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## REGULAR ARTICLE

# Biomass yield, nutritive value and accumulation of minerals in *Trifolium subterraneum* L. as affected by fungal endophytes

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## Abstract

*Background and aims Trifolium subterraneum* is a legume species which is valuable for feeding livestock and frequently used as a forage crop in countries with temperate or Mediterranean-like climates. The aim of the present work was to evaluate the effect of six leaf fungal endophytes on biomass production, nutritive value and mineral status of *T. subterraneum* forage.
*Methods* Plants were inoculated with each of seven treatments (six endophytes + control) at two different growth stages. After inoculation, two experiments (under greenhouse and field conditions) were established.
*Results* Endophytes affected biomass yield, nutritive value and mineral status of *T. subterraneum* forage, but effects varied between experiments and depended on fungal species. E202 (*Chaetosphaeronema* sp.) increased forage productivity by around 80 % in the field. *Fusarium lateritium* and E244 (Pleosporales) reduced Al concentration, and *Epicoccum nigrum* reduced Pb of the forage in the greenhouse experiment. An increase in essential nutrients, such as Zn, was mainly produced by *Stemphylium globuliferum*.
*Conclusions* This study demonstrated that inoculation with endophytes can increase forage productivity and help reduce potential nutrient deficiencies and/or potential mineral toxicity in *T. subterraneum*.

**Keywords** Subterranean clover · Mycoflora · Herbage · Protein · Fiber · Pb toxicity

## Introduction

*Trifolium subterraneum* L. (subterranean clover), an annual self-regenerating pasture legume of Mediterranean origin, occurs naturally in grasslands and *dehesas* (i.e., grasslands with scattered trees and well-developed herbaceous understory) in southwestern Spain on acidic soils. This species produces a relatively abundant and high quality forage for feeding livestock, with amounts of 2330–3550 kg ha$^{-1}$ dry matter and values of crude protein around 15–19 % (García-Criado et al. 1986). In addition, subterranean clover forage exhibits a high palatability and digestibility due to its relatively low content in fiber and lignin, with average values of NDF (neutral detergent fiber), ADF (acid detergent fiber) and ADL (acid detergent lignin) of 26.8, 21.8, and 3.23 %, respectively (García-Ciudad et al. 1985).

For these reasons, *T. subterraneum* is one of the most important legumes used alone or in mixtures in sown pastures in Spain and many other countries with temperate or Mediterranean-like climates (Frame et al. 1998).

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Many factors affect growth, biomass production, quality traits, persistence and regeneration of *T. subterraneum*. Climatic conditions, especially precipitation during the growing season, are undoubtedly the most important factor (Bolger et al. 1993), but soil fertility (Saul et al. 1999), competition with other plant species (Conning et al. 2011), shading levels (Kyriazopoulos et al. 2012), livestock management (Ates et al. 2013), and pest and diseases (Simpson et al. 2011) can also have an important influence on these parameters.

In addition to these well-known factors, fungal endophytes, defined as organisms which for part or all of the life cycle invade the living tissues of plants without causing any disease symptoms, have been shown to affect widely the production and performance of other pasture species. Many studies have reported increases in biomass production and in re-growth capacity in endophyte-infected plants in comparison with uninfected ones (Hesse et al. 2005; Thom et al. 2013; Mahendra et al. 2014). The nutrive value of *Festuca rubra* L., *F. arundinacea* Schreb. and *Lolium perenne* L., measured through digestibility of organic matter, has also been shown to be improved by the endophyte *Epichloë festucae* Leuchtm. et al. (Zabalgogeazcoa 2008). Likewise, several foliar endophytes have been found either to increase the concentration of some minerals such as P, Ca or Mg (Zabalgogeazcoa et al. 2006), or to reduce the concentration of others such as Cu (Dennis et al. 1998; Zabalgogeazcoa et al. 2006), Al (Malinowski and Belesky 1999) or Zn (Monnet et al. 2001).

These effects could be a consequence of the substantial benefits that endophytic fungi may confer on hosting plants, such as drought tolerance (Hall et al. 2014), resistance to herbivory and pathogens (Thom et al. 2013; Romeralo et al. 2015), enhanced nutrient uptake (Yang et al. 2014), and increased competitive ability (Vázquez-de-Aldana et al. 2013). However, this influence appears to be variable and clearly dependent on the species of endophyte, the host genotype and environmental conditions (Ahlholm et al. 2002). For this reason, it is common also to find studies in which fungal endophytes have neutral or negative effects (Zabalgogeazcoa et al. 2006; Faeth et al. 2010). Consequently endophyte-plant interactions should be tested specifically for each combination of plant host, endophytic species and environmental conditions.

Most of the studies dealing with such plant-endophyte interactions, such as those already cited above, have been performed on grasses species with clavicipitaceous fungi, which are systemic fungal endophytes defined according to the classification given by Rodriguez et al. (2009). Conversely, studies dealing with non-clavicipitaceous endophytes in legume species are very limited (Dudeja et al. 2012), most of them focusing on endophytic diversity, such as those conducted on peanut (Ferreira de Souza et al. 2014), soybean (Impullitti and Malvick 2013), or on several sand dune wild legumes (Seena and Sridar 2004), rather than endophyte effects on plant production. With the aim of testing the hypothesis that non-clavicipitaceous endophytes also affect biomass production and quality of an important forage legume, this study evaluated the effect of six foliar fungal endophytes on the biomass production (herbage and root biomass), nutritive value (protein, fiber, and lignin) and mineral status of *T. subterraneum* forage.

## Materials and methods

### Fungal and plant material

Six fungal endophytes, previously isolated from pasture species and identified in our laboratory, were used in the experiments (Table 1). Endophyte identification was first based on morphologic characteristics and then on comparison to ITS sequences in GenBank with a similarity criterion >97 %. Two months before the experiments, fungi were grown at 25 °C in the dark in 1.5-L flasks containing 1 L culture medium PDB (Potato Dextrose Broth) to obtain sufficient inoculum for plant inoculations. Seeds of *T. subterraneum* cv 'Valmoreno' were surface-disinfected by immersion for 5 min in 2.5 % NaClO, and then washed three times with sterilized distilled water. Five sterilized seeds per pot were sown in 7x7x6 cm plastic pots containing soil substrate consisting of a 1:1 (*vol*/*vol*) mix of perlite and a commercial growing medium composed of peat, perlite, lime, root activator and fertilizer NPK (COMPO SANA Universal, COMPO GmbH & Co. KG, Münster Germany). On four dried and homogenized soil substrate samples, pH was determined using a calibrated pH meter (ratio 10 g soil: 25 ml deionized $H_2O$). Electrical conductivity (EC) was determined using an

