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Comparative study of growth parameters on diaspores and seedlings between populations of *Bromus setifolius* from Patagonia, differing in *Neotyphodium* endophyte infection

Maria Victoria Novas, Agustina Gentile & Daniel Cabral*

Lab. de Micología., PRHIDEB-CONICET, Dpto. Biodiversidad y Biol. Exp., Fac. de Cs. Exactas y Naturales, Universidad de Buenos Aires, Pab. II, 4º piso, Cdad. Universitaria, Buenos Aires, Argentina

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Summary

Bromus setifolius Presl., a native perennial grass, is widely spread in the harsh environment of Patagonia, Argentina. This grass can be associated with *Neotyphodium* Glenn, Bacon et Hanlin endophytes which are related to aerial tissues of these plants and never show external signs of infection. Endophyte presence of certain grasses improves abiotic and biotic stress resistance, productivity or reproduction in their hosts.

To compare and contrast natural populations of *B. setifolius* differing in endophyte infection diaspore weight of seven populations was registered. In addition, emergence and growth parameters of seedlings, dry weight, leaf length and mortality rate were measured. For this purpose, two independents assays were conducted under controlled environmental conditions, using an infected (E+) and an uninfected (E–) population in each one.

Most of the analysed parameters differed between E+ and E– populations, being significantly greater in E+ populations. Populations with percentage of endophyte infection between 0% and 100% presented intermediate mean diaspore weight. These results could suggest an adaptive advantage of infected plants as a consequence of endophyte presence.

Key words: *Neotyphodium*, *Bromus setifolius*, diaspores, seedlings, emergence, dry weight, leaf length

Introduction

Patagonia, is a broad region in South America that encompasses different ecosystems, from forest to grasslands, shrub-steppes and semideserts (Aguilar & Sala 1998). The extra-andean Patagonia shows precipitation below 300 mm, strong winds that cause high evaporation rates, low mean annual temperature and extreme cold winter that create severe restrictions to plant growth and result in short growing seasons (Ravetta & Soriano 1998).

Bromus setifolius J. Presl is a perennial grass with an extensive distribution in Patagonia and we have found it present in all the previously mentioned ecosystems. This grass, as many cool-season grasses, could be associated with *Neotyphodium* endophytes conforming a symbiosis. This type of symbiosis is known as constitutive mutualism (Carroll 1988).

Neotyphodium endophytes develop an intercellular systemic infection throughout the aerial tissues of the host. They are clavicipitaceous (Clavicipitaceae, Balansieae) fungi, most of them in their anamorphic state. *Neotyphodium* endophytes never show external signs of infection in the host and it is considered that they are transmitted only by diaspores.

Considering the vertical transmission, host plants can only lose endophyte infections but can never gain them (Schulthess & Faeth 1998). Therefore the persistence of *Neotyphodium* at high levels within and among populations strongly suggests that the endophyte in-

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* **Corresponding author:** Daniel Cabral, Lab. de Micología., PRHIDEB-CONICET, Dpto. Biodiversidad y Biol. Exp., Fac. de Cs. Exactas y Naturales, Universidad de Buenos Aires, Pab. II, 4º piso, Cdad. Universitaria, Buenos Aires, Argentina, e-mail: cabral@bg.fcen.uba.ar

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crease survival and/or reproduction of host plants due to antiherbivore properties or other beneficial effects (Clay 1989).

Endophyte infection in certain hosts increases tillering, reproduction and growth of the plant, increases drought tolerance, enhances nematode resistance and mineral phosphate uptake and decreases the plant's susceptibility to insect feeding (Bacon et al. 1977; Johnson et al. 1985; Latch et al. 1985; Siegel et al. 1987; Bacon & Siegel 1988; Arechevaleta et al. 1989; Kimmons et al. 1990; Lyons et al. 1990). Therefore, some infected grasses may be enhanced in their ability to survive under stressful environments.

During the last ten years we have been studying the endophytes of native grasses in Argentina. So far we had recognised 33 native grasses associated with *Neotyphodium* endophyte (Lugo et al. 1998, Cabral et al. 1999) and we have never noticed the presence of sexual forms.

Considering that there are no records of toxicosis caused by grazing *B. setifolius* in Santa Cruz province, the question comes up what benefits offers the endophyte to the host when there is no clear evidence of the role of the fungus symbiont.

The aim of this study was to compare natural populations of *B. setifolius* from Santa Cruz province, Patagonia, Argentina, differing in the presence of *Neotyphodium* endophytes. To achieve this we analysed growth parameters of diaspores and seedlings from infected and uninfected populations.

