

# Effect of Fungal Endophytes on Biomass Yield, Nutritive Value and Accumulation of Minerals in *Ornithopus compressus*

Oscar Santamaria<sup>1</sup> · Santiago Lledó<sup>1</sup> · Sara Rodrigo<sup>1</sup> · Maria J. Poblaciones<sup>1</sup>

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**Abstract** Yellow serradella (*Ornithopus compressus*), a valuable pasture species in Mediterranean areas, presents a high diversity of endophytic mycoflora. In the present work, the hypothesis of a significant effect of fungal endophytic species on the parameters of forage production, nutritive value and mineral status of herbage was tested. *O. compressus* plants were inoculated with each of seven endophytes (four in 2012/2013 and three in 2013/2014). After inoculation, two experiments (under greenhouse and field conditions) were established. Results evidenced a certain influence of several endophytes on herbage yield, nutritive value and mineral status of *O. compressus* forage. *Byssochlamys spectabilis* increased herbage biomass yield by around 42% in the field experiment. *Stemphylium* sp. improved the nutritive value of forage either by increasing crude protein, digestibility and the concentration of essential minerals (such as B, Mo, P or S) or by reducing the concentration of toxic elements such as Al or Pb. In conclusion, the results presented here provide evidence that plant inoculation with endophytes could be a suitable strategy to increase forage yield and its nutritive value or to deal with potential nutrient deficiencies or potential mineral toxicity in forage.

**Keywords** Yellow serradella · Mycoflora · Herbage · Protein and fibre · Pb toxicity

## Introduction

Yellow serradella (*Ornithopus compressus* L.) is an annual pasture species of Mediterranean origin. It is endemic to grasslands in southwestern Spain due to its adaptation to the Mediterranean climate and tolerance of sandy and acidic soils containing low levels of organic matter. The legume *O. compressus* has been widely used for recovering areas with degraded soils, as well as for amending soil structure and fertility [29]. Although extremely variable due to the inter-annual irregularity typical of the Mediterranean climate, herbage yield ranges between 2200 and 6000 kg dry matter (DM) ha<sup>-1</sup> [11, 28]. This species produces forage with an excellent nutritive value, high in crude protein (CP) concentration and digestibility. Values reported at full bloom stage are CP of 217–290 g kg<sup>-1</sup> DM, acid detergent fibre (ADF) of 188–231 g kg<sup>-1</sup> DM and levels of metabolizable energy (ME) of 11.2–12.0 MJ kg<sup>-1</sup> DM [11]. For this reason, *O. compressus* is commonly used for animal feeding. It has been used as a sown fodder crop in its native European area, but it has also been introduced to other Mediterranean climate areas, mainly Chile [10] and Australia [5, 23]. Its use is mostly seen in Australia where it is cultivated alone or mixed with pink or French serradella (*O. sativus* Brot.), subterranean clover (*Trifolium subterraneum* L.), and/or other clover and *Biserrula* species [2, 12].

Forage nutritive value, mineral content and forage yield of pasture species can be affected by soil, climate, plant phenology and diseases [25, 34]. Fungal endophytes, organisms which inhabit plant tissues without causing any disease symptoms, have also been shown to affect some of the productive parameters in other pasture species, such as *Festuca rubra* L. [37]. These endophytic fungi frequently have beneficial effects on the host plants, increasing their adaptive value, especially under stress conditions, and protecting them against

✉ Oscar Santamaria  
osantama@unex.es

<sup>1</sup> Departamento de Ingeniería del Medio Agronómico y Forestal, Universidad de Extremadura, Avda, Adolfo Suárez s/n, 06007 Badajoz, Spain

different plant pathogens [8, 19, 31, 36]. These positive effects caused by endophytes have shown to bring about increments in biomass production and/or in regrowth of the plant host, as has previously been observed in endophyte-infected plants by using different endophyte-host combinations, such as *Neotyphodium* spp.-*Lolium perenne* L. or *Chaetosphaeronema* sp.-*Trifolium subterraneum* L. [13, 16].

Similarly, several endophytes, such as *Epichloë festucae* Leuchtm., Schardl and Siegel, have been found to increase the nutritive value of forage, for instance by increasing the organic matter digestibility in different hosts such as *F. rubra* [37]. Moreover, the uptake capacity of host plants and later accumulation of minerals in forage can be modified by fungal endophytes [15]. In some cases, the fungus was able to increase uptake of some minerals such as P, Ca or Mg [37]. However, the literature reports also the opposite effect, i.e. a decrease in uptake of other minerals such as Al [18] or Zn [16].

Nevertheless, the effects caused by fungal endophytes on their hosts appear to be variable and clearly dependent on species of endophyte, host genotype and environmental conditions [1]. Therefore, if the final objective is improvement of the productive parameters of forage crops by using fungal endophytes, each endophyte-host system should be specifically studied for each environmental condition. To date, most studies dealing with effects of fungal endophytes on plant biomass productive parameters have been carried out on grasses, especially on *Lolium* and *Festuca* species. Few studies on endophyte influence have been performed on leguminous species, and none on serradella. Therefore, the objective

of this study was to evaluate the effect of seven fungal endophytes, isolated from pasture species, on biomass production (herbage and root biomass), nutritive value (protein, fibre, lignin and ash content) and mineral status of *O. compressus* forage.

## Materials and Methods

### Fungal and Plant Material

The effect of fungal endophytes on biomass production, nutritive value and mineral status of *O. compressus* forage was evaluated after the inoculation of each fungus into 2-month-old seedlings of this plant host. The seven fungal endophytes used in the experiments were previously isolated from the herbage of pasture species and identified in our laboratory by morphologic and molecular procedures (Table 1). The isolation and identification process was similar to that described by Lledó et al. [17]. Endophyte identification was first based on morphologic characteristics and then on comparison to ITS sequences in GenBank with a similarity criterion  $\geq 99\%$ . Four of the endophytes (E060: *Fusarium* sp., E071: *Sordaria fimicola*, E140: *Stemphylium* sp. and E636: *Sporormiella intermedia*) were selected because they had been quite frequently isolated from several pasture hosts [17], a fact which could reflect an eventual important ecological role. Selection of the other three species (E063: *Mucor hiemalis*, E346: *Fusarium equiseti* and E408: *Byssoschlamys spectabilis*) was

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

Isolate number	GenBank accession no.	Plant host	Geographic origin	UTM coordinates (zone 29 north datum) (m)	Identification <sup>a</sup>
E060	KP698325	<i>Ornithopus compressus</i> L.	Moheda de Olalla (Cáceres)	X = 704,216 Y = 4,349,073	<i>Fusarium</i> sp.
E063	KP899388	<i>O. compressus</i> L.	La Barrosa (Cáceres)	X = 692,853 Y = 4,357,660	<i>Mucor hiemalis</i> Wehmer
E071	KP698327	<i>O. compressus</i> L.	Jerez de los Caballeros (Badajoz)	X = 685,420 Y = 4,243,932	<i>Sordaria fimicola</i> (Rob. ex Desm.) Ces. & De Not.
E140	KP698338	<i>O. compressus</i> L.	Haza (Cáceres)	X = 748,510 Y = 4,428,827	<i>Stemphylium</i> sp.
E346	KP899431	<i>Medicago polymorpha</i> L.	Valdesequera (Badajoz)	X = 685,365 Y = 4,325,603	<i>Fusarium equiseti</i> (Corda) Sacc.
E408	KP899436	<i>Biserrula pelecinus</i> L.	Higuera de Vargas (Badajoz)	X = 675,686 Y = 4,258,595	<i>Byssoschlamys spectabilis</i> (Udagawa & Shoji Suzuki) Houbraken & Samson
E636	KP698330	<i>O. compressus</i> L.	Abadía (Cáceres)	X = 751,761 Y = 4,461,076	<i>Sporormiella intermedia</i> (Auersw.) S.I. Ahmed & Cain ex Kobayasi