**Table 1** Origin and identification of the endophyte species used in the experiments

| Isolate no. | GenBank accession No | Plant host | Geographic origin | UTM Coordinates (Zone 29 North Datum) (m) | Identification[a] |
|---|---|---|---|---|---|
| E060 | KP698325 | *Biserrula pelecinus* L. | Moheda de Olalla (Cáceres) | X=704,216 Y=4,349,073 | *Fusarium lateritium* Nees |
| E071 | KP698327 | *Bromus mollis* L. | Cheles (Badajoz) | X=653,013 Y=4,267,740 | *Sordaria fimicola* (Rob. ex Desm.) Ces. & De Not. |
| E140 | KP698338 | *Trifolium subterraneum* L. | Haza (Cáceres) | X=748,510 Y=4,428,827 | *Stemphylium globuliferum* (Vestergr.) |
| E202 | KP698370 | *Biserrula pelecinus* L. | Haza (Cáceres) | X=748,510 Y=4,428,827 | *Chaetosphaeronema* sp. |
| E244 | KP698359 | *Lolium rigidum* Gaud. | Haza (Cáceres) | X=748,510 Y=4,428,827 | Pleosporales |
| E631 | KP698340 | *Ornithopus compressus* L. | Cheles (Badajoz) | X=653,013 Y=4,267,740 | *Epicoccum nigrum* Link |

[a] Based on morphological characters and on comparison to ITS sequences in GenBank with a similarity >97 %

EC-meter. Total N was determined using the Kjeldahl method (Bremner 1996), with the aid of a Kjeltec™ K350 destillation Unit (Buchi Ltd., Flawil, Switzerland). Extractable P was determined by the Olsen procedure, and Ca and K were extracted with ammonium acetate (1 N) and quantified by atomic absorption. Total Al, B, Ca, Cu, Fe, K, Li, Mg, Mn, Mo, Na, P, Pb, S, Se and Zn concentrations were determined by the Ionomics Service of CSIC (Spanish High Centre for Science and Research) by means of inductively coupled plasma optical emission spectrometry (ICP-OES).

Because the age of the plant seems somehow to affect fungal endophyte colonization (Arnold and Herre 2003), two different planting dates were established in order to obtain more robust and reliable results. The first planting date was in early December 2012, and the second 3 weeks later. After planting, pots were placed in a greenhouse and watered to field capacity every 2 to 3 days. Maximum and minimum temperatures and relative humidity in the greenhouse during the experiments are reported in Supplemental Information 1. When the youngest plants were 1 month old, all of them were treated with the systemic fungicide AMISTAR XTRA® (Syngenta, Madrid). Two additional treatments, ten days apart, were also applied. In each treatment, around 1 mL per pot of a solution obtained by adding 1 mL of fungicide to 1 L of distilled water was sprayed as foliar application. In the third application, 1 mL per pot of the solution was additionally applied to the soil substrate.

Inoculations

In order to verify that plants were free of culturable endophytes, just before inoculation, four plants were randomly selected and taken to the laboratory. Plants were then surface-disinfested by immersion for 30 s in 70 % ethanol, followed by one immersion for 1 min in 2 % NaClO plus two drops Tween-80 per litre, and washed three times with sterilized distilled water. Then five 5-mm-long segments were cut from different parts of each plant and placed on PDA in a Petri dish. Endophytic colonies were not observed growing out of any of the plant segments. Five weeks after the last fungicide application, plants were wounded by puncturing their leaves and stems with a home-made tool composed of two arms, one of them ending in a multiple needle structure, and the other in a smooth surface (Supplemental Information 2). In this way plants were wounded sufficiently to facilitate fungal infection without serious plant damage. For fungal inoculation of the wounded plants, the actively growing mycelium of each endophyte was homogenized with the culture medium by blending. Inoculation was carried out with the aid of a hand sprayer in two doses: one half of the homogenized

inoculum (i.e., 500 mL) just after plant wounding, and the other half 3 days later. During the 48 h following the inoculations, plants were maintained in a high humidity atmosphere in order to maximize fungal infection. Ten pots or experimental units (containing five plants each) per planting date were inoculated with each of the endophytes. Ten additional pots or experimental units per planting date were inoculated only with culture medium to be used as control.

Greenhouse experiment

One half of the pots containing inoculated plants, i.e., five pots per endophyte and planting date (a total of 70 pots), were arranged in the greenhouse following a completely randomized design. Pots were placed on greenhouse benches separated at least 5 cm apart, without any direct contact, to avoid secondary infections. In order to check the effectiveness of the inoculations, approximately 1 month later, plant samples of each treatment were taken to the laboratory for re-isolations following the procedure described above. After the re-isolation process, the six endophytes were positively re-isolated and identified in culture medium. This might mean that the inoculation method was sufficiently effective to cause infection, and consequently the differences observed between treatments could be attributed to the inoculation. Three months after inoculations (i.e., early May 2013), herbage and roots were harvested and taken to the laboratory for processing. Roots were carefully washed with tap water to remove soil substrate.

Field experiment

Approximately 1 month after the inoculations, the second half of the pots were transported to an experimental area located in the *Dehesa* "Valdesequera" owned by the regional government of Extremadura. This *Dehesa* is located in Badajoz, south-west Spain (UTM Coordinates, Zone 29 North Datum: X=685,365 m; Y=4,325,603) in an Alfisol Xeralft soil (according to USDA classification). The soil of the study site had a sandy loam texture, determined gravimetrically on four representative soil samples taken before transplanting at 30 cm depth, which is the usual rooting depth of the clover (Pearson and Jacobs 1995). On these soil samples, all the edaphic characteristics were determined as explained above for the substrate samples. During the experiment, the climatic data, which were taken from a

weather station located close to the study site, were the following: mean temperature 11.3, 14.3, and 16.9 °C; for March, April and May, respectively. Monthly precipitation was 190.5, 22.1, and 27.6 mm for March, April and May, respectively (Supplemental Information 1).

Before transplanting, conventional tillage was applied to prepare an appropriate environment for plants. Transplanting was made in late February and the experimental units (a set of five plants) were arranged by following a completely randomized design with a planting layout of 50 cm×50 cm. Two months and a half after transplanting (i.e., mid-May 2013), herbage was harvested and taken to the laboratory for processing.

