1 ***Epichloë* endophytes affect the nutrient and fiber content of *Lolium***
2 ***perenne* regardless of plant genotype**

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15 **Abstract**

16 *Background and Aims:* *Epichloë* endophytes inhabit aerial grass tissues but they can modify belowground
17 processes that might affect host nutrient balance. We aimed to determine the effects of endophyte status
18 (E+=endophyte-infected; E-=non-infected) and three *Epichloë* morphotypes (M1,M2,M3) on growth and
19 nutrient content of a heterogeneous set of naturally infected asymptomatic plants of *Lolium perenne*. In
20 addition, plant parameters were compared between asymptomatic E+ and plants with choke disease.

21 *Methods:* A field experiment was conducted with 194 plants obtained from six natural populations (97E+,
22 97E-). For each E+ plant, the endophyte morphotype it hosted was known.

23 *Results:* Endophyte-infected plants had significantly lower P, Ca, S, B, neutral detergent fiber and lignin
24 contents, and higher Mn and digestibility than E-, independently of plant origin. Biomass production was
25 affected by plant origin but not by endophytes. No effect of *Epichloë* morphotypes in any parameter was
26 found. However, asymptomatic E+ and choke diseased plants differed in nutrients, fibers, and
27 digestibility.

28 *Conclusions:* An endophyte effect was detected in nutrient and fiber content, in spite of the heterogeneous
29 constitution of the plant and fungal material used. The results obtained indicate that *Epichloë* may affect
30 above and possibly underground processes involved in nutrient absorption, as well as plant quality, what
31 may potentially affect litter decomposition processes.

32
33 **Key words**

Epichloë endophytes; choke disease; mineral content; fibers; digestibility; perennial ryegrass

Introduction

The nutrient content of grasses can be affected by soil properties, climate, plant factors (species, genotype, phenological stage), and also by symbiotic relationships (Jones and Thomas 1987; Nelson and Moser 1994). Symbiotic associations of plants with fungi are ubiquitous in nature. For instance, those occurring with arbuscular mycorrhizae have been known for long and positively affect nutrient acquisition in plant hosts (Jeffries et al. 2003). More recent studies indicate that most plant species harbor a rich and diverse mycobiota composed of endophytes, fungi that infect plant tissues without causing disease symptoms. An increasing number of examples is showing that some symbioses of plants with endophytes are beneficial, providing increased plant growth and stress tolerance (Rodriguez et al. 2009).

Today *Epichloë* (= *Neotyphodium*) is probably the most studied genus of fungal endophytes (Saikkonen et al. 2006; Leuchtman et al. 2014; Tadych et al. 2014). Hyphae of *Epichloë* endophytes colonize systemically the intercellular space of aerial organs like leaves and stems of the host plant, but there are differences among species of this genus in their life cycles. Some species (e.g. *Epichloë festucae* var. *lolii* = *Neotyphodium lolii*) are transmitted vertically to the seeds, this type of spread is asexual and clonal, and the same fungal genotype that infects a host plant will be transmitted to its seeds. Other *Epichloë* species are exclusively sexual (e. g. *Epichloë typhina*), they are not vertically transmitted to seeds and develop a tubular stroma that wraps the inflorescence primordium in host plant reproductive stems, inhibiting panicle development, a disorder known as “choke disease” of grasses. Ascospores released from fertilized stromata of *E. typhina* can infect florets of other healthy plants, giving rise to infected seeds (Clay and Schardl 2002).

Although *Epichloë* endophytes are only present in aerial parts, they can alter belowground components and processes. Changes in root biomass and morphology, root exudates and mycorrhizal colonization have been observed in *Epichloë* infected plants (reviewed by Omacini et al. 2012). Thus, endophyte-mediated alterations of roots can affect host plant nutrition. For instance, roots of infected tall fescue plants release phenolic compounds with Fe^{3+} reducing and P solubilizing activity (Malinowski et al. 1998; Malinowski and Beleski 1999), and endophyte-infected plants showed an increased capability to bind copper in the rhizosphere (Malinowski et al. 2004). An increase of phenolic compounds in roots of endophyte-infected plants have been reported in several other grass species (Ponce et al. 2009; Vazquez

de Aldana et al. 2011), increasing the competitive ability of infected versus non-infected plants due to root-mediated allelopathic interactions (Shutherland et al. 1999; Vazquez de Aldana et al. 2013b). Changes in plant nutrient content might alter the quality of litter produced by senescent grass shoots, which is one of the main factors that control decomposition rates (Meier and Bowman 2008). Therefore, *Epichloë* endophytes might modify litter decomposition rates, and consequently nutrient cycling, by altering the quality of the litter produced by infected plants, and/or by changing the composition of invertebrate detritivores and microbial decomposers (Franzluebbers et al. 1999; Omacini et al. 2004; Lemons et al. 2005).