<sup>a</sup> Based on morphological characters and on comparison to ITS sequences in GenBank with a similarity  $\geq 99\%$

based on the observation of some interesting properties related to plant protection in previous experiments carried out in our laboratory (data not yet published). In order to obtain sufficient inoculum for the seedling infection, 2 months before the experiments, two plugs (5-mm diameter) of the actively growing mycelia of each fungus growing on potato dextrose agar (PDA) plates were added to 1.5-L flasks containing 1 L culture medium potato dextrose broth (PDB) and incubated at 25 °C in the dark. Flasks were shaken manually every 3 days during 5 min.

Seeds of *O. compressus* cv ‘Mazagón’ 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 7 × 7 × 6 cm plastic pots containing sterilized (1 h at 121 °C, twice) soil substrate consisting of a 1:1 (v/v) mixture of perlite and peat (COMPO SANA Universal, Compo GmbH & Co. KG, Münster, Germany). On dried and homogenized soil substrate samples, pH was determined using a calibrated pH meter (ratio 10 g soil 25 mL deionized H<sub>2</sub>O), extractable P by Olsen procedure [27]; Ca and K were extracted with ammonium acetate (1N) and quantified by complexometric titration method [7] and using a K-ion-selective electrode (ISE 96 61, Crison Instruments S.A., Barcelona, Spain), respectively. Electrical conductivity (EC) was determined using an EC-meter. Total N was determined using the Kjeldahl method [6], with the aid of a Kjeltect™ K350 distillation Unit (Buchi Ltd., Flawil, Switzerland). Total Al, B, Ca, Cu, Fe, K, Li, Mg, Mn, Mo, Na, P, Pb, S, Se and Zn were determined by the Ionomics Service of the CSIC (Spanish High Centre for Science and Research) by means of inductively coupled plasma optical emission spectrometry (ICP-OES) after digestion with HNO<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> in UltraClave Microwave Milestone. The soil substrate properties are described in Table 2.

The experiment was carried out over 2 years, 2012/2013 (for endophytes E060, E071, E140 and E636) and 2013/2014 (for endophytes E063, E346 and E408). In both study years, the sowing date was in early December. After sowing, 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 Fig. 1. In order to avoid interactions between the evaluated endophytes and those previously established naturally, which could have some effect on our results, plants were treated with a systemic fungicide before inoculations. The first fungicide treatment was applied on 1-month-old plants and was repeated twice every 7 days. Around 1 mL per pot of solution obtained by adding 1 mL of fungicide (Amistar Xtra®, Syngenta, Madrid, Spain) 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. This procedure was similar to that used in previous works with similar objectives [37].

**Table 2** Soil substrate (greenhouse experiment) and soil (field experiment) properties expressed as mean ± standard error (SE) from four samples ( $n = 4$ )

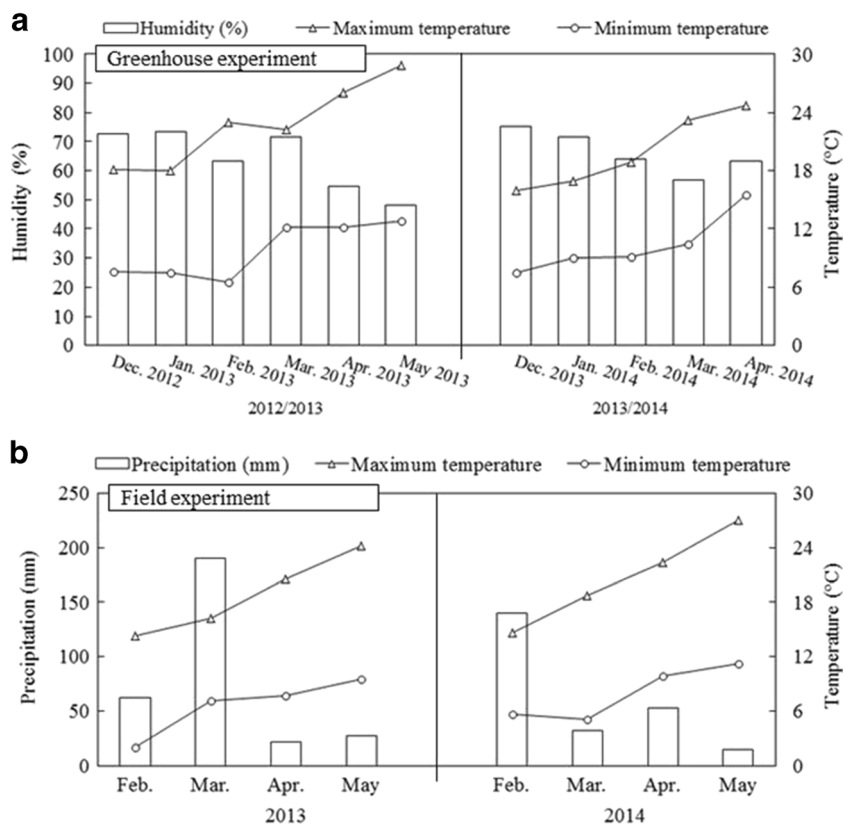
Parameter	Soil substrate	Field soil
pH (1:2.5)	4.43 ± 0.01	5.35 ± 0.12
EC (S m <sup>-1</sup> ) (1:5)	0.05 ± 0.01	0.86 ± 0.06
N <sub>tot</sub> (g kg <sup>-1</sup> )	9.6 ± 0.2	1.28 ± 0.11
P <sub>Olsen</sub> (mg kg <sup>-1</sup> )	5.11 ± 0.30	10.03 ± 1.65
K (mg kg <sup>-1</sup> )	238.51 ± 0.01	115.3 ± 27.9
Ca (g kg <sup>-1</sup> )	7.23 ± 0.07	0.68 ± 0.09
Al <sub>tot</sub> (g kg <sup>-1</sup> )	0.53 ± 0.03	17.94 ± 0.89
B <sub>tot</sub> (mg kg <sup>-1</sup> )	3.19 ± 0.17	5.52 ± 0.12
Ca <sub>tot</sub> (g kg <sup>-1</sup> )	5.16 ± 0.50	0.83 ± 0.04
Cu <sub>tot</sub> (mg kg <sup>-1</sup> )	5.19 ± 0.64	6.84 ± 0.31
Fe <sub>tot</sub> (g kg <sup>-1</sup> )	0.44 ± 0.08	11.40 ± 0.49
K <sub>tot</sub> (g kg <sup>-1</sup> )	1.21 ± 98.98	3.39 ± 0.12
Li <sub>tot</sub> (mg kg <sup>-1</sup> )	4.61 ± 0.74	17.04 ± 0.82
Mg <sub>tot</sub> (g kg <sup>-1</sup> )	0.52 ± 0.01	1.03 ± 0.05
Mn <sub>tot</sub> (mg kg <sup>-1</sup> )	26.55 ± 2.51	539.50 ± 39.46
Mo <sub>tot</sub> (mg kg <sup>-1</sup> )	< 0.01	< 0.01
Na <sub>tot</sub> (g kg <sup>-1</sup> )	3.86 ± 0.67	0.28 ± 0.01
P <sub>tot</sub> (mg kg <sup>-1</sup> )	286.39 ± 13.46	175.70 ± 7.87
Pb <sub>tot</sub> (mg kg <sup>-1</sup> )	2.07 ± 0.18	16.09 ± 0.85
S <sub>tot</sub> (mg kg <sup>-1</sup> )	765.59 ± 13.46	77.73 ± 5.57
Se <sub>tot</sub> (mg kg <sup>-1</sup> )	< 0.01	0.82 ± 0.16
Zn <sub>tot</sub> (mg kg <sup>-1</sup> )	6.86 ± 56.51	18.99 ± 0.66