Response-variables determinations

In the laboratory, plant samples were oven dried (70 °C) until constant weight and then dry matter (DM) of herbage and root biomass production was recorded. From part of the herbage samples, the following parameters were determined: total N content using the Kjeldahl method (Kjeltec™ 8200 Auto Distillation Unit. FOSS Analytical. Hilleroed, Denmark), crude protein (CP) by multiplying the biomass $N \times 6.25$ (protein on average contains 16 % N) as conversion factor (Sosulski and Imafidon 1990), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) by means of a fiber analyzer (ANKOM 8–98, ANKOM Technology, Macedon, NY), by following the official procedures (AOAC 2006). Another part of the herbage samples was sent to the Ionomics Service of CSIC (Spanish High Centre for Science and Research) for mineral determinations with an ICP-OES as explained above for the soil samples.

Statistical analysis

The effect of the endophyte (seven treatments, including controls) and planting date (young and old), as well as their interaction on herbage dry matter (HDM) and root dry matter (RDM) (RDM only in the greenhouse experiment), nutritive value parameters (CP, NDF, ADF, and ADL) and mineral concentration (Al, B, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, and Zn) was evaluated by two-way ANOVA. Fisher's protected least significant difference (LSD) test for multiple comparison was used when significant differences ($P < 0.05$)

were found in the ANOVA. In order to normalize variable distribution and to stabilize the variance of residuals the following variable transformation was performed: Ln (x+1) for Al, B, Cu, Fe, K, Li, Mn, P, Pb, and Zn in the greenhouse experiment, and Fe, Mn, and Zn in the field experiment; $^2\sqrt{x}$ for HDM in the field experiment; $^3\sqrt{x}$ for HDM and RDM in the greenhouse experiment, and Cd, Li, Na, P, and Pb in the field experiment; and $^5\sqrt{x}$ for Cd, Cr, Mo, and Ni in the greenhouse experiment, and ADL, Al, Cr, Mo, Ni, and Se in the field experiment. All these analyses were performed with the Statistix v. 8.10 package.

## Results

### Differences between experimental soils

Both types of soils, i.e., the substrate used in the greenhouse experiment and the soil used in the field experiment, presented quite low pH values (4.43±0.01 and 5.35±0.12, respectively). However, electrical conductivity (EC) was 17-fold higher in the field soil than in the greenhouse substrate. Because the commercial substrate had been amended with NPK fertilizer and lime, the values of total N, Ca and P and extractable Ca and K were much higher in the greenhouse soil in comparison with the field soil (Supplemental Information 3). Regarding essential micronutrients for plants and/or livestock, such as Fe, Li, Se or Zn, or toxic elements, such as Al or Pb, the field soil presented much higher values than the greenhouse substrate (Supplemental Information 3).

### Effects on biomass yield (herbage and root)

Endophyte inoculation, regardless of the species, did not cause plant disease symptoms during the experiment. In the greenhouse experiment, herbage yield was significantly affected ($P <0.05$) by the two main effects considered (endophyte treatment and planting date) but not by the interaction (Table 2). The inoculation with the endophytes E631 (*Epicoccum nigrum*) and E202 (*Chaetosphaeronema* sp.) prompted the highest herbage production in the *T. subterraneum* forage, although without significant differences compared to the control plants. Conversely, two endophytes E244 (Pleosporales) and E140 (*Stemphylium globuliferum*) caused a significant reduction in herbage yield in comparison with

**Table 2** Two-way ANOVA showing the effect of endophyte, planting date (age) and their interaction on each parameter evaluated in both experiments (under greenhouse and field conditions). DF, degree of freedom; F values, including the level of significance (* $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$) are shown in the rest of the rows

| | Endophyte | Age | Endo*age |
|---|---|---|---|
| Greenhouse experiment | | | |
| DF | 6 | 1 | 6 |
| HDM | 6.05*** | 18.75*** | 1.50 |
| RDM | 2.50* | 3.45 | 2.28* |
| CP | 1.30 | 0.08 | 0.66 |
| NDF | 3.15* | 39.16*** | 1.13 |
| ADF | 3.09* | 47.42*** | 0.62 |
| ADL | 8.81*** | 11.80** | 1.13 |
| Al | 12.81*** | 0.53 | 3.36* |
| B | 2.22 | 1.90 | 3.15* |
| Ca | 9.29*** | 4.91* | 0.92 |
| Cd | 8.34*** | 20.10*** | 2.94* |
| Cr | 3.92** | 1.53 | 1.78 |
| Cu | 3.77** | 3.97 | 1.26 |
| Fe | 4.73** | 2.01 | 2.09 |
| K | 2.61* | 12.63** | 1.74 |
| Li | 0.88 | 13.64** | 1.81 |
| Mg | 1.83 | 5.55* | 0.48 |
| Mn | 3.41* | 9.22** | 1.39 |
| Mo | 3.47* | 2.17 | 1.20 |
| Na | 6.43** | 2.22 | 2.69* |
| Ni | 0.53 | 0.69 | 0.70 |
| P | 1.56 | 6.39* | 2.39 |
| Pb | 76.88*** | 3.33 | 0.84 |
| S | 2.26 | 4.63* | 1.29 |
| Zn | 2.83* | 2.87 | 1.70 |
| Field experiment | | | |
| DF | 6 | 1 | 6 |
| HDM | 2.37* | 0.43 | 1.38 |
| RDM | – | – | – |
| CP | 0.81 | 1.53 | 0.36 |
| NDF | 7.77*** | 1.82 | 1.70 |
| ADF | 3.99** | 2.99 | 1.51 |
| ADL | 19.62*** | 4.72* | 0.87 |
| Al | 2.71* | 3.63 | 1.11 |
| B | 2.58* | 0.00 | 0.57 |
| Ca | 1.54 | 1.12 | 0.53 |
| Cd | 1.34 | 0.24 | 0.31 |
| Cr | 2.23 | 3.37 | 0.57 |
| Cu | 3.87** | 0.00 | 0.51 |

**Table 2** (continued)

|  | Endophyte | Age | Endo*age |
|---|---|---|---|
| Fe | 2.57* | 4.02 | 1.26 |
| K | 2.11 | 0.36 | 1.22 |
| Li | 2.61* | 2.49 | 1.07 |
| Mg | 0.79 | 0.50 | 0.18 |
| Mn | 0.24 | 0.34 | 0.19 |
| Mo | 2.34 | 0.60 | 1.85 |
| Na | 1.40 | 1.92 | 0.47 |
| Ni | 1.44 | 2.56 | 0.31 |
| P | 2.30 | 0.08 | 0.63 |
| Pb | 5.22** | 1.96 | 1.13 |
| S | 1.38 | 1.22 | 0.56 |
| Zn | 2.65* | 0.70 | 0.74 |