While research on the alkaloid-mediated antiherbivore resistance or drought performance of endophyte infected plants is abundant (Bush et al. 1997; Malinowski and Belesky 2000; Clay and Schardl 2002; Kuldau and Bacon 2008; Saikkonen et al. 2013), the effects of endophytes on plant nutrient content have been much less explored. Several studies have shown that endophytes modify the nutrient status of host plants like tall fescue (*Festuca arundinacea* = *Schedonorus arundinaceus*) (Lyons et al. 1990; Malinowski et al. 1998; Malinowski and Belesky 1999; Rahman and Saiga 2005), *Festuca rubra* (Zabalgogezcoa et al. 2006; Vazquez de Aldana et al. 2013a), or *Achnatherum sibiricum* (Li et al. 2012). However, in spite of the economic importance of perennial ryegrass (*Lolium perenne*) as a forage crop, little is known about the effect of *Epichloë* endophytes on its nutrient content. To our knowledge, there is only one report about the effect of *Epichloë* infection on the content of several nutrients in ryegrass (Ren et al. 2009). Other reports focus on single elements like nitrogen (Lewis et al. 1996), phosphorus (Ren et al. 2007), or heavy metals (Malinowski et al. 2004; Monnet et al. 2005; Ren et al. 2006). The effect of *Epichloë* endophytes on fiber content and digestibility of *L. perenne* has also received scarce attention (Oliveira et al. 2004). Furthermore, all of these studies were carried out with one or two cultivars of *L. perenne* and their corresponding *Epichloë* genotypes; therefore, very limited variability among plants and endophytes was included.

Lolium perenne is one of the most important forage and turf grasses in the world. As a forage grass, it can produce good yields, has high digestibility, and withstands repeated defoliation. In addition, *L. perenne* can tolerate a broad range of environmental conditions, including widely fluctuating temperatures and soil moisture deficits. Its wide adaptability and good agronomic performance have contributed to its ecological success and worldwide spread (Peeters 2004). Perennial ryegrass is often infected by *Epichloë* endophytes (Hume and Sewell 2014), and in natural populations phenotypic

diversity is found among fungal isolates (Bony et al. 2001; Soto-Barajas et al. 2013). Some of these distinct *Epichloë* phenotypes correspond to particular rDNA nucleotide sequences, and might belong to distinct taxa (Soto-Barajas et al. 2013). In fact, at least three *Epichloë* taxa have been identified in plants of *L. perenne*: *E. festucae* var. *lolii*; *E. typhina*, the causal agent of choke disease; and an asexual hybrid of these two endophytes designated LpTG-2 (Moon 1999).

Given the phenotypic and possible species diversity of *Epichloë* endophytes found in wild populations of *L. perenne* (Moon 1999; Bony et al. 2001; Soto Barajas et al. 2013), our hypothesis was that the type of *Epichloë* endophyte hosted might influence the nutrient acquisition from soil, and this could alter the nutrient content of ryegrass plants. A field experiment was conducted with a set of perennial ryegrass plants naturally infected by different *Epichloë* endophytes and non-infected plants. These plants had been collected at six natural populations in different habitats. We aimed to identify endophyte effects on growth, mineral and fiber contents in a genetically heterogeneous set of *L. perenne* plants and *Epichloë* endophytes. Specific questions were to determine whether *Epichloë* endophytes affect plant tissue chemistry independently of plant origin, and to determine whether diverse *Epichloë* endophytes have different effects on the tissue chemistry of their plant hosts.

Materials and Methods

Plant material

In the spring of 2012, plants of *L. perenne* were collected at six locations in different habitats in Western Spain (Table 1). These habitats were not agricultural, and clumps or individual plants of perennial ryegrass occurred interspersed with other plant species. At each location, about 50 plants were collected leaving a distance of at least 10 m between each pair of samples. In some locations, plants with symptoms of choke disease were sampled (Fig. 1d). The plants were transported to the Institute of Natural Resources and Agrobiology in Salamanca (IRNASA-CSIC) and transplanted to 2 L pots containing a mixture of perlite and peat moss (1:1, v/v). The pots were kept outdoors in a wirehouse, watered regularly, and fertilized once a year with a liquid commercial fertilizer.

In a previous work we reported that on average, 44% of the plants of *Lolium perenne* were infected by *Epichloë* endophytes in several Spanish natural populations, including some whose plants were used for this study (Table 1) (Soto-Barajas et al. 2013). The endophytes isolated from asymptomatic plants in that study were classified into three morphotypes based in morphological characters observed in

potato dextrose agar cultures. The most common morphotype was M1, characterized by white cultures with strongly aggregated 'brain-like' mycelium, and the slowest growth rate of the three morphotypes (Fig. 1a). Isolates of the M2 morphotype were the least abundant, having white, cottony aerial mycelium, and the fastest growth rate (Fig. 1b). The M3 morphotype had tan, flat and smooth mycelium, and an intermediate growth rate (Fig. 1c). Endophytes isolated from symptomatic plants with choke disease, caused by *Epichloë typhina* (Fig. 1d) had M2 morphology, and were designated as M2S. Furthermore, rDNA nucleotide sequences of M2 and M2S morphotypes were identical to those of *Epichloë typhina*, while sequences of M1 and M3 morphotypes were identical to *E. festucae* sequences (Soto-Barajas et al. 2013).

Field experiment design

A field experiment was carried out with a total of 194 plants of *L. perenne* obtained from the six locations described in Table 1; 97 plants were naturally infected with *Epichloë* (E+), and 97 were not (E-). For each E+ plant, the morphotype it hosted (M1, M2, M3 and M2S) was known, and both asymptomatic plants bearing M1, M2 and M3 *Epichloë* morphotypes, and symptomatic plants with choke disease (M2S) were considered together as 'morphotype' factor. *Lolium perenne* is a cross-pollinated species in which genetic diversity occurs even within commercial cultivars (Peeters 2004). For instance, McNeilly and Roose (1984) reported that 10-year-old pastures originally sown with two cultivars of *L. perenne* could contain 40 to 50 different genotypes per 0.25 m². Therefore, in this field experiment we could expect to have a very heterogeneous set of plant genotypes because the plants were collected from different locations and habitats in natural populations, where they occurred in sympatry with other plant species, and sampled individuals were spatially distanced.

