) Response of soil food-web structure to defoliation of different plant species combinations in an experimental grassaland community. Soil Biol Biochem 33:205–214

Mikola J, Nieminen M, Ilmarinen K, Vestberg M (2005) Belowground responses by AM fungi and animal trophic groups to repeated defoliation in an experimental grassland community. Soil Biol Biochem 37:1630–1639. doi:10.1016/j.soilbio.2005.01.027

Mills KE, Bever JD (1998) Maintenance of diversity within plant communities: soil pathogens as agents of negative feedback. Ecology 79:1595–1601

Moore JC, Berlow EL, Coleman DC et al (2004) Detritus, trophic dynamics and biodiversity. Ecol Lett 7:584–600. doi:10.1111/j.1461-0248.2004.00606.x

Novas VM, Iannone LJ, Godeas AM, Cabral D (2008) Positive association between mycorrhiza and foliar endophytes in *Poa bonariensis*, a native grass. Mycol Prog 8:75–81. doi:10.1007/s11557-008-0579-8

Ohgushi T (2005) Indirect interaction webs: herbivore-induced effects through trait change in plants. Annu Rev Ecol Syst 36:81–105. doi:10.1146/annurev.ecolsys.36.091704.175523

Omacini M, Chaneton EJ, Ghersa CM, Müller CB (2001) Symbiotic fungal endophytes control insect host-parasite interaction webs. Nature 409:78–81. doi:10.1038/35051070

Omacini M, Chaneton EJ, Ghersa CM, Otero P (2004) Do foliar endophytes affect grass litter decomposition? A microcosm approach using *Lolium multiflorum*. Oikos 104:581–590. doi:10.1111/j.0030-1299.2004.12915.x

Omacini M, Chaneton EJ, Ghersa CM (2005) A hierarchical framework for understanding the ecosystem consequences of endophyte-grass symbioses. In: Robert CA, West CP, Spiers DE (eds) Neothyphodium cool seas. Blackwell Publishing Ldt, Grasses, pp 141–162

Omacini M, Eggers T, Bonkowski M et al (2006) Leaf endophytes affect mycorrhizal status and growth of co-infected and neighbouring plants. Funct Ecol 20:226–232. doi:10.1111/j.1365-2435.2006.01099.x

Omacini M, Chaneton EJ, Bush L, Ghersa CM (2009) A fungal endosymbiont affects host plant recruitment through seed- and litter-mediated mechanisms. Funct Ecol 23:1148–1156. doi:10.1111/j.1365-2435.2009.01582.x

Pietikäinen A, Mikola J, Verstbers M, Setälä H (2009) Defoliation effects on *Plantago lanceolata* resource allocation and soil decomposers in relation to AM symbiosis and fertilization. Soil Biol Biochem 41:2328–2335

Pinheiro J, Bates D (2000) Mixed-effects models in S and S-PLUS

Pinheiro J, Bates D, DebRoy S et al (2009) nlme: linear and nonlinear mixed effects models

Ponce MA, Bompadre MJ, Scervino JM et al (2009) Flavonoids, benzoic acids and cinnamic acids isolated from shoots and roots of Italian rye grass (*Lolium multiflorum* Lam.) with and without endophyte association and arbuscular mycorrhizal fungus. Biochem Syst Ecol 37:245–253. doi:10.1016/j.bse.2009.03.010

Reynolds HL, Packer A, Bever JD, Clay K (2008) Grassroots ecology: plant-microbe-soil interactions as drivers of plant community structure and dynamics. Ecology 84:2281–2291

Rodríguez AM, Jacobo EJ (2010) Glyphosate effects on floristic composition and species diversity in the Flooding Pampa grassland (Argentina). Agric Ecosyst Environ 138:222–231. doi:10.1016/j.agee.2010.05.003

Rudgers JA, Orr S (2009) Non-native grass alters growth of native tree species via leaf and soil microbes. J Ecol 97:247–255. doi:10.1111/j.1365-2745.2008.01478.x

Rudgers JA, Holah J, Orr SP, Clay K (2007) Forest succession suppressed by an introduced plant-fungal symbiosis. Ecology 88:18–25

Schardl CL, Young CA, Faulkner JR et al (2012) Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecol 5:331–344. doi:10.1016/j.funeco.2011.04.005