*HDM* herbage dry matter, *RDM* root dry matter, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *ADL* acid detergent lignin

controls (Fig. 1 left). In the case of root dry matter, the main effect of the endophyte treatment and the interaction between planting date and endophyte treatment were variables with a significant effect in the greenhouse experiment (Table 2). In this case, the endophytes E071 (*Sordaria fimicola*), E631 (*Epicoccum nigrum*) and E202 (*Chaetosphaeronema* sp.) yielded the highest root production in plants derived from the later planting date, i.e., those inoculated at an earlier growth stage and considered as 'young'; whilst again E202 produced the highest root biomass in plants from an earlier planting date (considered as 'old') (Supplemental Information 4). Regarding the effect of planting date, plants considered as 'old' had a higher herbage production than 'young'

**Table 3** Mean±standard error of the different parameters (HDM: herbage dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; and mineral status) significantly affected ($P <0.05$) by the main effect of planting date according to the two-way ANOVA performed in both greenhouse and field experiments

|  | Young | Old |
|---|---|---|
| Greenhouse experiment |  |  |
| HDM (g unit$^{-1}$) | 1.66±0.18 B | 2.57±0.20 A |
| NDF (%) | 31.55±0.74 B | 36.86±0.68 A |
| ADF (%) | 21.73±0.41 B | 25.39±0.45 A |
| ADL (%) | 3.88±0.22 B | 4.60±0.23 A |
| Ca (g kg$^{-1}$) | 21.56±0.74 A | 19.96±0.82 B |
| Cd (mg kg$^{-1}$) | 0.14±0.02 A | 0.06±0.02 B |
| K (g kg$^{-1}$) | 8.08±0.70 A | 5.42±0.55 B |
| Li (mg kg$^{-1}$) | 2.30±0.11 A | 1.79±0.11 B |
| Mg (g kg$^{-1}$) | 5.55±0.13 A | 5.10±0.15 B |
| Mn (mg kg$^{-1}$) | 209.1±13.4 A | 166.7±16.7 B |
| P (g kg$^{-1}$) | 2.29±0.14 A | 1.86±0.15 B |
| S (g kg$^{-1}$) | 5.98±0.30 A | 5.17±0.37 B |
| Field experiment |  |  |
| ADL (%) | 2.87±0.27 B | 3.32±0.32 A |

For each parameter, averages with different letter are significantly different according to LSD test at a significance level of 0.05

plants (2.57 and 1.66 g per experimental unit, respectively) (Table 3).

In the field experiment only herbage biomass (HDM) was studied. In this case, the ANOVA showed a significant influence of the endophyte on herbage production (Table 2). In those field conditions, plants inoculated with the endophyte E202 (*Chaetosphaeronema* sp.) had higher herbage production compared with any other treatment, including controls (Fig. 1 right).
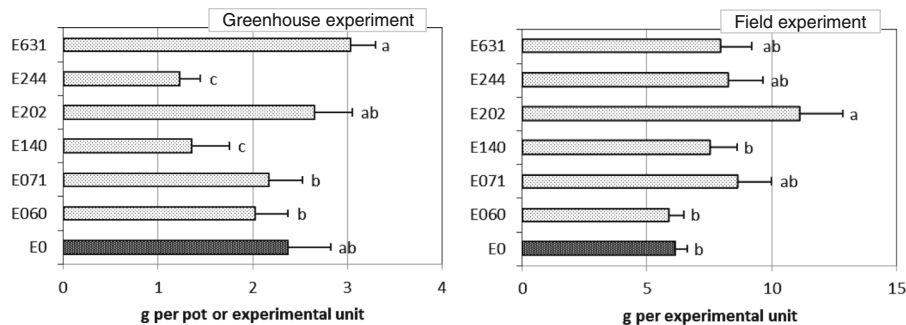


**Fig. 1** Herbage dry matter of *T. subterraneum* forage when inoculated with each endophyte in the greenhouse experiment (*left*) and in the field experiment (*right*). Horizontal bars indicate means and horizontal lines standard error. For each experiment, averages with the same letter are not significantly different according to LSD test at a significance level of 0.05. Although the LSD test was performed on transformed variables, back-transformed values are represented to ease interpretation

Effects on nutritive value parameters

In the greenhouse experiment, only the two main effects, endophyte treatment and planting date (age), affected significantly ($P <0.05$) neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) obtained in herbage (Table 2). In the case of NDF and ADF, none of the endophytes caused an increase of these parameters in the forage in comparison with controls. Conversely, inoculation with the endophytes E244 (*Pleosporales*) and E140 (*S. globuliferum*) provided forage with lower values of NDF and ADF, respectively, in comparison with those obtained in the controls (Fig. 2). By contrast, in the case of ADL, forage derived from plants inoculated with the endophyte E060 (*F. lateritium*) had significantly higher lignin values than those found in the controls (5.9 and 3.9 %, respectively) (Fig. 2). Regarding the effect of planting date, plants considered as 'old', i.e., those derived from the earliest planting date which were inoculated at a later growth stage, showed higher values of NDF, ADF and ADL (36.86, 25.39 and 4.60 %,

respectively) in comparison with 'young' plants, i.e., those derived from the latest planting date (31.55, 21.73 and 3.88 %, respectively) (Table 3).

