*SYMBIOSIS* (2005) 40, 23–30  ISSN 0334-5114

# Interaction between grass endophytes and mycorrhizas in *Bromus setifolius* from Patagonia, Argentina

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*(Received April 1, 2005; Accepted June 5, 2005)*

**Abstract**
The interaction between grass endophytes and mycorrhizas of *Bromus setifolius* from Patagonia, Argentina was examined. To determine effects of the endophyte (*Neotyphodium* sp.) on the colonisation of *B. setifolius* by arbuscular mycorrhizal fungi (AM), we analysed roots collected from the field and we also experimentally evaluated the association. Two populations of *B. setifolius* differing in endophyte colonisation were grown in either presence or absence of AM fungi using two different sources of soil. We also analysed the combined influence of these fungi on host growth. Roots of endophyte-colonised populations (E+) obtained from the field showed a higher frequency of colonisation by AM fungi than noncolonised populations (E–). The assay showed that there was a significant difference in the extent of colonisation of roots between the two populations used. E+ population roots were colonised more extensively than those of E– populations. The E+ population also showed increased growth characteristics in comparison to the E– population. The source of soil did not affect any of the host parameters analysed. For the first time, a positive interaction between *Neotyphodium* endophytes and arbuscular mycorrhiza is reported.

**Keywords**: Arbuscular mycorrhizal fungi, grass endophytes, fungal interaction, plant growth, *Neotyphodium*

## 1. Introduction

Grass endophytes and mycorrhizas are two symbioses with aerial and subterraneous plant tissue respectively. These two plant-fungal relationships are generally considered mutualistic (Clay, 1990; Hodge et al., 2001; Newsham et al., 1995).

Many cool-season grasses are infected by *Neotyphodium* endophytes. These anamorphic endophytes develop an intercellular systemic colonisation throughout the aerial tissues involving a substantial fungal biomass (Schardl and Clay, 1997; White, 1987). Although they are related to, and derived from, pathogenic, sexually-reproducing balansiaceous fungi, the anamorphic grass endophytes do not cause disease symptoms (Schardl and Clay, 1997; White, 1987). They are transmitted vertically by seeds, although conidia have been observed in the phylloplane of *Agrostis hiemalis* and *Poa rigidifolia* (White et al., 1996).

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Experimental attempts to initiate infections by conidia have been unsuccessful to date (White et al., 1996). Several beneficial effects of endophyte colonisation to host plant survival have been reported. Endophyte-colonised plants may show an increase ability to survive under stressful environments. Endophyte colonisation increases tillering, reproduction and growth of the plant, relieves drought tolerance and decreases the plant susceptibility to insect feeding (Arechevaleta et al., 1989; Bacon and Siegel, 1988; Johnson et al., 1985; Latch et al., 1985; Novas et al., 2003; Siegel et al., 1987; White et al., 2001). Arbuscular mycorrhizal (AM) fungi form symbiotic associations with the roots of the majority of herbaceous plant species (Harley and Smith, 1983; Trappe, 1987). The host plant gains several potential benefits from colonisation, including enhanced uptake and transport of poorly mobile soil nutrients, improved water relations, and reduced pathogenic infections (Abbott and Robson, 1984; Allen and Allen, 1986; Newman and Reddel, 1987; Newsham et al., 1995).

AM fungi are common mutualistic symbionts of plant roots from the grasslands to deserts (Brundrett, 1991), thus,

interactions between fungal endophytes and AM fungi could be common in grasses (Clay, 1992). However, most work conducted so far has focused on direct plant-fungal interaction (Clay, 1992), and the possible interaction between grass endophytes and mycorrhizas is poorly known (Barker, 1987; Chu-Chou et al., 1992; Guo et al., 1992; Gange, 2001; Vicari et al., 2002).

*Bromus setifolius* J. Presl is a perennial grass with an extensive distribution in Patagonia. As for many cool-season grasses, it could be associated with *Neotyphodium* endophytes. A survey of the endophyte incidence in *B. setifolius* has been carried out along 800 Km from Southeast to Northwest Patagonia steppe, Santa Cruz province, Argentina (Novas et al., 2000; Novas, 2004). *B. setifolius* association with AM fungi also has been reported in the Patagonian steppe (Fontela et al., 2001). The aim of this study was to examine the interaction between *Neotyphodium* endophytes and mycorrhizas of *B. setifolius* from Patagonia, Argentina and to determine the possible endophyte effect on the colonisation by mycorrhizal fungi in field-collected roots and in a greenhouse experiment. We also determined the influence of both symbiotic fungi on the host plant.