## Inoculations

In order to verify that plants were free of endophytes, just before inoculation, four plants were randomly selected and taken to the laboratory. These plants were then surface-disinfested by one immersion for 30 s in 95% ethanol, and then another immersion for 1 min in 2% NaClO plus two drops Tween-80 per litre. Next, five 5-mm-long segments were cut from different parts of each plant and placed in a Petri dish containing PDA supplemented with 50 mg/L chloramphenicol to suppress bacterial growth. Plates were incubated at 25 °C in the dark and observed daily during the first 2 weeks and weekly during the following 2 months.

The plants of ten pots (each containing a set of five plants) were inoculated with each endophyte. An additional ten pots were inoculated only with culture medium to be used as control (thus a total of 50 pots were inoculated in 2012/2013 and 40 pots were inoculated in 2013/2014). Inoculations were carried out by following the procedure indicated by Lledó et al. [16], which had been shown to produce effective infections. Four weeks after the last fungicide application, plants were wounded by puncturing their leaves and stems with a homemade tool composed of two arms, one of which had a multiple needle structure at the end and the other a smooth surface [16].

**Fig. 1** Mean relative humidity or monthly precipitation and mean maximum and minimum temperatures during the greenhouse (a) and field (b) experiments in the two study years (2012/2013 and 2013/2014)



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 at a medium speed during approximately 5 min. The homogenized inoculum was then placed in hand sprayer.

In order to evaluate the viability of the mycelium after blending, Petri dishes containing PDA were sprayed with the homogenized inoculum. New colonies of each fungus were observed after a few days. Inoculation was carried out with the aid of the 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. Each plant received the following amount of mycelia depending on the endophytic species inoculated: E060 (*Fusarium* sp.) 56.4 mg plant<sup>-1</sup>, E063 (*M. hiemalis*) 74.0 mg plant<sup>-1</sup>, E071 (*S. fomicola*) 143.2 mg plant<sup>-1</sup>, E140 (*Stemphylium* sp.) 815.4 mg plant<sup>-1</sup>, E346 (*F. equiseti*) 134.0 mg plant<sup>-1</sup>, E408 (*B. spectabilis*) 63.8 mg plant<sup>-1</sup>, E636 (*S. intermedia*) 282.6 mg plant<sup>-1</sup>. During 48 h following the inoculations, plants were maintained in a high humidity atmosphere in order to maximize fungal infection.

### Experimental Design and Response-Variable Determinations

The experiment was carried out under the greenhouse conditions described before. A set of five plants (i.e. one pot) was

considered as the experimental unit. The pots containing the inoculated plants were arranged on greenhouse benches by following a completely randomized design (with five replicates or pots per endophyte treatment), as no environmental variations in the experimental area were expected. Pots were placed at least 5 cm apart, without direct contact, to avoid secondary infections. In order to ensure the effectiveness of the inoculations, approximately 1 month later, plant samples of each treatment were taken to the laboratory for re-isolations following the same procedure as that carried out to verify that plants were free of endophytes.

In order to evaluate eventual pathogenic behaviour of the endophytes used in the present study on *O. compressus*, plants were visually examined weekly (over 8 weeks starting 1 week after inoculation) to check for disease symptoms (yellowing, drying, rotting leaves, blackish spots, etc.). According to the severity of these symptoms, the plants in each pot were assigned to one category on the following scale: 1 = healthy, 2 = slightly affected, 3 = moderately affected, 4 = severely affected and 5 = dead. Disease progress curves for each pot were constructed by plotting the values of disease severity over time. The area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids, considering one unit per period between two consecutive measurements. The AUDPC was used as response variable to evaluate disease severity.



Three months after inoculations (i.e. early May for both years of study, 2012/2013 and 2013/2014), herbage and roots were harvested and taken to the laboratory for processing. Roots were carefully washed with tap water to remove soil substrate. In the laboratory, samples were oven-dried (70 °C) until constant weight. After that, DM of herbage (HDM) and root (RDM) biomass production was recorded. From part of the herbage samples, the following parameters were determined: CP by multiplying biomass  $N \times 6.25$  (protein on average contains 16% N) as conversion factor (biomass N was obtained by Kjeldahl distillation (distillation unit K-350, Buchi, Flawil, Switzerland), neutral detergent fibre (NDF), ADF and acid detergent lignin (ADL) by means of a fibre analyzer (ANKOM 8-98, ANKOM Technology, Macedon, NY, USA), and total ash by ignition in a muffle furnace at 600 °C by following the official procedures [3]. The other part of the herbage samples was sent to the Ionomics Service of the CSIC for mineral determinations as described above for the soil samples.

Only five out of the ten pots inoculated per endophytic treatment were used in the greenhouse experiment. The remaining five pots were used to evaluate how transferrable the results obtained in the greenhouse were under field conditions. This evaluation was performed on the ‘herbage biomass yield’ response variable, as it is considered one of the most important parameters for farmers. Approximately 1 month after the inoculations, the second half of the pots were transported to an experimental area located in the grassland ‘Valdesequera’ owned by the regional government of Extremadura. This area is located in Badajoz, southwest Spain (UTM coordinates, zone 29 north datum  $X = 685,365$  m;  $Y = 4,325,603$ ) in Alfisol Xeralf 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 herbaceous legume species. On these soil samples, all the edaphic characteristics were determined as explained above for the substrate samples (Table 2). The climatic data during the experiments, which were taken from a weather station located close to the study site, can be observed in Fig. 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  $\times$  50 cm. Two months and a half after transplanting (i.e. mid-May for both study years, 2012/2013 and 2013/2014), herbage was harvested and taken to the laboratory for processing. The herbage DM production obtained in the field experiment (FDM) was determined as explained above for the HDM.