The experimental design was based on having the same number of E+ and E- plants from each location; however, the number of plants differed among locations because we used all E+ plants obtained at each location in order to include all the morphotype variability available in the experiment (Table 2). On October 2013, the ryegrass plants were transplanted in a randomized design, at the experimental farm Muñovela (Salamanca, Spain; 40°54'19" N, 5°46'28" W; 780 masl; annual precipitation 372 mm, and mean annual temperature 12.7 °C) in a clay eutric chromic cambisol (FAO/UNESCO 1998) with neutral pH on the surface, decreasing slightly with depth. This soil contains low concentrations of organic matter (1.26 %), total nitrogen (0.08 %), available phosphorus (16.6 mg kg⁻¹), potassium (107 mg kg⁻¹), and

calcium (984 mg kg⁻¹). Each plant was set at a distance of 50 cm from its neighbors. Plants were watered during their establishment in October but not thereafter. During the experiment, the plants were not fertilized and the plot was maintained free of weeds.

On May 19 2014, the plants were harvested at the flowering stage, cutting them 5.0 cm above the soil surface. Before harvesting, the number of tillers was counted in all plants, including those whose reproductive stems bore *E. typhina* stromata. All samples were dried at 60 °C in a forced air oven during 48 hours and the dry matter yield was determined.

Chemical analysis

All ryegrass samples were ground using a hammer mill (Fritsch 15303) fitted with a 0.5 mm screen. Each sample was analyzed for total N by digital colorimetry with a segmented flow system (AutoAnalyzer AA3, Bran Luebbe). For mineral content, plant samples were calcined (450 °C), for 8 hours, and ashes were dissolved in HCl:HNO₃:H₂O (1:1:8). The concentrations of P, K, Ca, Mg, S, Na, Mn, Zn, Cu, B, Mo and Co were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Varian 720-ES).

Neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin and dry matter digestibility (DMD) were evaluated using the filter bag technique, with an automated fiber analyzer Ankom A2000, based on the analytical method of Goering and Van Soest (1970).

Statistical analysis

A two-way ANOVA was performed to determine the effect of infection status (E+ and E-) and plant origin (CR, LVA, LVE, POR, TAB and VAF) on growth and chemical composition of *L. perenne*. For this statistical analysis, plants with choke disease were not included because their scarce biomass production was visually evident, and these plants were found only in one half of the locations (Table 2). When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test, adequate for the unbalanced design.

The effect of *Epichloë* morphotype on dry matter production and the number of tillers was assessed on asymptomatic infected plants hosting M1, M2 and M3 morphotypes by means of one-way ANOVA. When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test.

To evaluate the effect of each *Epichloë* morphotype on chemical composition of ryegrass, both asymptomatic plants (M1, M2, and M3) and plants with choke disease (M2S) were considered. When a significant effect was detected, differences between pairs of means were assessed using the Bonferroni test. SigmaPlot software version 13.0 (Systat Software, San Jose, CA, USA) was used for all the statistical analyses.

Results

Plant biomass and tiller number

The aboveground plant biomass, composed of leaves, stems and inflorescences, was measured at the flowering stage. The ANOVA did not detect a significant effect of endophyte infection on dry weight of ryegrass ($P=0.442$) neither an interaction between endophyte and plant origin ($P=0.126$). However, the location of origin of plants affected the dry matter yield ($P<0.001$). Plants from LVE, VAF and CR had the highest yield, and those from POR had the lowest dry weight (Fig. 2a).

The ANOVA detected a significant effect of the interaction between infection status and plant origin on the number of tillers ($P=0.039$). This effect was expressed only in plants from TAB, with a significantly higher tiller production in E⁻ (124.8 ± 13.6) than in E⁺ (63.5 ± 11.6) plants; in the remaining populations there were no significant differences in tiller number between E⁺ and E⁻ plants (Fig. 2b).

The effect of each endophyte morphotype of E⁺ asymptomatic plants on dry matter yield and tiller number was not significant ($P=0.411$ and $P=0.626$ respectively). Thus differences among plants infected with different endophyte morphotypes in dry matter production (M1= 50.9 ± 6.99 g plant⁻¹; M2= 49.8 ± 14.92 g plant⁻¹; M3= 36.24 ± 7.27 g plant⁻¹) and number of tillers (M1= 83 ± 7.4 ; M2= 88 ± 20.6 ; M3= 72 ± 9.7) were not statistically different.

In plants with choke disease, the average dry matter production was 9.5 ± 1.7 g plant⁻¹ and the number of tillers was 22 ± 10.4 .

Mineral content

The two-way ANOVA indicated a significant effect of endophyte and plant origin on the concentration of several mineral elements, with a significant interaction between those factors only for Co (see Table 3). The concentrations of P, Ca, S, and B were significantly greater in E⁻ than in E⁺ plants, and that of Mn was greater in E⁺ than in E⁻. The effect of plant origin was significant for the mineral

content of nine elements (N, P, K, Ca, Mg, Na, Zn, Cu and Co), but not for S, Mn, B and Mo. In general, plants from LVE and TAB had the highest concentrations of minerals and those from CR and VAF the lowest (Table 3). Regarding the interaction between endophyte and plant origin on Co, its concentration in E- plants ($0.414 \pm 0.036 \text{ g kg}^{-1}$) was significantly greater than in E+ ($0.251 \pm 0.038 \text{ g kg}^{-1}$), but only in plants from CR, in the other locations differences between E+ and E- were not statistically significant.