## 2. Materials and Methods

A survey of endophyte colonisation at 6 sites chosen from a previous study (Novas et al., 2000; Novas, 2004) was conducted in South Patagonia, Santa Cruz province, Argentina. At each site, approximately 2–3 culms of 20 different plants of *B. setifolius* were sampled at random. Populations were named P1 to P6. The vegetation typical of populations P1, P2, P3 and P4 was scattered tussock grasses as *Festuca magellanica*, *F. pallescens*, *Poa rigidifolia* and *Stipa* sp. interspersed with bare soil patches. While the vegetation of populations P5 and P6 was *B. setifolius* growing in association with *Mulinum spinosum* (Cav.) Pers., "neneo", a shrub species and other grasses as *Stipa* and herbaceous plants. The frequency of endophyte-colonised plants in these populations has been previously determined (Novas et al., 2000; Novas, 2004) by microscopical examination of parenchyma of leaf culms stained with aniline blue (Clark et al., 1983). Culms were identified as endophyte-infected if typical non-branching intercellular mycelium was evident among plant parenchyma tissues (White, 1987). The populations studied differed in the percentage of endophyte colonisation. P1 and P2 evidenced 0% of colonisation (E–), P3 and P4 presented 43% and 45% respectively (E+/E–), and P5 and P6, 100% and 72% of colonisation respectively (E+).

*Study system*

The immense territory in southern Argentina, known as Patagonia, is considered as a cool semi-desert (Soriano, 1983). The climate of the extra-Andean Patagonia is characterised by precipitation below 300 mm and also by strong winds that cause high evaporation rates (Soriano, 1983). In addition, low mean annual temperature and extreme cold winters create severe restrictions to plant growth and result in a short growing season (Ravetta and Soriano, 1998). Soils in Patagonia present characteristics mostly related to the arid condition under which they have evolved (Ares et al., 1990). *Bromus setifolius* J. Presl (nomenclature follows Cámara Hernández (1978) and Mathei (1986)) is a common tussock grass with an extensive distribution in Patagonia (Gutiérrez and Penseiro, 1998).

*Field-study*

In order to estimate the mycorrhizal colonisation level at field condition, roots of 10 *B. setifolius* plants were collected from each population. When possible, the whole root system was collected. They were washed to remove free soil and preserved in vials with FAA (10% formalin: 5% acetic acid: 50% ethanol). Later, the roots were stained in trypan blue (Phillips and Hayman, 1970) and 50 pieces per plant (each approximately 1 cm long) were selected at random. To determine the percentage of mycorrhizal colonisation, the slide method was used (Giovannetti and Mosse, 1980). To study differences in colonisation within populations one E+/E– population (P4) and one E+ population (P5) were selected at random. Differences in mycorrhizal colonisation percentages between populations and between infected plants and non-infected plants within populations P4 and P5 were analysed with a one-way ANOVA. All assumptions were tested and did not require transformation.

*Correlation between mycorrhizal colonisation and soil nutrients*

To study possible associations between soil nutrients and mycorrhizal colonisation, soil samples of the upper horizon (5–15 cm) were taken in four sites, P2, P3, P5 and P6. Populations were chosen considering endophyte colonisation level and differences in ecological characteristics of the sites, such as vegetation cover. Samples were subjected to the following analyses (according to Jackson (1981), unless indicated otherwise): pH in water 1:25; total C (Walkley-Black); total N (Kjeldahl, modified by Ritcher (1980)); C.E.C. (ammonium acetate 1 N, pH 7), $Ca^{++}$, $Mg^{++}$, $Na^{+}$ and $K^{+}$. Mycorrhizal colonisation percentage was correlated with soil nutrients by a Pearson correlation, considering mycorrhizal colonisation percentage as a dependent variable and amount of individual soil nutrients as independent variables.