Materials and methods

Diaspores infection status

A screening of the endophytes incidence in *B. setifolius* has been carried out from East to West in Santa Cruz province, in Southern Patagonia, Argentina (Novas et al. 2000). The frequency of infected plants was determined by microscopical examination of leaves and culms stained with aniline blue, for 20 plants harvested in each population (Clark et al. 1983).

Bromus setifolius caryopses enveloped by lemma and palea will be denominated diaspores. The diaspores were gathered from seven different natural populations. Populations were named P1–P7. At the populations P1 and P3, *B. setifolius* growths in association with a shrub species, *Mulinum spinosum* (Cav.) Pers., and other herbaceous plants. Habitats of the populations P2, P4, P5, P6 and P7 are characterized by scattered tussock grasses interspersed with bare soil patches.

Experimental design and variables

The assays were performed in a growth chamber at the Facultad de Ciencias Exactas y Naturales (UBA) under controlled environmental conditions. To study growth parameters of seedlings, four populations were chosen among the seven which we had analysed for endophyte infection. The criterion was to compare those populations that showed the greatest differences in frequency of infection. Two independent assays were performed, comparing an E+ and E– population in each one.

The variables studied were: diaspore weight, emergence, leaf length, shoot dry weight and mortality rate.

Diaspore weight: A random sample of 200 diaspores of each of seven populations was individually weighted to the nearest 0.1 mg on an electronic balance.

Emergence test: One hundred diaspores of each population were sown in order to compare emergence. Four trays (18 × 25 cm) per population were filled with vermiculite and then 25 diaspores were sown in each of them. The trays were placed in an environmental growth chamber with a diurnal light/temperature cycle (14 h light/29 °C, 10 h dark/26 °C) and the soil was kept constantly moist. Seedlings were considered emerged when they were two centimetres above ground. The number of daily emergence was recorded and it was evaluated.

Growth test: Seedlings obtained from the emergence assay were used for growth comparisons and mortality percentage. Each of 60 seedlings per population was placed in a 25 cm^3 pot filled with a sterile soil mixture (one part sterile soil, one part perlite). The pots were placed in trays, each one containing 20 pots. The trays were randomly arranged in the growth chamber and rotated weekly. All plants were watered daily. It was not necessary to use either fertiliser or insecticide. Dead plants were not replaced.

Leaf length and dry weight were measured after 6, 10 and 14 weeks, as we considered these ages as representative ones in the seedling development. Four plants per tray were randomly chosen for measurement of both characters. Length was considered as the longest leaf of each plant. Harvests were conducted by clipping 12 plants of each population at the soil surface. They were dried at 80 °C for 48 h and then weighted. The presence of *Neotyphodium* endophyte in the seedlings used in the assays was corroborated by microscopical examination of sheaths stained with aniline blue after the drying treatment.

Mortality: Dead plants were recorded at the end of each assay.

Statistical analysis

Diaspore weight was compared by means of a one-way analysis of variance between the populations and the Scheffe test was conducted to compare the means. The total emergence to total sowing percentage ratio was examined using chi-square tests corrected for continuity and the Kolmogorov-Smirnov test for two samples was conducted to study the evenness of the time distributions of accumulated emergence (Siegel & Castellan 1988). Leaf length and dry weight were compared by means of a two-way analysis of variance between the populations for each assay. The infection status of the plant and the harvest time were analysed as fixed main effects. Chi-square tests corrected for continuity were also utilised to compare the percentages of mortality of infected and uninfected plants.

Results

Endophyte infection

The populations P1 and P3 had 100% endophytic infection (E+) and the P2 and P4 had 0% (E–). Population P5, P6 and P7 showed an intermediate frequency of endophytic infection of 33%, 10% and 45% respectively. Populations used in the first assay were P1 (E+) and P2 (E–) and in the second one P3 (E+) and P4 (E–).

Diaspore weight

Average diaspore weights were 10 mg, 5.5 mg, 9.9 mg, 5.2 mg, 6.7 mg, 6.6 mg and 6.4 mg for populations P1, P2, P3, P4, P5, P6 and P7 respectively (Fig. 1). Differences in diaspore weight between populations were significant ($F = 362.84$, $P < 0.0001$). Average weights of P1 (E+) and P3 (E+) were almost twice of those from populations P2 (E–) and P4 (E–). Comparison of means revealed three groups in which the means were not significantly different from one another. Populations P1 and P3 formed one group, P5, P6 and P7 conformed a group with intermediate diaspore weight and P2 and P4 formed the third group (Fig. 1).