## Statistical Analysis

Under greenhouse conditions, the evaluated effects of the endophytes (five treatments, including controls in 2012/2013, and four treatments, including controls in 2013/2014) were as follows: disease severity (estimated as the area under the disease progress curve, AUDPC); herbage and root dry matter yield (HDM and RDM, respectively); nutritive value parameters (CP, NDF, ADF, ADL and ash) and herbage mineral concentration (Al, B, Ca, Cu, Fe, K, Li, Mg, Mn, Mo, Na, P, Pb, S, Se and Zn). Data were subjected to a one-way ANOVA for each year separately. Under field conditions, the effect of the endophyte treatment was also evaluated by one-way ANOVA on herbage dry matter yield (FDM). 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 AUDPC; and  $\sqrt[5]{x}$  for HDM, RDM and FDM. All these analyses were performed with the Statistix v. 8.10 package.

## Results

### Effects on Disease Severity and Biomass Yield

In the greenhouse experiment, none of the endophytes caused disease severity on the *O. compressus* plants, as the area under the disease progress curve (AUDPC) was not significantly affected by the endophyte treatment (Table 3). With regard to biomass yield, herbage production was not significantly affected by the endophyte treatment in the two study years (Table 3). Herbage dry matter ranged between (mean  $\pm$  standard error)  $0.68 \pm 0.13$  g pot<sup>-1</sup> and  $1.55 \pm 0.58$  g pot<sup>-1</sup> in 2012/2013, and  $0.65 \pm 0.10$  g pot<sup>-1</sup> and  $0.94 \pm 0.12$  g pot<sup>-1</sup> in 2013/2014 (Fig. 2a). In the case of root dry matter, this was significantly affected by the endophyte treatment in 2012/2013, but not in 2013/2014 (Table 3). In the case of 2012/2013, plants inoculated with the endophyte E060 (*Fusarium* sp.) presented a root biomass 47% lower than that of the controls (Fig. 2a).

In the field experiment, herbage yield (FDM) was not significantly affected by the endophyte treatment in 2012/2013, but it was in 2013/2014 (Table 3). In the latter case, the endophyte E408 (*B. spectabilis*) caused an increment of about 42% in the herbage yield in comparison with the controls (Fig. 2a). On average, the herbage yield obtained in 2013/2014 was much higher ( $9.37 \pm 0.54$  g pot<sup>-1</sup>) than in 2012/2013 ( $2.32 \pm 0.19$  g pot<sup>-1</sup>).

### Effects on Nutritive Value Parameters

The endophyte treatment affected significantly CP, lignin (ADL) and total ash obtained in herbage in 2012/2013 and

**Table 3** Summary of the one-way ANOVAs showing the effect of the endophytes on the AUDPC (area under the disease progress curve,  $\text{Ln}(x + 1)$  transformed), on the biomass production (root RDM and herbage HDM; both  $\sqrt[5]{x}$  transformed); CP crude protein, NDF neutral detergent fibre, ADF acid detergent fibre, ADL acid detergent lignin, and ashes in plants of *Ornithopus compressus* at the end of the greenhouse experiment, and on the herbage biomass (FDM,  $\sqrt[5]{x}$  transformed) of the *O. compressus* plants at the end of the field experiment in the two study years (2012/13 and 2013/14)

Source	Endophyte treatment					
	2012/2013			2013/2014		
	df	F	P value	df	F	P value
AUDPC	4	0.36	0.8341	3	0.92	0.4404
RDM (g DM pot <sup>-1</sup> )	4	3.07	0.0257	3	1.36	0.2711
HDM (g DM pot <sup>-1</sup> )	4	2.13	0.0922	3	2.31	0.0929
FDM (g DM unit <sup>-1</sup> )	4	0.38	0.8241	3	3.49	0.0255
CP (%)	4	6.37	0.0102	3	1.15	0.3862
NDF (%)	4	0.95	0.4773	3	3.11	0.0884
ADF (%)	4	1.80	0.2053	3	5.16	0.0283
ADL (%)	4	3.51	0.0488	3	0.51	0.6886
Ashes (%)	4	17.68	0.0002	3	0.87	0.4941

df degrees of freedom, F F value. In italics P values  $\leq 0.05$

ADF in 2013/2014 (Table 3). In 2012/2013, inoculation with endophytes E060 (*Fusarium* sp.), E140 (*Stemphylium* sp.) or E636 (*S. intermedia*) provided forage with higher CP values in comparison with those obtained in the controls (87.54, 62.61 and 80.87%, respectively; Fig. 2b). The forage derived from plants inoculated with any of the same three endophytes (E60, E140 or E636) presented the lowest values of ADL and total ashes (Fig. 2c, d). Plants inoculated with endophyte E140 (*Stemphylium* sp.) presented ADL values significantly lower (18%) than those found in the controls (Fig. 2c). In the case of total ash content, the three endophytes provided forage with values approximately 64% lower than the controls (Fig. 2d). In 2013/2014, inoculation with any of the three endophytes studied, E063 (*M. hiemalis*), E346 (*F. equiseti*) or E408 (*B. spectabilis*), provided forage with higher ADF than that obtained in the control plants (12.67, 17.04 and 13.45%, respectively; Fig. 2c).

### Mineral Uptake and Accumulation in Herbage

The ANOVA showed that, while in 2012/2013, the endophyte treatment affected significantly the concentration in plants of Al, B, Fe, Mn, Mo, P, Pb, S and Zn (Table 4), in 2013/2014, it affected only the concentration of Al and Mg (Table 5). In 2012/2013, forage of the plants inoculated with any of the four studied endophytes presented higher concentrations of B, Mo, P, S and Zn (on average around 16, 114, 31, 35 and 38%, respectively) than in the controls (Table 4). From this