*Greenhouse-study*

*Experimental design and statistics*

To test the effect of endophyte status on the mycorrhizal

root colonisation, an experiment was performed in a greenhouse. Two populations of the six analysed in the field study P2 (E–) and P6 (E+) were used. The populations were chosen considering endophyte status, seed availability (for simplicity, cariopses enveloped by lemma and palea will also be denominated "seeds") and differences in ecological characters.

AM inoculum appeared to be more abundant in habitats composed of a higher biomass of vegetation, as in the shrub patches in association with grasses observed in P5 and P6, than in those with a lower biomass, such as grass patches detected in P1 to P4. Therefore, the differences in mycorrhizal colonisation found in the field-collected roots could be attributable to differences in natural inoculum and/ or properties of the soil. To determine if soil affected the frequency of mycorrhizal colonisation, seed was sown on native soil from two different sites and mycorrhizal colonisation rates were compared.

The experiment had a 3×2×2 design with mycorrhizal colonisation of the host (mycorrhizas/ microorganisms/ control) combined with endophyte status and with the native soil source from two different places (soil from P2 and P6). There were thus 12 different treatments, each with five replication pots.

The effects of endophyte status, AM status and soil source on the growth parameters of the host were recorded. The variables studied were leaf length, shoot and root dry weight and rate mortality. Length was considered as the longest leaf of each plant. Data for mycorrhizal colonisation were analysed using a two-way ANOVA. Means were compared with LSD tests. No transformation was required. Data sets for the host responses were analysed using a three-way ANOVA. All assumptions were tested. Root dry weight was log-transformed for normality and homogeneity of variances.

Results of ANOVA presented here are from transformed root weight. But the means presented in Fig. 3a are not transformed. Analysis of the leaf length did not require any transformation. After unsuccessful trials to transform the data so as to reach the requirement for a parametric test, the variable shoot dry weight was analysed using a Kruskal-Wallis non-parametric test.

Mortality rate was analysed studying the effect of each factor. Rate mortality due to mycorrhizal treatment was analysed by means of chi-square test with a 3×2 contingency table. Endophyte and soil treatment effects were examined using chi-square tests corrected for continuity.

*Soil and seedling preparation*

The AM inoculum was the native soil itself collected from P2 and P6. The soil was sieved (2 mm), the root fragments were cut in approximately 1 cm long pieces and then the soil was homogenised. Then, it was diluted with sterilised sand (1:3 v/v). In the mycorrhizal treatment, the control treatment was established by sterilising the soil at 100°C for 1h for three consecutive days. To assess the effect of nonmycorrhizal soil microbes, the steam-sterilised soil was inoculated with a soil extract [20 ml $pot^{-1}$ of soil/water mixture (1:10 v/v) filtered through a Whatman no. 1 paper] re-inoculating the native microbiota.

Seeds of both populations were surface-sterilised with a water-sodium hypochlorite (1:1 v/v) and then were germinated in moist paper towels inside Petri dishes. The seedlings were selected for uniformity in size (3 cm long) and transplanted into 12 cm × 12 cm pots filled with the soil appropriate to each treatment. The seedlings were grown in a greenhouse with temperatures between 18°C and 35°C and watered to saturation with distilled water once a week. The plants were harvested after six months.

At harvest time the length of the longest leaf was recorded. The dry weights of shoots and roots were recorded after drying in an oven at 80°C for two days (or constant weight). Before drying the roots, a third part of them was separated for staining. The percentage of root length infected by AM fungi was estimated by examining stained samples using the slide method (Giovannetti and Mosse, 1980). The presence of *Neotyphodium* endophyte in the seedlings used in the assays was corroborated by examination of sheaths with aniline blue after the drying treatment using a microscope.

## 3. Results

*Mycorrhizal colonisation in field-collected roots*

Arbuscular mycorrhizal fungal structures were observed in roots of the six populations examined. Most of the root systems of field-collected plants showed hyphae from AM fungi spreading cell to cell and forming coils in each cell without intercellular hyphae. The extent of root colonisation varied significantly between populations ($F=16.81$; $P=0.0000$). Roots of endophyte-colonised populations showed higher mycorrhizal colonisation. Mean comparisons by LSD test indicate the arrangement of the populations in three groups. Populations 1 (E–) and 6 (E+) presented the lowest and the highest frequency of mycorrhizal colonisation respectively, forming two groups.