In the field experiment, in the case of crude protein, although there were no significant differences between endophytes and/or planting dates (Table 2), values of CP were much higher than those obtained in the greenhouse experiment ($17.9\pm0.5$ and $8.9\pm0.3$ %, respectively). Similar to the greenhouse experiment, the main effect of endophyte had a significant influence ($P <0.05$) on NDF, ADF and ADL (Table 2). None of the endophytes caused a reduction in any of these parameters in the forage in comparison with controls. By contrast, the inoculation with two endophytes, E060 (*F. lateritium*) and E244 (*Pleosporales*) provided forage with higher values of NDF, ADF and ADL than those of controls. Additionally, endophyte E140 (*S. globuliferum*) produced herbage with the highest values of ADL (Fig. 2). The planting date (age) only affected significantly ADL (Table 2). In this case, plants considered as 'old' had a higher ADL than 'young' plants (5.17 and 2.87 %, respectively) (Table 3).
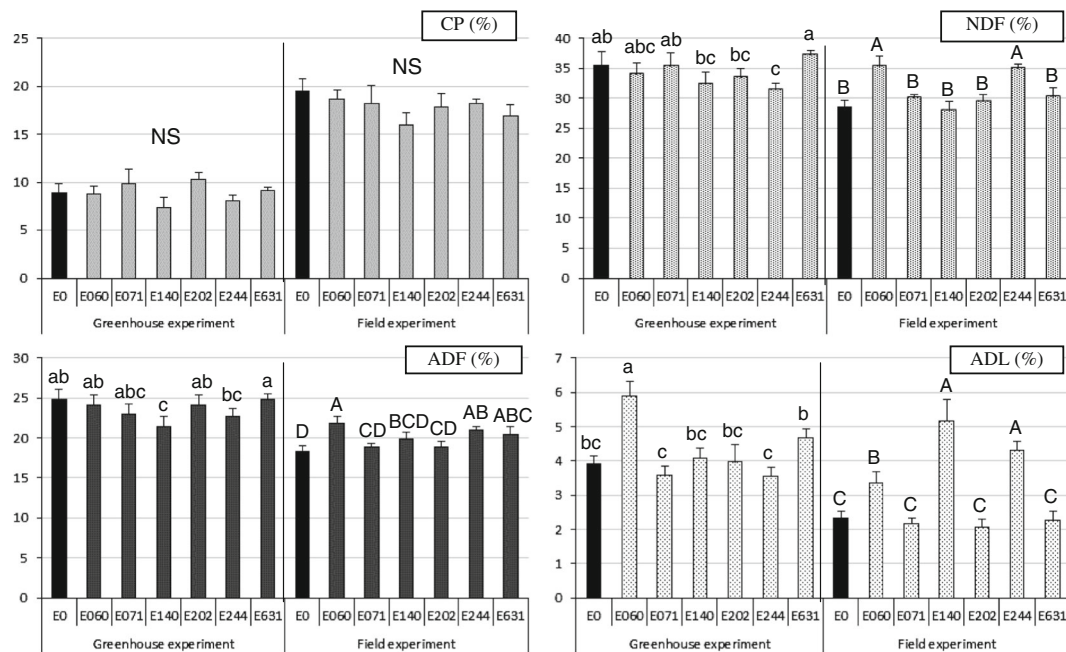


**Fig. 2** Quality parameters (CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin) of *T. subterraneum* forage when inoculated with each endophyte in the greenhouse and in the field experiments. Vertical bars indicate means and vertical lines standard error. For each parameter, averages with the same letter (lowercase letters in the greenhouse experiment and uppercase letters in the field experiment) are not significantly different according to LSD test at a significance level of 0.05. NS: Not significant according to ANOVA at a significance level of 0.05. Although the LSD test was performed on the transformed variable in the case of ADL, back-transformed values are represented to ease interpretation

Uptake and accumulation in the herbage
of the different minerals

In the greenhouse experiment, inoculation with an en-
dophyte significantly affected the concentration in
plants of Al, Ca, Cd, Cr, Cu, Fe, K, Mn, Mo, Na, Pb
and Zn (Table 2). Plants inoculated with endophyte
E060 (*F. lateritium*) had a lower concentration of Al,
Cr and Fe (approximately 66, 82 and 45 % lower,
respectively) and a higher concentration of Pb (more
than 3-fold higher) compared to the control (Table 4).
Likewise, *Stemphylium globuliferum* (E140) caused a
higher accumulation in the forage of Ca, Cd, Cu, Mn, Pb
and Zn (more than 31, 217, 66, 14, 305 and 60 %,
respectively) and lower concentrations of Al, Cr and
Na (approximately 73, 88 and 33 % lower, respectively)
in comparison with controls (Table 4). The concentra-
tion of Al was also lower (60 and 73 %, respectively) in
plants inoculated with endophytes E202
(*Chaetosphaeronema* sp.) and E244 (*Pleosporales*)
than in non-inoculated controls. However E244 also
caused a lower accumulation of Fe (48 % lower) and a
higher concentration of K and Pb (more than 72 and
225 % higher) compared to the control (Table 4).
Finally, E631 (*E. nigrum*) provided forage with lower
concentrations of Cd, Fe, Mo and Pb (100, 43, 82 and
80 %, respectively) in comparison with control plants.
When a significant influence of planting date in the
concentration of minerals was found (Table 2), in all
cases it was higher in 'young' plants than in 'old' plants
(Table 3).

In the field experiment, the concentration of minerals,
but only in the case of Al, B, Cu, Fe, Li, Pb and Zn, was
significantly affected only by the main effect endophyte
inoculation (Table 2). The pattern of the observed effect
was similar for Al, Fe and Li: E140 (*S. globuliferum*)
provided forage with the highest values of those min-
erals and E071 (*S. fimicola*) with the lowest values, but
in both cases without differences compared to the con-
trol plants (Table 4). For B and Cu, while none of the
endophytes increased their concentration in the forage in
comparison with controls, E140 (*S. globuliferum*)
caused lower accumulation of both than in con-
trols. *S. globuliferum* also provided the highest
values of Pb in the forage. Finally, the highest values
of Zn in the forage were provided by E202
(*Chaetosphaeronema* sp.), although without differences
with controls, and the lowest by E244 (Pleosporales)
(Table 4).

## Discussion

The influence of endophytes on herbage and root bio-
mass productivity has already been demonstrated in
several hosts (Hesse et al. 2005; Thom et al. 2013;
Mahendra et al. 2014). However, most of those experi-
ments were performed on grasses using clavicipitaceous
fungi, especially *Epichloë/Neotyphodium* species. The
use of non-clavicipitaceous fungal endophytes for this
purpose has been less frequently reported, and to our
knowledge the interaction between subterranean clover
and any of the fungal endophytes used in the present
work, has not been previously studied. In the field
experiment of our study, plants inoculated with the
endophyte E202 (*Chaetosphaeronema* sp.) produced
around 80 % more herbage as compared to the controls.
In the greenhouse experiment, although plants inoculat-
ed with this endophyte also showed a high herbage
production, there were no differences with control plants
(Table 5). The higher adaptive value that endophytes
can give to plant host (Malinowski and Belesky 2000)
may explain why in less favourable environments, such
as that found in the field in comparison with the green-
house, the eventual effect of a microorganism-plant
interaction might become more evident. Thus, many
studies carried out under non-limiting conditions for
plant growth (Fritz and Collins 1991; Faeth et al.
2010) have shown a neutral or negative effect of the
endophyte on biomass yield.