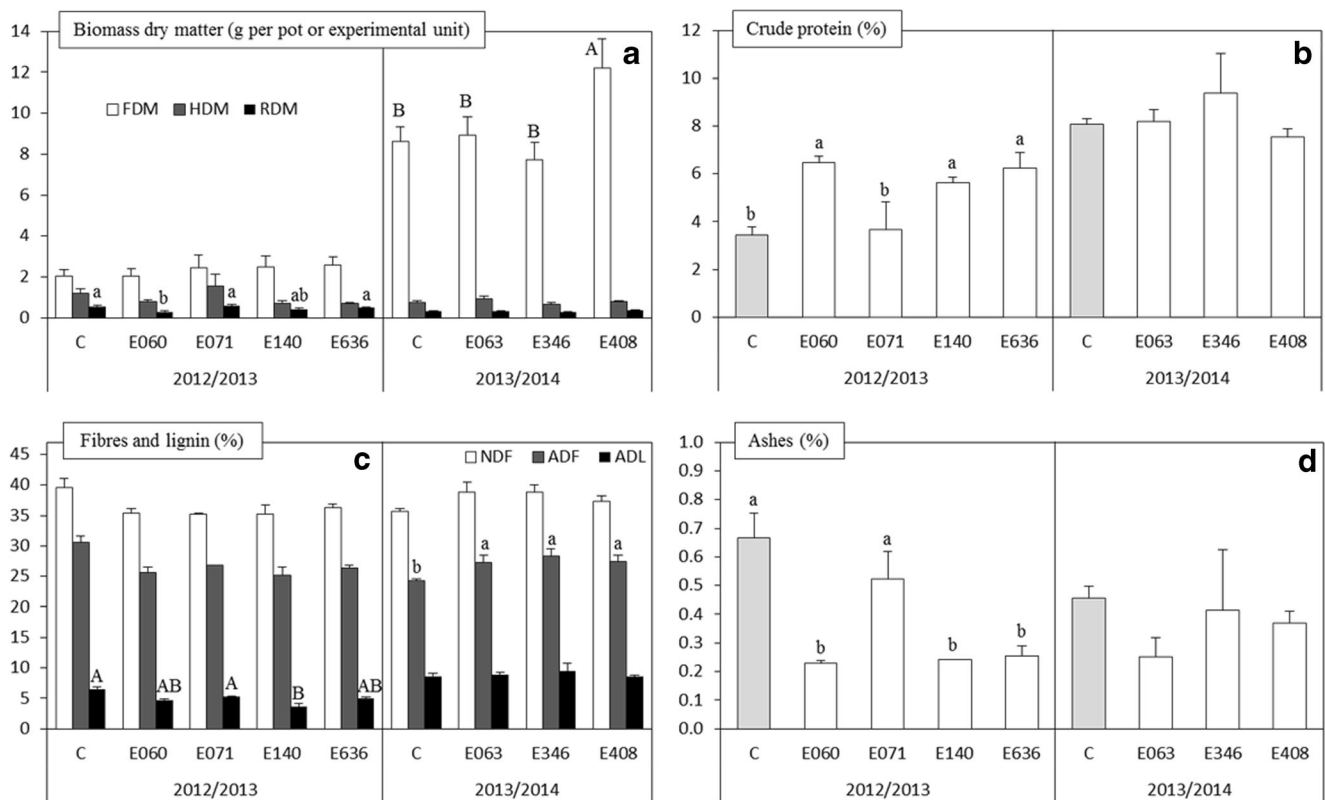
general pattern, only inoculation with *S. fimicola* (E071) and *Stemphylium* sp. (E140) did not cause significant differences in the concentration of P and Zn, respectively, in comparison with the controls. Regarding the concentration of Al, Fe and Pb, the pattern observed in plants inoculated with either E060 (*Fusarium* sp.), E140 (*Stemphylium* sp.) or E636 (*S. intermedia*) was quite similar. In the three cases, endophyte E140 caused a strong decrease in Al, Fe and Pb concentrations in the forage (around 74, 49 and 46%, respectively) than in the controls (Table 4). Inoculation with either endophyte E060 (*Fusarium* sp.) or E636 (*S. intermedia*) also resulted in a significant but more moderate decrease in concentration of Al, Fe and Pb (on average around 51, 22 and 25%, respectively) than in the controls (Table 4). Likewise, Pb concentration in the forage was also negatively affected by *S. fimicola* (E071), with values 25% lower than the controls. This fungus, in addition to E636 (*S. intermedia*), also provoked a higher concentration of Mn (37 and 24%, respectively) in comparison with the controls (Table 4).

In 2013/2014, the forage of the plants inoculated with any of the three studied endophytes presented a lower concentration of Al than in the controls; around 31, 37 and 26%, for E063 (*M. hiemalis*), E346 (*F. equiseti*) and E408 (*B. spectabilis*), respectively. In the case of the Mg concentration, only plants inoculated with the endophyte E063 (*M. hiemalis*) presented values 11% lower than those observed in the control plants (Table 5).

### Discussion

In the re-isolation process carried out before inoculations in order to check the efficiency of the systemic fungicide, no endophytic colonies were observed in the culture medium growing from any of the plant segments. The procedure used, based on fungal culturability, does not permit us to state with absolute certainty that plants were devoid of fungus, as quiescent cells or spores not culturable under the tested conditions could have persisted. However, if the fungicide treatment was able to remove the whole culturable mycobiota, which was that analysed, it seems likely that this treatment had also been efficient against the non-culturable fungi, at least at a level which could allow establishment and further spread of the inoculated endophyte. This is supported by the fact that all the isolates were positively re-isolated and identified in culture medium and also because when any of the endophytic species were inoculated, significant effects were found in at least one of the evaluated parameters (Table 6).

Removal of the pre-existing fungi was carried out to evaluate the actual effect of the studied endophytes on their host under the best possible controlled conditions, so that comparisons could be made between them. Untreated plants may have contained a high mycobiota load with a complex fungal



**Fig. 2** Effect of endophyte inoculation in 2012/2013 and 2013/2014 on a biomass yield (*FDM* herbage dry matter in the field experiment, *HDM* and *RDM* herbage and root DM, respectively, in the greenhouse experiment), **b** crude protein (*CP*), **c** fibre (*NDF* and *ADF*) and lignin (*ADL*) and **d** ashes. Vertical bars indicate means and vertical lines standard error. For each parameter and study year, averages with the

same letter are not significantly different according to LSD test at a significant level of 0.05. When letters do not appear, the influence of the endophyte treatment was not significant ( $\alpha > 0.05$ ) according to ANOVA. *C* control, *E060* *Fusarium* sp., *E063* *Mucor hiemalis*, *E071* *Sordaria fimicola*, *E140* *Stemphylium* sp., *E346* *Fusarium equiseti*, *E408* *Byssoschlamys spectabilis*, *E636* *Sporormiella intermedia*

diversity of a variable and undetermined nature. Therefore, the use of such untreated plants would have meant microbiologically uncontrolled conditions during the experiments. Likewise, the uninoculated controls would have been exposed to the effect of undetermined potentially active microbiota, thus possible either enhancing or counteracting the measured effects. It is clear that with this technique, we are creating an artificial environment where eventual competition has been mostly eliminated. Furthermore, in the present study, inoculations were performed after wounding the plants in order to facilitate infection of the endophytes. Again, with this procedure, we are creating an artificial route for endophytes to reach the inner part of plant tissues.

The results obtained, therefore, cannot be considered as directly transferrable to real in-field agriculture, since a future application of the new inoculants should not require fungicide treatment and/or previous plant wounding as a pre-requisite to observe the expected effects. As many of the endophytes used in the present study were selected because they had been very frequently isolated naturally from several pasture hosts, it seems likely that they have a strong competitive ability and a good aptitude to naturally colonize plant tissues. In any case, the study presented here should be understood as a previous

necessary search for endophytic candidates, requiring further investigation in order to be used in the future as growth-promoting inoculants for plants. Future experiments should include natural conditions, to evaluate endophytes' ability to enter plants by natural mechanisms and to produce the required effects in environments subjected to natural competition with pre-existing microorganisms. In addition, different inoculation procedures or the development of endophyte-based products (which could eventually produce the same effects) should also be tested in order to optimize the effectiveness of the application. Another interesting line of research which could be explored is the possibility of vertical transmission (i.e. from mother plant to offspring, via seeds) in the studied endophytes, since this phenomenon has been found to occur more frequently and in more non-clavicipitaceous endophyte species than commonly thought [14]. In this way, the use of endophytes in in-field agriculture on a large scale might become more feasible, as no further treatments other than seeding with already-infected seeds would be necessary for farmers.