According to the one-way ANOVA with morphotype factor, asymptomatic plants infected with M1, M2 or M3 endophytes did not differ in their concentration of minerals (Table 4). On the other hand, plants with stromata (M2S) had significantly greater concentrations of N, K, Ca, Mg, S, Mn, Zn, Cu and B than those infected by asymptomatic endophytes (Table 4). The concentrations of Zn, Cu and B in plants with M3 were not statistically different from plants with M2S.

Fiber content and digestibility

Results of the two-way ANOVA showed a significant effect of endophyte status and plant origin on neutral detergent fiber (NDF), lignin, and dry matter digestibility (DMD), and the interaction between both factors was not significant for any parameter (Table 5). The E+ plants had lower NDF and lignin ($P=0.060$), and higher DMD than E- plants. Differences in acid detergent fiber (ADF) between E+ and E- plants were not statistically significant. The effect of plant origin was significant for NDF, lignin and DMD contents. Plants from CR had the lowest NDF and lignin and the greatest DMD; on the contrary, samples from TAB had the highest NDF and lignin, and the lowest DMD. Plants from LVA, LVE, POR and VAF had similar percentages of NDF, lignin and DMD, and they were intermediate between those found in plants from CR and TAB.

Considering the *Epichloë* morphotypes evaluated, a significant effect was detected in ADF and NDF, but plants infected with asymptomatic endophytes (M1, M2 and M3) did not differ in these parameters (Table 6). Plants with choke disease (M2S morphotype) had lower ADF and NDF contents than asymptomatic E+ plants.

Discussion

In spite of the potentially large plant variability included in our experiment, with plants from six wild populations, we detected a significant effect of *Epichloë* endophytes on the concentration of five mineral elements, fibers and DMD in shoots, and it was independent of the plant origin. This result

indicates that *Epichloë* can alter the nutrient balance in perennial ryegrass by decreasing the concentrations of P, Ca, S, B, NDF and lignin, and increasing Mn content and DMD in comparison to E⁻ plants. This occurred, even though dry matter yield was not significantly altered.

Under our experimental conditions, infected plants had lower P, Ca, S and B content than those not infected. Ren et al. (2009) also reported lower B content in E⁺ than in E⁻ shoots of one ryegrass cultivar, with no significant effects on the concentration of the other nine elements that they analyzed (P, K, Ca, Mg, Na, Mn, Fe, Zn, Cu), and Ren et al. (2007) found a slight increase in P content in sheaths of E⁻ ryegrass plants growing with a low P supply, as compared to E⁺. In agreement with our results, an absence of endophyte effect for total N (Lewis et al. 1996) and Zn concentration (Monnet et al. 2005) was reported when a single genotype of ryegrass was evaluated. In contrast, our results showed an endophyte-mediated increase in Mn, an essential element for some lignin-degrading enzymes such as Mn peroxidases (Fioretto et al. 2005). Increased nutrient content due to endophyte was also observed for Zn and Mo in perennial ryegrass (Malinowski et al. 2004), and for P, Ca or Zn in other grasses like *Festuca rubra* (Zabalgoeazcoa et al. 2006; Vazquez de Aldana et al. 2013a) and tall fescue (Malinowski et al. 1998, 2000).

Nutrient uptake in plants through the root surface is mainly mediated by three mechanisms: mass flow, diffusion, and root interception (Jungk 2002). Mass flow occurs when nutrients are transported to the root surface by the movement of water in the soil (i.e. percolation, transpiration, or evaporation). Any change produced by *Epichloë* endophytes in leaf area or tiller structure in their grass host could affect the evapotranspiration rate and consequently the nutrient acquisition. In plants with choke disease (MS2), high evaporation from the surface of stomata maximizes the flow of nutrients needed by the fungus for reproduction (White et al. 1997), and those elements which move by mass flow (N, Ca, Mg, S, Mn and Mo) were found in higher concentrations. In contrast, in asymptomatic plants (hosting M1, M2 and M3 *Epichloë* endophytes) concentrations of these elements were almost identical.

The movement of nutrients by diffusion in soils implicates concentration gradients. This kind of flow is particularly susceptible to any chemical change in the rhizosphere and is responsible for the acquisition of P, K, Fe and Zn. For instance, release of root exudates (phenolic compounds) in E⁺ tall fescue was linked to an increase of P uptake in aboveground tissues (Malinowski et al. 1998). In perennial ryegrass, Ren et al. (2007) found a greater content of total phenolic compounds in roots of E⁺ plants, but P concentration was superior in sheaths of E⁻ plants than in E⁺, which is in accordance with our results.

This suggests that root exudates of perennial ryegrass may be of different nature than those of tall fescue, and thus mechanisms of nutrient acquisition could be different in both grass species, as suggested Malinowski et al. (2004).