The rest of the populations comprised one group with intermediate frequencies (Fig. 1). Differences in root colonisation percentage between E+ and E– plants within mixed populations, P4 ($F=0.97$; $P=0.3529$) and P5 ($F=3.68$; $P=0.0914$), were not significant.

*Nutrients correlation*

Mycorrhizal colonisation was significantly correlated with only 2 (C and N) out of the 10 chemical characters analysed (Table 1). However, the C/N ratio did not show any significant differences among populations.

Table 1. Pearson correlation coefficients (CC) between mycorrhizal colonisation and nutrients in soil, in populations P2, P3, P5 and P6.

| Nutrients | Root colonisation | |
|---|---|---|
| | CC | P |
| PH | 0.163 | 0.837 |
| C | 0.982 | 0.018 |
| N | 0.989 | 0.011 |
| C/N | −0.633 | 0.367 |
| P | −0.484 | 0.516 |
| CEC | 0.778 | 0.222 |
| Ca | 0.614 | 0.385 |
| Mg | −0.336 | 0.669 |
| Na | −0.399 | 0.6 |
| K | 0.838 | 0.161 |

Table 2. Analysis of Variance (two-way ANOVA) on the effects of *Neotyphodium* endophyte status and soil source on arbuscular mycorrhizal colonisation of *Bromus setifolius* plants.

| Source | df | Mean square | F | P |
|---|---|---|---|---|
| Endophyte status (A) | 1 | 12557.6 | 20.53 | 0.003 |
| Soil source (B) | 1 | 311.9 | 0.51 | 0.4854 |
| A*B | 1 | 163.4 | 0.27 | 0.6123 |
| Error | 16 | 611.5 | | |

Table 3. Analysis of Variance (three-way ANOVA) on the effects of *Neotyphodium* endophyte status, arbuscular mycorrhiza status and soil source on root dry weight and leaf length of *Bromus setifolius* plants. Root dry weight was log-transformed for normality and homogeneity of variances. Leaf length data did not require any transformation.

| Variable/Source | df | Mean square | F | P |
|---|---|---|---|---|
| **Root dry weight** | | | | |
| Mycorrhiza status (A) | 2 | 0.908 | 0.92 | 0.405 |
| Soil source (B) | 1 | 0.023 | 0.03 | 0.871 |
| Endophyte status (C) | 1 | 42.289 | 42.98 | <0.001 |
| A*B | 2 | 2.071 | 2.1 | 0.134 |
| A*C | 2 | 3.019 | 3.07 | 0.056 |
| B*C | 1 | 3.307 | 3.36 | 0.074 |
| A*B*C | 2 | 1.376 | 1.4 | 0.258 |
| Error | 43 | 0.984 | | |
| **Leaf length** | | | | |
| Mycorrhiza status (A) | 2 | 85.73 | 7.46 | 0.002 |
| Soil source (B) | 1 | 6.02 | 0.52 | 0.473 |
| Endophyte status (C) | 1 | 5688.97 | 495.15 | <0.0001 |
| A*B | 2 | 1.97 | 0.17 | 0.843 |
| A*C | 2 | 4.84 | 0.42 | 0.658 |
| B*C | 1 | 15.75 | 1.37 | 0.248 |
| A*B*C | 2 | 0.24 | 0.02 | 0.978 |
| Error | 42 | 11.48 | | |

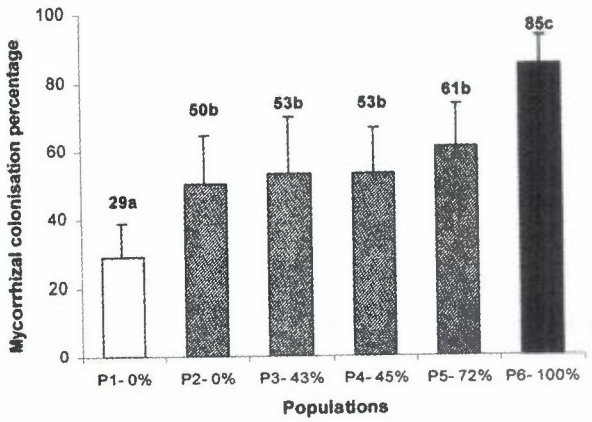


Figure 1. Mycorrhiza colonisation percentage between native populations of *Bromus setifolius* differing in *Neotyphodium* endophyte colonisation. Different letters above bars indicate significant differences between soil sources (P<0.05, LSD test). Percentage following population number indicates *Neotyphodium* endophyte frequency.