For inoculum production, all the endophytes tested were incubated under the same conditions for 2 months. Since each endophyte presented a different growth rate, the amount of

**Table 4** Concentrations (mean  $\pm$  standard error) of each mineral in the herbage obtained from the greenhouse plants inoculated with each endophyte in 2012/2013

Mineral	Treatment					ANOVA summary		
	Control (C)	<i>Fusarium</i> sp. (E060)	<i>Sordaria fimicola</i> (E071)	<i>Stemphylium</i> sp. (E140)	<i>Sporormiella intermedia</i> (E636)	<i>df</i>	<i>F</i>	<i>P</i> value
Al (mg kg <sup>-1</sup> )	91.5 $\pm$ 13.7 a	41.4 $\pm$ 5.0 bc	74.2 $\pm$ 6.4 a	23.5 $\pm$ 1.2 c	48.2 $\pm$ 11.1 b	4	14.50	<0.001
B (mg kg <sup>-1</sup> )	23.4 $\pm$ 1.3 b	28.3 $\pm$ 1.9 a	27.2 $\pm$ 0.6 a	26.8 $\pm$ 0.7 a	26.5 $\pm$ 0.5 a	4	3.62	0.050
Ca (g kg <sup>-1</sup> )	15.5 $\pm$ 0.1	14.3 $\pm$ 0.6	13.3 $\pm$ 1.7	14.2 $\pm$ 0.4	14.6 $\pm$ 0.5	4	1.24	0.354
Cu (mg kg <sup>-1</sup> )	5.0 $\pm$ 0.5	5.9 $\pm$ 0.2	5.6 $\pm$ 1.4	6.7 $\pm$ 0.2	6.9 $\pm$ 0.3	4	2.01	0.169
Fe (mg kg <sup>-1</sup> )	112.5 $\pm$ 10.4 a	83.9 $\pm$ 2.1 b	98.5 $\pm$ 9.5 ab	57.2 $\pm$ 3.1 c	91.1 $\pm$ 4.2 b	4	13.68	0.001
K (g kg <sup>-1</sup> )	13.3 $\pm$ 1.7	18.6 $\pm$ 2.3	13.4 $\pm$ 4.7	18.2 $\pm$ 1.2	18.3 $\pm$ 0.6	4	1.76	0.214
Li (mg kg <sup>-1</sup> )	3.4 $\pm$ 0.2	3.6 $\pm$ 0.3	2.7 $\pm$ 0.5	3.3 $\pm$ 0.2	3.6 $\pm$ 0.4	4	2.17	0.147
Mg (g kg <sup>-1</sup> )	4.5 $\pm$ 0.3	5.0 $\pm$ 0.3	5.3 $\pm$ 0.1	5.2 $\pm$ 0.2	5.1 $\pm$ 0.1	4	2.73	0.097
Mn (mg kg <sup>-1</sup> )	223.8 $\pm$ 25.8 c	260.2 $\pm$ 3.1 bc	307.4 $\pm$ 17.5 a	258.4 $\pm$ 7.8 bc	278.1 $\pm$ 2.8 ab	4	5.85	0.013
Mo (mg kg <sup>-1</sup> )	1.1 $\pm$ 0.3 b	2.3 $\pm$ 0.5 a	2.0 $\pm$ 0.0 a	2.5 $\pm$ 0.2 a	2.6 $\pm$ 0.0 a	4	6.15	0.012
Na (g kg <sup>-1</sup> )	6.2 $\pm$ 0.7	7.0 $\pm$ 0.5	6.5 $\pm$ 0.4	5.9 $\pm$ 0.2	6.0 $\pm$ 0.3	4	1.58	0.254
P (g kg <sup>-1</sup> )	4.2 $\pm$ 0.5 b	5.3 $\pm$ 0.4 a	4.9 $\pm$ 0.0 ab	5.5 $\pm$ 0.3 a	5.7 $\pm$ 0.1 a	4	3.73	0.047
Pb (mg kg <sup>-1</sup> )	2.6 $\pm$ 0.3 a	1.9 $\pm$ 0.2 b	1.9 $\pm$ 0.1 b	1.4 $\pm$ 0.1 c	2.0 $\pm$ 0.2 b	4	6.58	0.007
S (g kg <sup>-1</sup> )	8.2 $\pm$ 0.9 b	10.9 $\pm$ 0.9 a	10.3 $\pm$ 0.5 a	11.0 $\pm$ 0.6 a	12.0 $\pm$ 0.2 a	4	6.12	0.012
Se (mg kg <sup>-1</sup> )	0.52 $\pm$ 0.2	0.86 $\pm$ 0.1	0.69 $\pm$ 0.0	0.57 $\pm$ 0.1	0.57 $\pm$ 0.1	4	2.22	0.147
Zn (mg kg <sup>-1</sup> )	66.0 $\pm$ 8.0 b	86.6 $\pm$ 9.6 a	90.3 $\pm$ 2.0 a	79.9 $\pm$ 4.7 ab	96.4 $\pm$ 6.3 a	4	4.03	0.038

Averages in the same row, with *different lowercase letters*, are significantly different ( $\alpha \leq 0.05$ ) according to LSD test. When letters do not appear, differences were not significant according to the *P* value obtained from the ANOVA (in *italics P* values  $\leq 0.05$ )

*df* degrees of freedom, *F* *F* value

inoculum produced for each endophyte after 2 months of incubation was different. However, we decided to use the same incubation time for all of the endophytes rather than the same inoculum amount, in order to establish the comparisons between them by focusing on the inoculum production process. In any case, the inoculum produced by all of the endophytes after 2 months of incubation could be considered sufficient to infect the plant. The eventual effect caused by a fungus on the host, once it has spread inside the plant, is expected to be due to its specific aptitude to produce this effect rather than to the initial amount of inoculum of the endophyte. This is supported by studies carried out by Spiering et al. [32], which show that the effects caused by a fungal endophyte on its host were independent of *in-planta* endophyte concentration.

Several studies have shown a significant and positive effect of endophytes on herbage and root biomass productivity in several hosts (e.g. Assuero et al. [4], Hesse et al. [13], Lledó et al. [16]). However, most of those experiments were performed under certain limitations for plant growth: water stress, deficiencies in mineral nutrition, soil mineral toxicity, pest or disease infections, etc. Therefore, it would seem to be clear that since the endophyte can give a higher adaptive value to the plant host, the effect of the microorganism-plant interaction might be more evident under stressful conditions for plants. This fact may explain why in our study, under good environmental conditions in the greenhouse, none of the

endophytes affected herbage biomass yield. However, under the less favourable conditions of the field experiment, inoculation with the endophyte E408 (*B. spectabilis*) produced around 42% more herbage than the controls.