The third possible plant mechanism to acquire nutrients from soil solution is root interception. It occurs when a nutrient comes into physical contact with the root surface, and is responsible for a considerable amount of Ca uptake, and to lesser extent Mg, Zn and Mn (Jungk 2002). The plant-endophyte interaction can alter rhizospheric conditions that affect the density and activity of different soil organisms, like arbuscular mycorrhizal fungi (AMF) which in turn can enhance root interception. Thus, *Epichloë* infected plants have shown a reduction in colonization and sporulation of mycorrhizae, as compared to plants without *Epichloë* (Chu-Chou et al. 1992; Müller 2003; Omacini et al. 2006; Antunes et al. 2008; Liu et al. 2011). On the contrary, a positive association between *Epichloë* endophytes and AMF has been found in wild native grasses (Novas et al. 2005, 2012; Vignale et al. 2015). Although we did not analyze AMF in our ryegrass plants, a reduction in AMF in E+ plants could explain the lower P content found in plant tissue, but not the greater concentration of Mn, an element immobile in soil which can be mobilized by mycorrhizas. This suggests that other root endophytic and epiphytic species than AMF might be linked to nutrient absorption processes in plant roots (Sanchez-Marquez et al. 2010).

Effects of endophytes on plant fiber contents have received scarce attention. We found that the presence of *Epichloë* in ryegrass plants decreased NDF and lignin and increased DMD, with independence of plant origin. The NDF measures all the forage fiber (hemicellulose, cellulose and lignin), and a high NDF content decreases forage quality and intake by ruminants. Our results showed that *Epichloë* endophytes boost the digestibility of *L. perenne*, and this cannot be only attributed to the observed decrease in the lignin fraction. A similar endophyte effect on NDF and DMD was reported for ryegrass (Oliveira et al. 2004; Rasmussen et al. 2008) and red fescue (Zabalgogazcoa et al. 2006). In contrast, most studies done with tall fescue and *Epichloë coenophiala* (= *Neotyphodium coenophialum*) have shown no effect of endophyte infection in the concentration of NDF, ADF, lignin, or digestibility (Fritz and Collins 1991; Humphry et al. 2002; Johnson et al. 2012). The positive effect of *Epichloë* on digestibility of *L. perenne* could be explained, according to Rasmussen et al. (2012), because the intercellular endophytic hyphae promotes a carbohydrate hydrolysis to use cell wall components as a supplementary source of C, and this process might reduce the content of hemicellulose and NDF in plant.

Changes induced by *Epichloë* endophytes on fiber and mineral contents of *L. perenne* maintained in senescent shoots might alter the quality of litter and consequently its decomposition rate as well as recycling of nutrients. The degradation of cellulose and lignin, the most abundant components of litter, is slower than that of other plant components, thus, the lower lignin content of E+ ryegrass plants would imply faster litter decomposition compared to E- plants. On the other hand, litter Ca has been related to increased microbial activity (Berg et al. 2003), and the lower concentration of this element in E+ plants could imply slower decomposition rates. Other aspects of litter quality also affect decomposition processes. The N:P ratio of litter contributes to determine the relative importance of bacteria and fungi in the decomposition process, with low N:P ratios promoting bacteria and high values fungi (Güsewell et al. 2009). Thus, differences in P content due to endophyte could affect the structure of the mycobiota involved in decomposition. Experiments of litter decomposition with tall fescue and *Lolium multiflorum* have shown that decomposition was slower for endophyte-infected litter, related to the fact that endophyte infection tended to reduce the N content of litter (Omacini et al. 2004), and to changes in the composition of associated decomposers (Lemons et al. 2005). Thus, differences in litter quality among species could imply differences in decomposition rates (Meier and Bowman 2008).

Due to the variability of the plant material and associated *Epichloë* endophytes used in this study, we expected to find differences in the chemical composition of plant tissues among plants infected by different fungal morphotypes. However, we did not find differences among asymptomatic *Epichloë* endophytes (M1, M2 and M3) in any parameter related to plant growth or chemical composition. Instead, significant differences in nutrient content were detected between asymptomatic and choked plants. In plants infected by the choke pathogen *Epichloë typhina*, the development of reproductive stems is arrested by fungal stromata. Flowering stems have greater fiber and lignin and lower mineral contents than leaves, thus, differences in the leaf:stem ratio might partly explain why plants with choke disease had the lowest NDF, and the greatest DMD and mineral content. In addition, an improvement in photosynthesis efficiency in plants infected with the choke pathogen *E. typhina* was observed in *Dactylis glomerata* (Rozpadeck et al. 2015), and this could imply an alteration in the assimilation of nutrients.

The biomass production of *L. perenne* was not affected by the endophyte infection status (E+, E-) or by different *Epichloë* morphotypes (M1, M2, M3), but a significant variation in yield occurred among plants from different origin. Our results agree with those of other studies showing no significant endophyte effect on dry matter yield of ryegrass (Lewis 1996; Barker et al. 1997; Oliveira et al. 2004).

Other studies reported an interactive effect between endophyte and plant genotype affecting plant production, both in greenhouse and field assays (Cheplick and Cho 2003; Hesse et al. 2004; Kane 2011). In our field trial, a significant interaction between infection and plant origin on the number of tillers produced was observed, but it was limited to plants from TAB, with more tillers in E- than in E+ plants. Similarly, among 13 genotypes of ryegrass, a negative effect of *Epichloë* endophytes on tillering under hydric stress conditions was reported by Cheplick et al. (2000), although differences were not detected under regular watering. On the contrary, a positive tiller response to *Epichloë* infection has been reported in some genotypes of *L. perenne* growing under hydric stress (Ravel et al. 1997; Kane 2011).