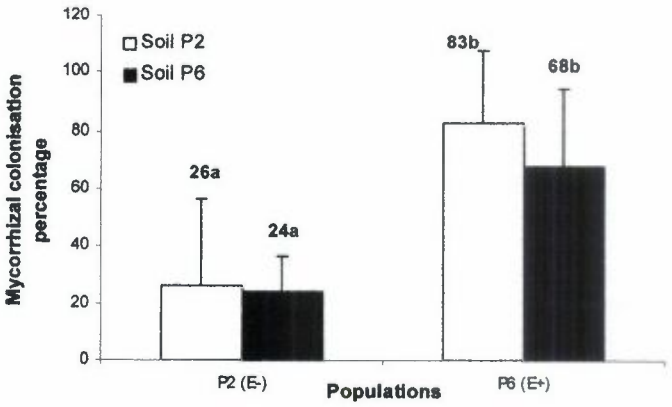


Figure 2. Effects of *Neotyphodium* endophyte status and soil source on arbuscular mycorrhizal colonisation of *Bromus setifolius*. Different letters above bars indicate significant differences between soil sources (P<0.05, LSD test) on arbuscular mycorrhizal colonisation of *B. setifolius*. Endophyte status: E+ = endophyte colonised plants; E– = endophyte free plants.

*Mycorrhizal colonisation assay*

*Endophyte status and soil effects on mycorrhizal colonisation*

Arbuscular mycorrhizal fungus hyphae and vesicles were observed in roots of both E+ and E– population plants of *B. setifolius* in the mycorrhizal treatment, but no evidence of mycorrhizal fungi was found in the roots of the non-mycorrhizal treatments.

The extent of the colonisation of root systems was significantly higher (F=20.53; P=0.003) in the E+