To explain this increase in herbage yield caused by endophytes, several authors have proposed that these organisms could affect photosynthesis and CO<sub>2</sub> fixation [22, 32], which could produce a higher growth rate in the plant host. On the other hand, fungal endophytes have also been found to cause a lengthening of the vegetative growth period of their plant host [37], which is the period with a more active biomass accumulation. Nevertheless, the mechanisms involved in this aspect are not fully understood. In some cases, endophyte occurrence could prevent or delay inflorescence emergence, thus postponing the beginning of the reproductive period, in which biomass production is considerably reduced. Other authors such as Assuero et al. [4] 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 that it may promote root growth, giving plants a higher capacity to absorb water and minerals. Regarding the production of hormone-like substances, *B. spectabilis* has been shown to be an important source of secondary metabolites (as summarized by Mioso et al. [20]); it has been found to produce cornexistin, a secondary metabolite with herbicidal activity [21], and several other bioactive compounds, such as viriditoxin or



**Table 5** Concentrations (mean  $\pm$  standard error) of each mineral in the herbage obtained from the greenhouse plants inoculated with each endophyte in 2013/2014

Mineral	Treatment				ANOVA summary		
	Control (C)	<i>Mucor hiemalis</i> (E063)	<i>Fusarium equiseti</i> (E346)	<i>Byssoschlamys spectabilis</i> (E408)	<i>df</i>	<i>F</i>	<i>P</i> value
Al (mg kg <sup>-1</sup> )	79.6 $\pm$ 2.2 a	55.0 $\pm$ 8.6 b	49.6 $\pm$ 5.1 b	58.6 $\pm$ 5.8 b	3	6.62	0.019
B (mg kg <sup>-1</sup> )	28.5 $\pm$ 0.5	26.4 $\pm$ 0.7	30.3 $\pm$ 1.9	29.3 $\pm$ 1.0	3	3.04	0.092
Ca (g kg <sup>-1</sup> )	17.4 $\pm$ 0.2	16.5 $\pm$ 0.5	18.0 $\pm$ 0.3	17.4 $\pm$ 0.5	3	3.95	0.053
Cu (mg kg <sup>-1</sup> )	5.3 $\pm$ 0.0	4.9 $\pm$ 0.3	5.6 $\pm$ 0.3	5.1 $\pm$ 0.2	3	2.52	0.132
Fe (mg kg <sup>-1</sup> )	121.4 $\pm$ 0.9	98.4 $\pm$ 9.8	116.5 $\pm$ 15.2	103.4 $\pm$ 12.5	3	1.44	0.301
K (g kg <sup>-1</sup> )	15.9 0.3 $\pm$	13.7 $\pm$ 1.7	17.5 $\pm$ 2.4	15.4 $\pm$ 1.0	3	1.46	0.297
Li (mg kg <sup>-1</sup> )	2.9 $\pm$ 0.3	2.2 $\pm$ 0.3	1.9 $\pm$ 0.3	2.1 $\pm$ 0.2	3	2.97	0.097
Mg (g kg <sup>-1</sup> )	5.4 $\pm$ 0.1 a	4.8 $\pm$ 0.1 b	5.3 $\pm$ 0.2 a	5.4 $\pm$ 0.2 a	3	4.83	0.033
Mn (mg kg <sup>-1</sup> )	267.8 $\pm$ 4.4	208.8 $\pm$ 0.6	259.2 $\pm$ 34.8	262.9 $\pm$ 25.0	3	2.44	0.139
Mo (mg kg <sup>-1</sup> )	1.4 $\pm$ 0.1	1.3 0.3 $\pm$	1.3 $\pm$ 0.4	1.4 $\pm$ 0.2	3	0.22	0.878
Na (g kg <sup>-1</sup> )	8.1 $\pm$ 0.4	8.6 $\pm$ 0.8	7.8 $\pm$ 1.1	7.9 $\pm$ 0.6	3	0.37	0.779
P (g kg <sup>-1</sup> )	4.9 $\pm$ 0.1	4.3 $\pm$ 0.5	4.6 $\pm$ 0.4	4.9 $\pm$ 0.0	3	1.31	0.336
Pb (mg kg <sup>-1</sup> )	0.3 $\pm$ 0.0	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1	3	0.93	0.472
S (g kg <sup>-1</sup> )	8.2 $\pm$ 0.2	6.8 $\pm$ 0.4	8.0 $\pm$ 0.7	7.9 $\pm$ 0.3	3	2.94	0.099
Se (mg kg <sup>-1</sup> )	< 0.1	< 0.1	< 0.1	< 0.1			
Zn (mg kg <sup>-1</sup> )	94.2 $\pm$ 2.1	78.2 $\pm$ 6.1	88.1 $\pm$ 11.7	92.2 $\pm$ 4.5	3	1.53	0.278

Averages in the same row, with *different lowercase letters*, are significantly different ( $\alpha \leq 0.05$ ) according to LSD test. When letters do not appear, differences were not significant according to the *P* value obtained from the ANOVA (in *italics P* values  $\leq 0.05$ )

*df* degrees of freedom, *F* *F* value

varioxepine, with antimicrobial properties [26, 38]. The ability of the multiple compounds produced by this fungus to generate physiological responses in its plant hosts has not been investigated. Therefore, we are not able to know with certainty whether any of these compounds could act as a hormone-like substance or whether they could promote the production of phytohormones by plant hosts. Further experiments should be specifically designed to clarify the mechanisms of this increase in herbage biomass yield.

It may also be that the increase in herbage biomass yield caused by the endophyte is due to a fertilizer effect of the inoculum applied (composed by mycelia growing in 1 L of PDB medium), rather than to the direct action of the living endophyte interacting with its host plant. However in this case, this possibility seems to be unlikely for several reasons: first, if the inoculum applied had a fertilizer effect, a higher amount of inoculum applied would have resulted in greater growth, but this was not the case. The increase in herbage production was only observed for one of the endophytes, and it was not that which had highest inoculum production during the incubation time before inoculations. Secondly, the effect on plant growth was not observed until plants were transferred to the field (Table 6); if the inoculum applied had a fertilizer effect, such an influence would also have appeared, and more intensively, in the greenhouse experiment, where

plants were growing in small pots with a limited amount of substrate. Once plants were transplanted into the field, the importance of the substrate contained in the pots in the nutrition of plants might be lesser than that in the field soil, which did not receive additional nutrients. Consequently, the most probable explanation is that the observed effects on plant growth could be attributed to the activity of the living endophyte inside the plant, rather than to a fertilizer effect of the inoculum applied.