Scherber C, Eisenhauer N, Weisser WW et al (2010) Bottom-up effects of plant diversity on multitrophic interactions in a biodiversity experiment. Nature 468:553–556. doi:10.1038/nature09492

Schipper LA, Degens BP, Sparling GP, Duncan LC (2001) Changes in microbial heterotrophic diversity along five plant successional sequences. Soil Biol Biochem 33:2093–2103

Schon NL, Mackay AD, Yeates GW, Minor MA (2010) Separating the effects of defoliation and dairy cow treading pressure on the abundance and diversity of soil invertebrates in pastures. Appl Soil Ecol 46:209–221. doi:10.1016/j.apsoil.2010.08.011

Shafir S, Wiegmann DD, Smith BH, Real LA (1999) Risk-sensitive foraging: choice behaviour of honeybees in response to variability in volume of reward. Anim Behav 57:1055–1061

Shiba T, Sugawara K, Arakawa A (2011) Evaluating the fungal endophyte *Neotyphodium occultans* for resistance to the rice leaf bug, *Trigonotylus caelestialium*, in Italian ryegrass, *Lolium multiflorum*. Entomol Exp Appl 141:45–51. doi:10.1111/j.1570-7458.2011.01162.x

Siegrist JA, McCulley RL, Bush LP, Phillips TD (2010) Alkaloids may not be responsible for endophyte-associated reductions in tall fescue decomposition rates. Funct Ecol 24:460–468. doi:10.1111/j.1365-2435.2009.01649.x

Sorensen LI, Mikola J, Kytöviita M (2008) Defoliation effects on plant and soil properties in an experimental low arctic grassland community – the role of plant community structure. Soil Biol Biochem 40:2596–2604. doi:10.1016/j.soilbio.2008.06.021

Sosa O, Martín B, Zerpa G, Montico S (2006) Evolución anual de la broza en diversas undidades forrajeras de un pastizal natural. Rev Cient Agropecu 10:33–45

Striker GG, Insausti P, Grimoldi AA, Leon RJC (2006) Root strength and trampling tolerance in the grass *Paspalum dilatatum* and the dicot *Lotus glaber* in flooded soil. Funct Ecol 20:4–10. doi:10.1111/j.1365-2435.2006.01075.x

Suding KN, Harpole WS, Fukami T et al (2013) Consequences of plant – soil feedbacks in invasion. J Ecol 101:298–308. doi:10.1111/1365-2745.12057

Sugawara K, Inoue T, Yamashita M, Ohkubo H (2006) Distribution of the endophytic fungus, *Neotyphodium occultans* in naturalized Italian ryegrass in western Japan and its production of bioactive alkaloids known to repel insect pests. Grassl Sci 52:147–154. doi:10.1111/j.1744-697X.2006.00060.x

Tognetti PM, Chaneton EJ, Omacini M et al (2010) Exotic vs. native plant dominance over 20 years of old-field succession on set-aside farmland in Argentina. Biol Conserv 143:2494–2503. doi:10.1016/j.biocon.2010.06.016

Van der Putten WH, Bardgett RD, Bever JD et al (2013) Plant – soil feedbacks: the past, the present and future challenges. J Ecol 101:265–276. doi:10.1111/1365-2745.12054

Veen GFC, De Vries S, Bakker ES et al (2014) Grazing-induced changes in plant – soil feedback alter plant biomass allocation. Oikos 123:800–806. doi:10.1111/j.1600-0706.2013.01077.x

Vignale MV, Iannone LJ, Scervino M, Novas MV (2014) Efecto de los exudados de endofitos Epichloë sobre el desarrollo in vitro de HMA. XIII Congr. Argentino Micol. Primera Reunion de la Asociación Micológica Carlos Spegazzini, Buenos Aires, Argentina, p 1

Walkley A, Black IA (1934) An examination of the digestion method for determining organic carbon in soils: effect of variations in digestion conditions and of inorganic soil constituents. Soil Sci 63:251–263

Wardle DA, Yeates GW, Williamson W, Bonner KI (2003) The response of a three trophic level soil food web to the identity and diversity of plant species and functional groups. Oikos 102:45–56

Wyatt R (1982) Inflorescence architecture: how flower number, arrangement, and phenology affect pollination and fruit-set. Am J Bot 585–594