In conclusion, in this work a heterogeneous set of plants of *L. perenne* and their associated *Epichloë* endophytes was used to study the effect of endophyte infection on host plant growth and nutrient content. The biomass production was affected by plant origin but not by endophyte infection. In contrast, a strong endophyte effect, independent of plant origin, was detected in mineral content (P, S, Mn and B) and fiber contents (NDF, lignin and DMD). The results suggest that *Epichloë* might alter belowground processes that influence nutrient acquisition in the host plant, although the mechanism is not clear and several processes might be involved. In spite of the variability of *Epichloë* endophytes infecting ryegrass plants, plant growth or chemical plant tissue did not vary among different *Epichloë* morphotypes. However, plants infected by the choke pathogen, *Epichloë typhina*, showed significant changes in nutrient content and fiber composition with respect to those infected by asymptomatic *Epichloë* endophytes.

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Table 1 Characteristics of the locations where *Lolium perenne* plants were collected

Location	Habitat	Altitude (masl)	Coordinates		Precipitation (mm/year)	Mean annual temperature (°C)
			Latitude	Longitude		
Ciudad Rodrigo	(CR) Riverbank	625	40°34'48"N	6°30'58"W	531	13.3
La Vecilla	(LVE) Rural path	879	42°42'20"N	5°23'2"W	556	11.2
Los Valles	(LVA) Dehesa grassland	813	40°56'20" N	6°7'36"W	531	12.1
Porqueriza	(POR) Dehesa grassland	807	40°58'18"N	5°57'24"W	531	13.3
Tábara	(TAB) Oak forest	766	41°50'15"N	5°58'40"W	379	12.3
Valle Fuentes	(VAF) Low woodland	1133	42°56'33"N	5°14'18"W	556	13.3

Table 2 Number of *Lolium perenne* plants used in the experimental trial, and distribution of infected plants according to the morphotype of their endophyte at each location of plant origin (percentage of plans in parentheses). The morphotypes were: M1= slow growth rate with ‘brain-like form’; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and with the choke disease M2S = M2 from plants with stromata (see Fig. 1).

Plant origin	Total number of plants ¹	<i>Epichloë</i> morphotype			
		M1	M2	M3	M2S
CR	26	2/13 (15.3)	1/13 (7.6)	6/13 (46.1)	4/13 (30.7)
LVE	28	14/14 (100)	0/14 (0.0)	0/14 (0.0)	0/14 (0.0)
LVA	40	9/20 (45.0)	3/20 (15.0)	1/20 (5.0)	7/20 (35.0)
POR	24	5/12 (41.6)	2/12 (16.6)	3/12 (25.0)	2/12 (16.6)
TAB	44	11/22 (50.0)	0/22 (0.0)	11/22 (50.0)	0/22 (0.0)
VAF	32	11/16 (68.7)	1/16 (6.2)	4/16 (25.0)	0/16 (0.0)
Total	194	52/97 (53.6)	7/97 (7.2)	25/97 (25.7)	13/97 (13.4)

(1) One half of the plants from each location were E+ and the other half were E-

Table 3 Mineral contents of *Lolium perenne* plants as affected by endophyte status (E+ = infected; E- = non-infected) and location of plant origin. The E+ sample set did not include plants with choke disease. Values are means $\pm$ standard error.