Taking into account that the quality of a forage is directly proportional to the protein content and to its digestibility (which is inversely correlated to fibre and lignin content), inoculation with the endophytes E60 (*Fusarium* sp.), E140 (*Stemphylium* sp.) or E636 (*S. intermedia*) provided forage with a higher nutritive value than that obtained in the controls (Table 6). These three fungi produced either a higher crude protein or reduced lignin content in the resulting forage. Authors such as Zabalgoceazcoa et al. [37], who also found a positive influence of endophytes (in this case *E. festucae*) on the digestibility of the organic matter in plants of *Festuca rubra*, *F. arundinacea* Schreb and *L. perenne*, have suggested endophytes to cause a delay in the maturity of the plant to explain this fact. Since throughout the plant life cycle, fibre and lignin values increase whereas protein values decrease [30], such a delay may have produced the higher protein

**Table 6** Summary of the effects of each endophyte on the different parameters evaluated. Symbols (+) and (-) indicate whether the endophyte significantly ( $P < 0.05$ ) increased or reduced, respectively, the corresponding parameter in comparison with the non-inoculated controls

Parameter	E060	E063	E071	E140	E346	E408	E636
AUDPC							
RDM	(-)						
HDM							
FDM						(+)	
CP	(+)			(+)			(+)
NDF							
ADF		(+)			(+)	(+)	
ADL				(-)			
Ashes	(-)			(-)			(-)
Al	(-)	(-)		(-)	(-)	(-)	(-)
B	(+)		(+)	(+)			(+)
Ca							
Cu							
Fe	(-)			(-)			(-)
K							
Li							
Mg		(-)					
Mn			(+)				(+)
Mo	(+)		(+)	(+)			(+)
Na							
P	(+)			(+)			(+)
Pb	(-)		(-)	(-)			(-)
S	(+)		(+)	(+)			(+)
Se							
Zn	(+)		(+)				(+)

AUDPC area under the disease progress curve, RDM root dry matter biomass, HDM herbage dry matter biomass, FDM herbage dry matter biomass in the field experiment, CP crude protein, NDF neutral detergent fibre, ADF acid detergent fibre, ADL acid detergent lignin; ashes content in the herbage; and mineral status), E060 *Fusarium* sp., E063 *Mucor hiemalis*, E071 *Sordaria fimicola*, E140 *Stemphylium* sp. E346 *Fusarium equiseti*, E408 *Byssosclamyces spectabilis*, E636 *Sporormiella intermedia*

content and the lower lignin content observed in the present study. However, further experiments including an exhaustive analysis of the exact growth stage of the plant after inoculations should be performed in order to confirm this hypothesis.

Changes in the nutritional status of the forage derived from plants infected with fungal endophytes has also been observed in *T. subterraneum* or *Poa pratensis* L. growing under good and controlled conditions [15, 16]. In these studies, two of the isolates evaluated in the present work, E60 (*Fusarium* sp.) and E140 (*Stemphylium* sp.), were also used. In both cases, E140 (*Stemphylium* sp.) was also found to cause a decrease in fibre or lignin content of the forage, but E60 (*Fusarium* sp.) did not. This might indicate, as suggested by other authors [1], that a

significant influence of the endophyte might occur only if the proper combination of endophyte species, host genotype and environmental conditions occurs.

Considerable differences were found between the study years in several of the parameters analysed, especially in herbage biomass yield obtained in the field experiment and in protein content of the forage. It could be that these differences were due to the different endophytes inoculated in each study year. However, the same differences were also observed between the controls. To explain this fact, it is important to bear in mind that the field experiment was carried out in an area with a typical Mediterranean climate, characterized by a great inter-annual irregularity, especially in precipitations which widely affect herbage yield. The climatic conditions of 2013/2014 (Fig. 1), with higher rainfall in February and April, seemed to favour plant growth in comparison with the conditions of 2012/2013. This high inter-annual irregularity highlights the importance of the controls to be included each year and prevents comparison between the years. Furthermore, temperatures greatly affect the plant growth cycle, producing a shortening in the plant vegetative stage as temperatures increase. As is already known, protein values of herbage can decrease during the plant life cycle [30]. This fact might explain the rather lower protein content of the forage in 2012/2013, in which the temperatures were considerably warmer than in 2013/2014 (Fig. 1).

With regard to mineral concentration contained in the herbage, Al is not essential for plants and animals. In fact, high concentrations of Al in the soil, which are mainly found when pH is low (as in the present study), may be toxic for plants and animals. Consequently, in those conditions, a lower Al uptake and later accumulation in forage may be highly desirable. Although most of the endophytes were able to reduce Al in forage (Table 6), endophyte E140 (*Stemphylium* sp.) was especially effective in this aspect, reducing the Al concentration by almost 75% in comparison with the controls. This endophyte was also the most effective in reducing accumulation of Pb in forage when it was inoculated. Lead poisoning is one of the most frequently reported causes of poisoning in farm livestock, cattle being the most commonly affected species [33]. Therefore, in areas with high lead concentration in the soil, due for example to lead-mining activity, the use of this fungus could reduce uptake and later accumulation of Pb in the forage to a suitable level for animal feeding, which should be lower than 5 mg kg<sup>-1</sup> DM according to the maximum permitted level [24]. The ability of *Stemphylium* sp. to reduce Al and Pb concentrations in the host plant has also been evidenced in other forage legume species such as *T. subterraneum* L. [16]. This fact seems to suggest that this endophyte may not present high host specificity, since it is able to establish effective association with different hosts and to consistently produce the indicated effect regardless of the host. This is an interesting aspect as it may allow use of this fungus for this purpose at a broader range.

Similarly, this species (*Stemphylium* sp.), and also E60 (*Fusarium* sp.), E071 (*S. fimicola*) and E636 (*S. intermedia*), produced forage with concentrations of B, Mo, P, S and Zn higher than in the controls in most of the cases (Table 6). These minerals are essential nutrients for both plants and animals. Therefore, although the concentration obtained in the present work even in the controls was higher than the recommended minimum concentration in pasture dry matter for grazing cattle and sheep [33], the increase in accumulation caused by the endophyte should be considered as positive, at least up to the maximum tolerable levels, established as 150 mg B kg<sup>-1</sup> DM, 5 mg Mo kg<sup>-1</sup> DM, 6 g P kg<sup>-1</sup> DM, 5 g S kg<sup>-1</sup> DM and 300 mg Zn kg<sup>-1</sup> DM [24]. Several species, closely related to the studied endophytes, such as *Stemphylium globuliferum* (Vestergr.), *Fusarium lateritium* Nees or *Paecilomyces variotii* Bainier, have been reported to be pathogenic in several plant species in which they produce metabolites derived from their activity, which can have phytotoxic effects [9, 20, 35]. This is an important issue with regard to a future eventual application of the studied endophytes, because if they showed pathogenicity or had phytotoxic effects, their use would have to be avoided. In the present case, none of the endophytes inoculated caused disease symptoms in plants or a loss in forage productivity, thus indicating a lack of pathogenicity of the isolates used and a lack of phytotoxic effects. An additional basic requirement to an eventual in-field application of these endophytes is that they should not produce toxic forage for livestock. Although according to the literature the studied endophytes do not seem to produce toxic compounds for animals, this fact should be further verified before use.

In conclusion, the results presented here provide evidence that endophytes can affect herbage yield, nutritive value and nutrient content of yellow serradella forage. According to our results, plant inoculation with endophytes could be a suitable strategy to increase forage yield and nutritive value, or to deal with potential nutrient deficiencies or with potential mineral toxicity in forage. The suitability of each endophyte might depend on the required effect. Of the endophytes studied, *B. spectabilis* in particular might increase herbage biomass yield, and *Stemphylium* sp. might improve the nutritive value of forage either by increasing crude protein, digestibility and the concentration of essential minerals, or by reducing the concentration of toxic elements such as Al or Pb.

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