To explain this higher herbage yield in infected
plants, several authors have proposed that endophytes
could cause a lengthening of the vegetative growth
period (Zabalgogeazcoa et al. 2006), which is an impor-
tant factor governing biomass production and
accumulation. However, the mechanisms involved in
this lengthened growth period are not fully known. In
some cases endophytic infections could prevent or delay
spike emergence, thus postponing the beginning of the
reproductive period, in which the biomass production is
considerably reduced. Other authors such as Assuero
et al. (2006) have suggested that endophyte presence
may promote plant growth by producing some
hormone-like substances which could delay the growth
cycle of the plant, or it may promote root growth giving
plants a higher capacity to absorb water and minerals.
This hypothesis is not strongly supported by our data
because, although the endophytes which caused the
highest herbage production tended also to produce the
largest root system, differences with controls were not

**Table 4** Mineral concentration (mean ± standard error) of the forage obtained from each endophyte-inoculated plants in each experiment (greenhouse and field)

| Mineral | Experiment | Control | E060 | E071 | E140 | E202 | E244 | E631 |
|---|---|---|---|---|---|---|---|---|
| Al (g kg$^{-1}$) | Greenhouse | 0.15±0.06 ab | 0.05±0.01 de | 0.15±0.02 a | 0.04±0.00 de | 0.06±0.00 cd | 0.04±0.00 e | 0.07±0.01 bc |
| | Field | 1.60±0.39 ABC | 1.21±0.19 BC | 0.94±0.26 C | 3.01±1.00 A | 1.73±0.28ABC | 1.03±0.10 BC | 1.99±0.54 AB |
| B (mg kg$^{-1}$) | Greenhouse | 18.9±2.3 | 18.4±2.8 | 18.4±1.9 | 23.7±2.4 | 17.2±1.9 | 22.3±2.6 | 16.3±1.2 |
| | Field | 21.3±0.7 A | 20.0±0.7 AB | 21.9±0.6 A | 17.9±1.2 B | 21.1±0.6 A | 19.9±0.6 AB | 21.0±1.2 A |
| Ca (g kg$^{-1}$) | Greenhouse | 19.9±1.4 b | 21.4±1.0 b | 20.1±0.7 b | 26.1±1.5 a | 16.4±0.9 c | 21.6±1.2 b | 19.9±0.6 b |
| | Field | 16.1±0.6 | 16.9±1.1 | 17.4±0.9 | 14.6±1.0 | 15.6±0.9 | 17.0±0.9 | 15.2±0.7 |
| Cd (mg kg$^{-1}$) | Greenhouse | 0.06±0.04 bc | 0.11±0.06 c | 0.08±0.02 ab | 0.19±0.05 a | 0.07±0.03 abc | 0.16±0.04 ab | 0.00±0.00 d |
| | Field | 0.04±0.01 | 0.10±0.04 | 0.07±0.02 | 0.09±0.03 | 0.06±0.02 | 0.18±0.04 | 0.08±0.02 |
| Cr (mg kg$^{-1}$) | Greenhouse | 1.28±0.57 a | 0.23±0.07 bc | 1.41±0.23 a | 0.15±0.07 c | 0.99±0.18 a | 0.61±0.05 ab | 1.13±0.43 ab |
| | Field | 16.2±12.2 | 16.2±3.1 | 1.8±0.4 | 11.0±1.0 | 34.4±27.2 | 5.0±2.3 | 54.6±37.8 |
| Cu (mg kg$^{-1}$) | Greenhouse | 7.8±1.4 bc | 7.2±1.3 c | 8.5±1.2 bc | 13.0±1.8 a | 7.0±0.7 c | 10.3±1.4 ab | 6.4±0.5 c |
| | Field | 10.7±0.5 ABC | 9.9±0.6 BCD | 10.6±0.7 ABC | 9.2±0.3 D | 11.7±0.4 A | 9.7±0.2 CD | 11.2±0.4 AB |
| Fe (mg kg$^{-1}$) | Greenhouse | 139.8±45.8 ab | 76.2±6.3 c | 146.6±13.7 a | 97.6±10.3 bc | 87.8±3.4 bc | 73.1±9.5 c | 79.8±8.2 c |
| | Field | 1076±293 ABC | 775±115 BC | 590±157 C | 1817±589 A | 1179±274 AB | 633±62 BC | 1461±490 AB |
| K (g kg$^{-1}$) | Greenhouse | 5.61±1.31 bc | 7.30±1.83 abc | 5.87±1.03 bc | 8.25±1.32 ab | 5.55±1.06 bc | 9.67±1.52 a | 5.00±0.39 c |
| | Field | 23.7±1.5 | 22.3±1.2 | 25.4±1.2 | 22.7±2.0 | 24.6±1.6 | 24.2±1.2 | 19.9±0.8 |
| Li (mg kg$^{-1}$) | Greenhouse | 1.99±0.37 | 2.02±0.20 | 2.06±0.17 | 2.33±0.29 | 1.81±0.27 | 2.20±0.27 | 1.91±0.11 |
| | Field | 2.04±0.38 ABC | 1.68±0.23 BC | 1.53±0.28 C | 3.26±0.87 A | 2.21±0.23ABC | 1.57±0.20 C | 2.60±0.44 AB |
| Mg (g kg$^{-1}$) | Greenhouse | 5.18±0.42 | 5.34±0.12 | 5.13±0.19 | 5.92±0.35 | 4.84±0.27 | 5.33±0.36 | 5.53±0.08 |
| | Field | 2.97±0.14 | 3.09±0.16 | 3.27±0.22 | 2.83±0.18 | 3.04±0.11 | 3.17±0.14 | 3.01±0.16 |
| Mn (mg kg$^{-1}$) | Greenhouse | 160.9±34.0 b | 158.2±17.4 b | 178.2±17.8 b | 291.0±41.0 a | 171.4±24.5 b | 198.0±28.0 b | 157.6±2.8 b |
| | Field | 218.3±22.6 | 218.6±30.8 | 198.7±19.6 | 222.7±27.5 | 211.8±22.8 | 218.1±21.0 | 237.0±21.6 |
| Mo (mg kg$^{-1}$) | Greenhouse | 0.51±0.17 a | 0.30±0.13 a | 0.44±0.13 a | 0.50±0.16 a | 0.26±0.06 a | 0.77±0.22 a | 0.09±0.06 b |
| | Field | 0.59±0.07 | 0.83±0.25 | 0.30±0.08 | 0.45±0.15 | 0.46±0.14 | 0.34±0.21 | 0.68±0.12 |
| Na (g kg$^{-1}$) | Greenhouse | 6.74±0.51 a | 6.01±0.40 a | 6.05±0.40 a | 4.50±0.30 b | 6.12±0.29 a | 6.49±0.39 a | 6.68±0.32 a |
| | Field | 1.51±0.17 | 2.47±0.46 | 2.00±0.42 | 1.62±0.35 | 1.70±0.16 | 1.96±0.52 | 3.00±0.70 |
| Ni (mg kg$^{-1}$) | Greenhouse | 1.00±0.22 | 0.65±0.07 | 0.66±0.16 | 0.75±0.15 | 0.80±0.13 | 0.83±0.06 | 0.84±0.31 |
| | Field | 8.44±4.57 | 10.10±1.71 | 2.99±0.49 | 6.00±0.94 | 14.75±9.73 | 4.95±0.94 | 22.31±13.49 |
| P (g kg$^{-1}$) | Greenhouse | 1.61±0.32 | 2.22±0.42 | 2.07±0.20 | 2.44±0.35 | 1.96±0.23 | 2.38±0.37 | 1.85±0.10 |
| | Field | 1.80±0.12 | 1.59±0.12 | 1.80±0.15 | 1.39±0.17 | 1.92±0.17 | 1.49±0.09 | 1.80±0.14 |
| Pb (mg kg$^{-1}$) | Greenhouse | 0.36±0.07 c | 1.18±0.08 b | 0.35±0.03 c | 1.46±0.14 a | 0.37±0.03 c | 1.17±0.11 b | 0.07±0.02 d |
| | Field | 1.55±0.31 BC | 2.03±0.15 B | 1.08±0.20 C | 3.85±0.90 A | 1.71±0.24 BC | 2.02±0.09 B | 2.04±0.55 B |