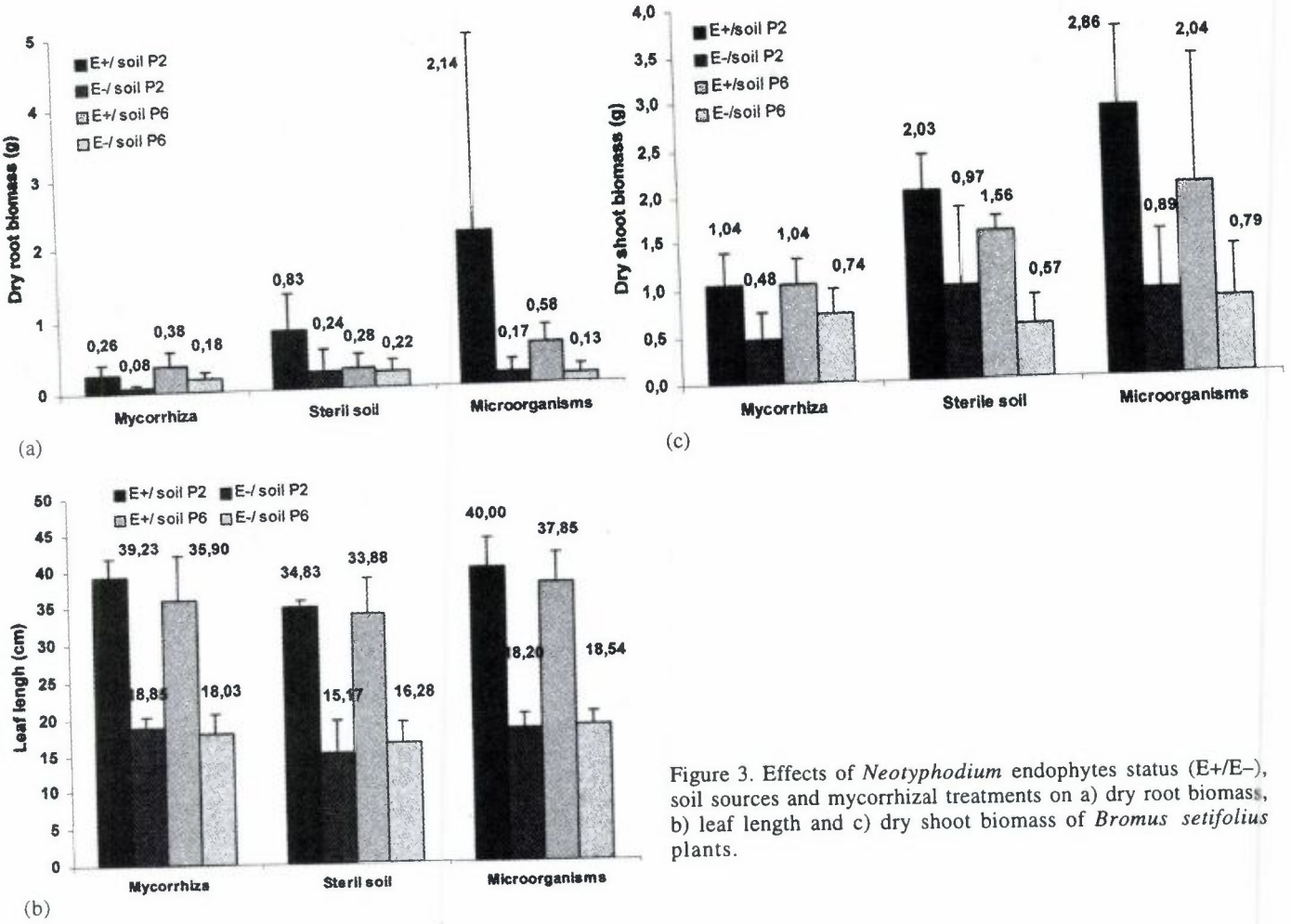


Figure 3. Effects of *Neotyphodium* endophytes status (E+/E–), soil sources and mycorrhizal treatments on a) dry root biomass, b) leaf length and c) dry shoot biomass of *Bromus setifolius* plants.

population than in the E– population, but it was not affected by the soil type (F=0.51; P=0.48) (Table 2, Fig. 2). There was no interaction between the two main effects.

The presence of *Neotyphodium* endophyte in the seedlings was analysed when the assay was completed. Frequency of colonisation was the same as that determined at field-collected culms at the beginning of the study.

*Host growth responses*

E+ plants presented higher root dry weight than E– plants (F=42.98; P<0.0001). Neither the source of the soil nor the mycorrhizal status, resulted in significant root weight differences from the control (Table 3, Fig. 3a). There was no interaction between the three main effects.

E+ plants presented longer leaves than E– plants in every combination of the mycorrhizal and soil treatments. E+ plants grown with mycorrhiza and soil microorganisms were longer that those from sterilised soil, regardless of which the source of soil was (Table 3, Fig. 3b). There was no interaction between the three main effects.

E+ plants showed higher shoot dry weights than E– plants in all treatments with a maximum score in the microorganism treatment (H=23.22; P<0.0001).

Mycorrhizal plants showed lower weights than those in the microorganism and the control treatments status (H=7.56; P=0.0229). Shoot dry weight was not significantly affected by the soil source (H=0.816; P=0.3661) (Fig. 3c).

*Mortality*

The mortality rate was not significant different between the two populations used ($\chi^2$=1.15; P=0.2845); neither mycorrhizal status ($\chi^2$=2.21; P=0.3308) nor soil source ($\chi^2$=1.15; P=0.2845) affected significantly this variable.

## 4. Discussion

Based on four studies of the interaction between *Neotyphodium* endophytes and AM fungi (Barker, 1987; Chu-Chou et al., 1992; Guo et al., 1992; Vicari et al., 2002) which suggested an antagonistic relationship between both fungal symbionts, we predicted a negative relation in *B. setifolius* plants from Patagonia.

However, our results did not confirm this hypothesis. We found that the *Neotyphodium* endophyte colonisation

was positively correlated with AM colonisation in *B. setifolius* populations in field samples.