		N	P	K	Ca	Mg	S	Na
		(g/kg)	(g/kg)	(g/kg)	(g/kg)	(g/kg)	(g/kg)	(g/kg)
Endophyte	E+	13.46 $\pm$ 0.24	2.70 $\pm$ 0.05	10.37 $\pm$ 0.18	2.34 $\pm$ 0.06	1.06 $\pm$ 0.02	1.62 $\pm$ 0.05	0.405 $\pm$ 0.033
	E-	13.45 $\pm$ 0.23	2.99 $\pm$ 0.05	10.39 $\pm$ 0.17	2.51 $\pm$ 0.05	1.05 $\pm$ 0.01	1.83 $\pm$ 0.05	0.476 $\pm$ 0.032
Plant origin	CR	12.54 $\pm$ 0.50 ab	2.51 $\pm$ 0.11 a	9.52 $\pm$ 0.38 a	2.27 $\pm$ 0.13 ab	0.92 $\pm$ 0.04 a	1.60 $\pm$ 0.11	0.788 $\pm$ 0.071 c
	LVA	13.27 $\pm$ 0.37 ab	2.62 $\pm$ 0.08 a	10.16 $\pm$ 0.28 a	2.65 $\pm$ 0.09 ab	1.09 $\pm$ 0.03 ab	1.74 $\pm$ 0.08	0.313 $\pm$ 0.052 ab
	LVE	14.16 $\pm$ 0.37 bc	3.20 $\pm$ 0.08 b	11.65 $\pm$ 0.28 b	2.48 $\pm$ 0.09 ab	1.14 $\pm$ 0.03 b	1.68 $\pm$ 0.08	0.365 $\pm$ 0.052 ab
	POR	13.51 $\pm$ 0.47 ab	2.84 $\pm$ 0.10 ab	10.04 $\pm$ 0.36 a	2.41 $\pm$ 0.12 ab	1.07 $\pm$ 0.04 ab	1.79 $\pm$ 0.10	0.519 $\pm$ 0.065 bc
	TAB	15.25 $\pm$ 0.34 c	3.06 $\pm$ 0.07 b	11.00 $\pm$ 0.26 ab	2.74 $\pm$ 0.09 b	1.10 $\pm$ 0.03 ab	1.86 $\pm$ 0.07	0.420 $\pm$ 0.048 ab
	VAF	11.98 $\pm$ 0.34 a	2.84 $\pm$ 0.07 ab	9.89 $\pm$ 0.26 a	2.06 $\pm$ 0.09 a	0.96 $\pm$ 0.03 a	1.68 $\pm$ 0.07	0.237 $\pm$ 0.048 a
P (ANOVA)	Endophyte(E)	0.958	<0.001	0.937	0.048	0.952	0.005	0.132
	Plant origin (P)	<0.001	<0.001	<0.001	<0.001	<0.001	0.340	<0.001
	E x P	0.440	0.910	0.250	0.303	0.508	0.945	0.807
		Mn	Zn	Cu	B	Mo	Co	
		(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	
Endophyte	E+	30.88 $\pm$ 1.08	18.47 $\pm$ 0.39	4.65 $\pm$ 0.13	5.41 $\pm$ 0.31	1.01 $\pm$ 0.06	0.043 $\pm$ 0.001	
	E-	27.86 $\pm$ 1.05	18.55 $\pm$ 0.38	4.58 $\pm$ 0.12	7.11 $\pm$ 0.29	1.16 $\pm$ 0.06	0.041 $\pm$ 0.001	
Plant origin	CR	27.83 $\pm$ 2.29	16.76 $\pm$ 0.83 a	4.20 $\pm$ 0.26 a	5.72 $\pm$ 0.64	1.03 $\pm$ 0.14	0.033 $\pm$ 0.003 a	
	LVA	31.43 $\pm$ 1.72	17.69 $\pm$ 0.62 ab	4.46 $\pm$ 0.19 a	6.27 $\pm$ 0.47	0.96 $\pm$ 0.10	0.042 $\pm$ 0.002 ab	
	LVE	30.35 $\pm$ 1.72	19.00 $\pm$ 0.62 ab	5.29 $\pm$ 0.18 b	6.43 $\pm$ 0.45	1.32 $\pm$ 0.09	0.042 $\pm$ 0.002 ab	
	POR	28.11 $\pm$ 2.12	19.78 $\pm$ 0.76 ab	4.57 $\pm$ 0.24 ab	6.28 $\pm$ 0.59	1.18 $\pm$ 0.12	0.042 $\pm$ 0.002 ab	
	TAB	32.36 $\pm$ 1.55	19.83 $\pm$ 0.56 b	4.70 $\pm$ 0.17 ab	7.02 $\pm$ 0.43	1.05 $\pm$ 0.09	0.046 $\pm$ 0.002 b	
	VAF	26.24 $\pm$ 1.57	17.46 $\pm$ 0.56 ab	4.35 $\pm$ 0.17 a	5.79 $\pm$ 0.43	0.98 $\pm$ 0.09	0.041 $\pm$ 0.002 ab	
P (ANOVA)	Endophyte(E)	0.049	0.645	0.670	<0.001	0.094	0.975	
	Plant origin (P)	0.074	0.003	0.002	0.431	0.135	0.016	
	E x P	0.891	0.269	0.163	0.651	0.438	0.024	

In each column, values with different letters are statistically different at $P < 0.05$

Table 4 Mineral content in *L. perenne* plants infected by different asymptomatic *Epichloë* morphotypes: M1= slow growth rate with ‘brain-like form’; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; and in plants with choke disease M2S = M2 from plants with stromata (see Fig. 1). Values are means $\pm$ standard error.

Morphotype	N (g/kg)	P (g/kg)	K (g/kg)	Ca (g/kg)	Mg (g/kg)	S (g/kg)	Na (g/kg)
M1	13.59 $\pm$ 0.36 a	2.79 $\pm$ 0.08	10.64 $\pm$ 0.31 a	2.41 $\pm$ 0.10 a	1.04 $\pm$ 0.04 a	1.70 $\pm$ 0.07 a	0.362 $\pm$ 0.049
M2	13.07 $\pm$ 0.78 a	2.56 $\pm$ 0.09	10.16 $\pm$ 0.25 a	2.18 $\pm$ 0.09 a	0.97 $\pm$ 0.04 a	1.45 $\pm$ 0.05 a	0.387 $\pm$ 0.113
M3	14.09 $\pm$ 0.50 a	2.75 $\pm$ 0.08	10.29 $\pm$ 0.25 a	2.44 $\pm$ 0.13 a	1.05 $\pm$ 0.04 a	1.60 $\pm$ 0.06 a	0.406 $\pm$ 0.072
M2S	16.65 $\pm$ 0.70 b	2.95 $\pm$ 0.14	12.25 $\pm$ 0.34 b	3.28 $\pm$ 0.11 b	1.44 $\pm$ 0.07 b	2.32 $\pm$ 0.19 b	0.583 $\pm$ 0.181
<i>P</i> (ANOVA)	0.002	0.399	0.016	<0.001	<0.001	<0.001	0.388