**Table 4** (continued)

| Mineral | Experiment | Control | E060 | E071 | E140 | E202 | E244 | E631 |
|---|---|---|---|---|---|---|---|---|
| S (g kg⁻¹) | Greenhouse | 4.80±0.76 | 5.93±0.67 | 5.32±0.36 | 7.01±0.81 | 5.03±0.54 | 6.29±0.86 | 4.66±0.13 |
| | Field | 1.97±0.05 | 1.95±0.06 | 2.05±0.11 | 1.73±0.16 | 2.00±0.10 | 1.91±0.05 | 2.05±0.11 |
| Zn (mg kg⁻¹) | Greenhouse | 47.3±8.3 bc | 52.6±7.7 abc | 50.1±6.1 bc | 76.0±10.7 a | 48.4±8.3 bc | 64.3±6.2 ab | 39.7±0.3 c |
| | Field | 31.1±3.1 AB | 26.0±1.1 BC | 30.3±1.4 AB | 25.7±1.6 BC | 34.8±3.5 A | 24.3±1.5 C | 31.1±4.4 AB |

For each parameter, averages with the same letter (lowercase letters for greenhouse experiment and uppercase letters for field experiment) are not significantly different according to LSD test at a significance level of 0.05. When letters do not appear in a row, differences between endophytes were not significant according to ANOVA at a significance level of 0.05. Although the LSD test was performed on transformed variables, back-transformed values are represented to ease interpretation

significant. Fungal endophytes have also been found to affect photosynthesis and $CO_2$ fixation (Newman et al. 2003; Spiering et al. 2006), which could also explain the different growth rate. However further research should be done to determine whether our endophytes are capable of producing such an effect.

Bearing in mind that the quality of a forage in terms of nutritive value is directly proportional to its protein content and its digestibility (which is inversely correlated to fiber and lignin content), in the present study none of the endophytes was able to improve in general terms such quality in comparison with the controls. Several positive aspects of forage quality derived from the inoculation with endophytes can be found in the present study, but they were not consistent throughout the experiments (Table 5). For instance, in the greenhouse experiment, the endophyte E244 (Pleosporales) was able to reduce the neutral detergent fiber (NDF) content of the forage, but this effect was not consistent in the field experiment. Also in the greenhouse experiment, endophyte E140 (*S. globuliferum*) reduced acid detergent fiber (ADF), but in the field experiment the inoculation with this fungus provided forage with the highest values of lignin (ADL). The most interesting endophytes, at least from a herbage-yield promotion point of view, such as endophyte E202 (*Chaetosphaeronema* sp.), did not cause a deterioration in the quality of the forage, as the values of protein, fiber and lignin were similar to those of controls in both greenhouse and field experiments (Table 5).

Scarce information, and mainly focused on clavicipitaceous endophytes in grasses species, exists on the influence of endophytes on forage quality traits such as crude protein (CP) or neutral detergent fiber (NDF) content in the biomass. Even so, the lack of influence of endophytes on total CP content obtained in the present study had been previously reported by Oliveira et al. (2004) in perennial ryegrass. However, a significant effect of the endophyte infection, in this case on tall fescue, on total CP content and on distribution of the different CP fractions, was found by Newman et al. (2003). Regarding fiber, Zabalgogeazcoa et al. (2006) reported a significant reduction in NDF in grass biomass when an endophyte (*Epichloë* sp.) was present in plants. Another study (Cripps and Edwards 2013) on the faeces of animals fed with endophyte-infected forage also reported a reduction in NDF. This reduction in NDF could be explained by the production of hemicellulolytic enzymes by endophytes, as has already been reported in maize by Bischoff et al. (2009) working with the endophyte *Acremonium*

**Table 5** Summary of the effects of each endophyte on the different parameters evaluated (HDM: herbage dry matter; RDM: root dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; and mineral status) in both experiments, under greenhouse (G) and field (F) conditions. (+)(−) indicates if the endophyte significantly ($P < 0.05$) increased or reduced, respectively, the corresponding parameter in comparison with the non-inoculated controls

| Parameter | E060 G | E060 F | E071 G | E071 F | E140 G | E140 F | E202 G | E202 F | E244 G | E244 F | E631 G | E631 F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HDM | − | − | − | − | (−) | − | − | (+) | (−) | − | − | − |
| RDM | − | ** | − | ** | − | ** | − | ** | − | ** | − | ** |
| CP | − | − | − | − | − | − | − | − | − | − | − | − |
| NDF | − | (+) | − | − | − | − | − | − | (−) | (+) | − | − |
| ADF | − | (+) | − | − | (−) | − | − | − | − | (+) | − | (+) |
| ADL | (+) | (+) | − | − | − | (+) | − | − | − | (+) | − | − |
| Al | (−) | − | − | − | (−) | − | (−) | − | (−) | − | − | − |
| B | − | − | − | − | − | (−) | − | − | − | − | − | − |
| Ca | − | − | − | − | (+) | − | (−) | − | − | − | − | − |
| Cd | − | − | − | − | (+) | − | − | − | − | − | (−) | − |
| Cr | (−) | − | − | − | (−) | − | − | − | − | − | − | − |
| Cu | − | − | − | − | (+) | (−) | − | − | − | − | − | − |
| Fe | (−) | − | − | − | − | − | − | − | (−) | − | (−) | − |
| K | − | − | − | − | − | − | − | − | (+) | − | − | − |
| Li | − | − | − | − | − | − | − | − | − | − | − | − |
| Mg | − | − | − | − | − | − | − | − | − | − | − | − |
| Mn | − | − | − | − | (+) | − | − | − | − | − | − | − |
| Mo | − | − | − | − | − | − | − | − | − | − | (−) | − |
| Na | − | − | − | − | (+) | − | − | − | − | − | − | − |
| Ni | − | − | − | − | − | − | − | − | − | − | − | − |
| P | − | − | − | − | − | − | − | − | − | − | − | − |
| Pb | (+) | − | − | − | (+) | (+) | − | − | (+) | − | (−) | − |
| S | − | − | − | − | − | − | − | − | − | − | − | − |
| Zn | − | − | − | − | (+) | − | − | − | (−) | − | − | − |