Similar AM colonisation percentages between E+ and E– plants within the same population (P4 and P5) could be a consequence of below-ground links through mycorrhiza. It has been suggested that grassland plants may be connected to each other by hyphae of mycorrhizal fungi and nutrients may flow between plants via these hyphal bridges (Chiariello et al., 1982).

We also found a positive correlation between soil nutrients (C and N) and mycorrhizal colonisation. Mendoza et al. (2002) did not find any correlation between these variables, but they did find a positive and significant correlation between the amount of spores and most of the soil characteristics. It was suggested that a fertile soil may be associated with a smaller number of spores (Egerton-Warburton and Allen, 2000; Hayman, 1970; Hayman et al., 1975). Mendoza et al. (2002) proposed that considering the particular soil and climate properties of the steppes of Tierra del Fuego, Argentina, the classic model based on other climates, soils, latitudes and plant communities might not be applied. As Tierra del Fuego steppes share some characteristics with south Santa Cruz, those results may be extrapolated to ours.

We conducted an assay to study the soil effect on AM colonisation, due to its chemical properties or to differences in inoculum amount, and the endophyte status. We used soil collected at the same sites where the seeds were gathered, and then we planted them using all possible combinations and treatments. The results, showed in Fig. 2, revealed that the colonisation of roots by AM fungi was significantly higher in E+ plants independent of soil source.

The colonisation of root systems by AM fungi, in our assay, was significantly affected by endophyte status. This is consistent with the results obtained in six field populations (Fig. 1). This suggests a positive association between AM fungi and *Neotyphodium* fungi colonising *B. setifolius* plants. We have worked with native populations therefore we have not eliminated genotype effects of the hosts that could have some influence upon the results we are presenting here. However, we believe that the host genotype influence would not be significant considering that populations with intermediate endophyte incidence showed intermediate AM colonisation in the field study.

These findings are contrary to previous studies, where plants infected with *Neotyphodium* endophytes showed reduced colonisation and sporulation by AM fungi (Chu-Chou et al., 1992; Guo et al., 1992). Previous studies employed *Neotyphodium*-infected *Festuca arundinacea* plants instead of a native grass as we did.

Guo et al. (1992) suggested that the toxic alkaloids produced by endophytes in tall fescue are responsible for the suppressive effect of leaf endophytes on AM fungi. We did not find inhibitory effects in this study.

*Growth responses*

All the parameters analysed in the present assay, except for mortality rate, showed an increased host growth when the plants were colonised by *Neotyphodium* endophytes. These results agree with a previous study, which proposed that the endophyte presence promotes host development (Novas et al., 2003). Other endophyte-colonised grasses which increased growth under controlled environmental conditions have also been reported (Clay, 1987; Latch et al., 1985; Stovall and Clay, 1988).

Mycorrhizal effect varied depending the parameter analysed. Unlike differences in root dry weight, differences in leaf length and shoot dry weight were significant. Leaf length was higher in plants under the mycorrhizal and the microorganism treatment when compared to those of the sterile soil. Plants colonised by AM fungi produced less biomass than those grown in the sterile soil or in microorganism treatments. This has been previously reported and has been attributed to competition for nutrients (Hetrick et al., 1986; 1988 a,b; Hetrick et al., 1989).

Plants colonised by both leaf endophytes and AM fungi produced less shoot biomass than those that grew in sterilised soil and in the microorganism treatments. This phenomenon could be attributed to the cost of harbouring fungal symbionts. Some studies estimate that the amount of C required below ground by a mycorrhizal plant over that of a non-mycorrhizal plant could range from 4 to 20% of fixed C (Douds et al., 2000). This results in a relocation of carbon from shoots to roots. The presence of the endophytic mycelium in leaves likely exacerbates the effect of the nutrient relocation to roots. The source of soil had no significant effect on any of the host growth parameters analysed.

## Acknowledgements

We want to thank Prof. Dr. J.E. Wright for improving the English of the manuscript. Unfortunately we suffered the loss of his company last January. This work was supported by Universidad de Buenos Aires and Consejo de Investigaciones Científicas y Técnicas (CONICET). PRHIDEB publication no. 165.

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