Morphotype	Mn (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	B (mg/kg)	Mo (mg/kg)	Co (mg/kg)
M1	31.18 $\pm$ 1.72 a	18.43 $\pm$ 0.62 a	4.71 $\pm$ 0.21 a	5.27 $\pm$ 0.26 a	0.96 $\pm$ 0.07	0.045 $\pm$ 0.002
M2	27.23 $\pm$ 3.36 a	17.48 $\pm$ 0.65 a	4.29 $\pm$ 0.32 a	5.25 $\pm$ 0.49 a	0.87 $\pm$ 0.13	0.037 $\pm$ 0.005
M3	33.13 $\pm$ 2.22 a	19.05 $\pm$ 0.82 ab	4.67 $\pm$ 0.30 ab	5.85 $\pm$ 0.41 ab	1.21 $\pm$ 0.14	0.042 $\pm$ 0.002
M2S	46.41 $\pm$ 3.83 b	22.27 $\pm$ 1.16 b	5.82 $\pm$ 0.43 b	7.21 $\pm$ 0.67 b	1.25 $\pm$ 0.14	0.047 $\pm$ 0.005
<i>P</i> (ANOVA)	<0.001	0.025	0.016	0.022	0.123	0.291

P= significance level for one-way ANOVA.

In each column, values with different letters are statistically different at *P*<0.05.

Table 5 Mean contents $\pm$ standard error of acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, and dry matter digestibility (DMD) of *L. perenne* plants as affected by endophyte status (E+ = infected; E- = non-infected) and location of plant origin. The E+ sample set did not include plants with stromata.

		ADF (%)	NDF (%)	Lignin (%)	DMD (%)
Endophyte	E+	28.05 $\pm$ 0.24	48.30 $\pm$ 0.35	5.61 $\pm$ 0.14	59.73 $\pm$ 0.36
	E-	27.67 $\pm$ 0.23	49.46 $\pm$ 0.33	6.02 $\pm$ 0.14	57.67 $\pm$ 0.35
Plant origin	CR	27.06 $\pm$ 0.51	47.17 $\pm$ 0.74 a	5.53 $\pm$ 0.31 ab	59.89 $\pm$ 0.77 b
	LVA	27.26 $\pm$ 0.38	48.62 $\pm$ 0.55 ab	5.68 $\pm$ 0.23 ab	58.83 $\pm$ 0.57 ab
	LVE	28.02 $\pm$ 0.38	49.66 $\pm$ 0.55 ab	6.20 $\pm$ 0.22 ab	57.47 $\pm$ 0.54 ab
	POR	28.40 $\pm$ 0.47	49.66 $\pm$ 0.68 ab	5.47 $\pm$ 0.29 a	59.65 $\pm$ 0.71 ab
	TAB	28.44 $\pm$ 0.35	49.86 $\pm$ 0.50 b	6.46 $\pm$ 0.21 b	56.71 $\pm$ 0.52 a
	VAF	27.95 $\pm$ 0.35	48.33 $\pm$ 0.50 ab	5.52 $\pm$ 0.21 ab	59.83 $\pm$ 0.52 ab
<i>P</i> (ANOVA)	Endophyte (E)	0.266	0.019	0.060	<0.001
	Plant origin (P)	0.115	0.022	0.008	<0.001
	E x P	0.231	0.501	0.663	0.776

P= significance level for one-way ANOVA.

In each column, values with different letters are statistically different at *P*<0.05.

Table 6 Mean contents $\pm$ standard error of acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin and dry matter digestibility (DMD) of *L. perenne* plants infected by different asymptomatic *Epichloë* morphotypes: M1= slow growth rate with ‘brain-like form’; M2= faster growing rate with cottony aerial mycelium; M3= intermediate growth rate with tan, smooth aerial mycelium; ; and with the choke disease M2S = M2 from plants with stromata (see Fig. 1).

<i>Epichloë</i> morphotype	ADF (%)	NDF (%)	Lignin (%)	DMD (%)
M1	28.18 $\pm$ 0.29 b	48.46 $\pm$ 0.41 b	5.47 $\pm$ 0.25	58.95 $\pm$ 1.00
M2	28.25 $\pm$ 0.69 b	49.87 $\pm$ 1.21 b	5.11 $\pm$ 0.23	60.25 $\pm$ 1.02
M3	27.52 $\pm$ 0.55 b	47.24 $\pm$ 0.86 b	5.46 $\pm$ 0.25	60.48 $\pm$ 0.67
M2S	21.72 $\pm$ 0.50 a	37.89 $\pm$ 0.85 a	5.87 $\pm$ 0.38	61.09 $\pm$ 0.75
<i>P</i> (ANOVA)	<0.001	<0.001	0.302	0.079

In each column, values with different letters are statistically different at $P < 0.05$.

Fig. 1 Cultures of the three morphotypes of *Epichloë* endophyte isolated from plants of *Lolium perenne* (a) M1 morphotype, slow growth rate with “brain-like” mycelium; (b) M2 morphotype, faster growth rate with white cottony aerial mycelium; (c) M3 morphotype intermediate growth rate with tan, smooth and flat mycelium; (d) A stroma of *Epichloë typhina* in ryegrass, a typical symptom of ‘choke disease’

Fig. 2 (a) Dry matter yield of *L. perenne* according to locations of plant origin. (b) Number of tillers per plant as affected by plant origin, and infection state: E+ = *Epichloë*-infected; E- = Non-infected plants. Bars are means + standard error

A,B,C: different letters indicate significant differences at $P < 0.05$

*Significant differences between E+ and E- (Infection status x Plant origin; $P = 0.039$)