** Root dry matter was determined only in the greenhouse experiment

*zeae*. However this was not observed consistently in our study. This lack of consistency between experiments (Table 5) and this lack of strong influence of endophytes on quality traits of forage could be explained by our environmental conditions, which might not be the most suitable to prompt such an effect. It has frequently been reported that significant effects of fungal endophytes might occur only under particular conditions, such as drought stress (Hesse et al. 2005) or high nutrient conditions (Newman et al. 2003), or if the proper combination of endophyte species, host genotype and environmental conditions takes place (Ahlholm et al. 2002).

Regarding mineral concentration contained in the herbage, there was also an evident lack of consistency between the results obtained in the greenhouse experiment and those in the field experiment (Table 5), which could be partially explained by the widely different concentration of these minerals in the substrate and soil

where the experiments were carried out respectively. Such lack of consistency, however, was not evidenced to the same extent in all the minerals and with all the endophytes. Regarding Al, as the substrate/soil values were much higher in the field than in the greenhouse, the Al concentration in plants was also much higher under field conditions. Aluminium is not essential for plants and animals. High concentrations of Al in soil, which mainly occurs with low pH (such as that found in the present study) might be toxic for plants, might reduce the uptake of P and Ca (Huang et al. 1992) and might have negative effects on lambs' appetite (Krueger et al. 1985). Consequently in those conditions, a reduction of Al uptake and later accumulation in forage may be highly desirable. Three endophytes, E140 (*S. globuliferum*), E202 (*Chaetosphaeronema* sp.) and E631 (*E. nigrum*), were able to reduce Al in forage in the greenhouse experiment, but not in the field. Therefore, those species

might not be completely suitable for this purpose. However, there were two species, E060 (*F. lateritium*) and E244 (Pleosporales) which were able two reduce Al concentration in both experiments (although in field conditions, not significantly in comparison with controls) and thus may deserve further experimentation in specific assays including different Al concentrations in soil.

*Fusarium lateritium* has been previously reported as an endophyte in wild grasses (Azliza et al. 2014), but also as a pathogen in several woody crops (Yun et al. 2013). Although in our study, inoculation with this species did not cause any disease symptoms, this pathogenic character, though small, could have been the cause of the lower accumulation of Cr and Fe, both essential nutrients for plant and animals. However, inoculation with *F. lateritium* also caused a higher accumulation of Pb in forage. Lead is one of the most frequently reported causes of poisoning in farm livestock, cattle being the most commonly affected species (Suttle 2010). Consequently, the effect caused by this fungal species, but also by E140 (*S. globuliferum*) and E244 (Pleosporales), might be clearly negative if this biomass is used with a feeding purpose. However such an effect could also have positive applications, for instance, it could be used in phytoremediation programs to gradually clean lead-contaminated areas, e.g., due to lead-mining activity, by means of subsequent culturing and removal of endophyte-infected plants. *S. globuliferum* was the endophyte which caused this effect most consistently, as was clearly observed under both greenhouse and field conditions. Conversely, endophyte E631 (*E. nigrum*) seemed to reduce the Pb values of the forage, but this effect was only observed under greenhouse conditions where the Pb levels in soil were much lower. The suitability and real potential of this eventual application of endophytes should be contrasted and further investigated in lead-contaminated areas or by designing specific experiments with different lead concentrations.

Several of the endophytes used in the present study, such as *S. globuliferum* or *F. lateritium*, have been reported to be pathogenic in several plant species where they produce metabolites derived from their activity, which can have phytotoxic effects (Debbab et al. 2009; Yun et al. 2013). For a future eventual application of endophytes in the field, farmers *a priori* may be reluctant to use fungal species reported as pathogens in other hosts. However, in our study, the inoculation of these fungi did not cause any symptoms of disease in *T. subterraneum* plants, which may indicate a lack of pathogenicity of the isolates used. This is an important basic requirement for an eventual application of these fungal endophytes. An additional basic requirement is that endophytes do not produce toxic forage for livestock. This fact should be further investigated as several associations of fungal endophyte-plant host have been shown to produce secondary metabolites, such as lolitrem B, ergovaline, peramine, etc., which can cause animal diseases (Fuchs et al. 2013; Thom et al. 2013).

Another interesting aspect regarding the influence of *Stemphylium globuliferum* was the increase in the accumulation of Zn in forage when the fungus was inoculated. Zinc, which is an essential micronutrient for humans and animals, is taken insufficiently in approximately 30 % of the world (Hotz and Brown 2004) because of the deficient Zn concentration in soils of many areas. Soils with Zn concentration lower than $25 \text{ mg kg}^{-1}$, such as those of the present study, are considered unsuitable to provide crops with sufficient Zn to accomplish intake requirements (Alloway 2009). Under these conditions, the use of fungal endophytes, which may increase Zn concentration in forage, could be a very promising strategy to remedy such deficiency and may deserve further research. In our study, the positive effect of Zn accumulation due to *S. globuliferum* was only observed in the greenhouse experiment, but not in the field. This fact could be explained by the three-fold higher Zn concentration in soils of field experiment in comparison with the substrate of greenhouse experiment. Because as has already been stated, in the microorganism-plant interaction the more unfavourable conditions for plants are, the more evident the observed effect (if any) might be.

In conclusion, the results presented here provide evidence that non-clavicipitaceous endophytes cause significant effects on the herbage production, nutritive value and mineral concentration of *Trifolium subterraneum* forage. The suitability of each endophyte may depend on the effect required. Of the endophytes studied, E202 (*Chaetosphaeronema* sp.) should be used to increase forage productivity. If a reduction in Al or Pb concentration is required, then either *Fusarium lateritium* or E244 (Pleosporales) and *Epicoccum nigrum*, respectively might be more adequate. An increase in essential nutrients for both plants and animals, such as Zn, was mainly produced by *Stemphylium globuliferum*.

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