Seedling emergence

Seedlings began to emerge five days after sowing, but most of them emerged approximately at the seventh day in all populations (Fig. 2). The emergence rate then slowly declined.

Emergence percentage was 87% and 62% for P1 (E+) and P2 (E–), respectively, during the first assay ($\chi_c{}^2 = 13.08$, $\chi^2_{1,0.05} = 3.84$), resulting in a significant difference. In the second assay the difference of 75% for P3 (E+) populations and 57% for P4 (E–) was also significant ($\chi_c{}^2 = 6.44$, $\chi^2_{1,0.05} = 3.84$) (Table 1).

Comparisons of the functions of accumulated emergence resulted in significant differences between populations P1 (E+) and P2 (E–) in the 1st assay (Dm = 0.270, $D_{87,64} = 0.224$), but not significant ones between populations P3 (E+) and P4 (E–) in the 2nd assay (Dm = 0.227, $D_{75,57} = 0.239$).

Leaf length and dry weight

Untransformed and log-transformed weight and length data of the longest leaves were analysed. Nevertheless, the results were similar for both types of variables, thus only results from untransformed variables are presented.

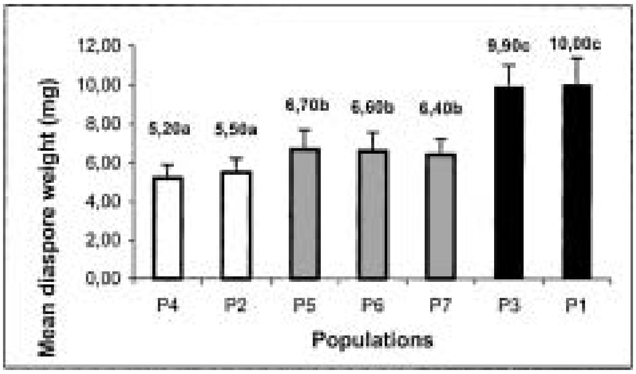

Fig. 1. Mean weight of 200 diaspores for each of the seven populations of *Bromus setifolius* gathered in Santa Cruz province. White bars: populations with 0% of endophyte infection; black bars: populations with 100% of endophyte infection; gray bars populations with intermediate infection. A one-way analysis of variance was conducted to compare differences in weight. For comparison of means the Scheffe test was used ($P < 0.05$). Bars not sharing a common letter are significantly different. Standard errors are shown.

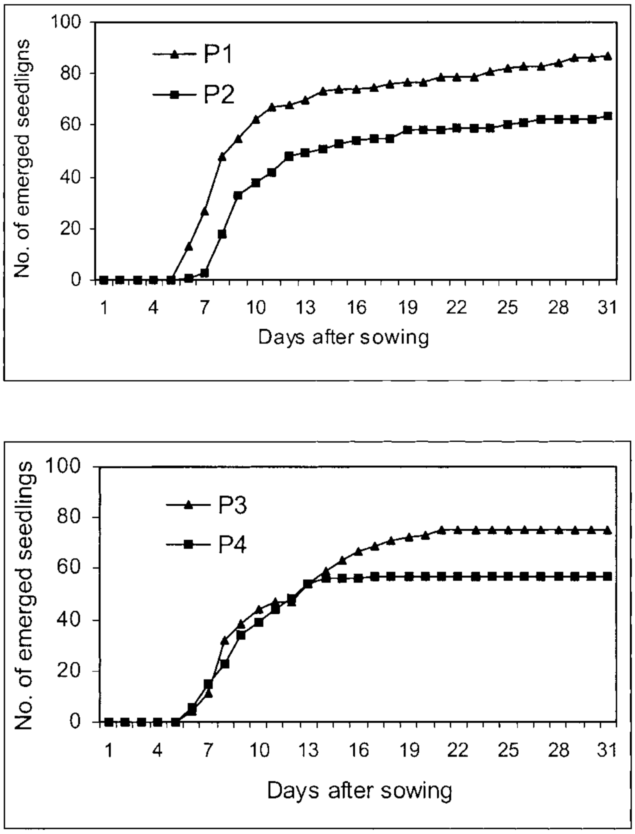

Fig. 2. Accumulated emergence curves of *Bromus setifolius* populations from Santa Cruz province.

First assay: The seedlings of population P1 (E+) presented longer leaves than those of P2 (E–) ($F = 395.91$, $P < 0.0001$). This variable ranked significantly higher for P1 (E+) at all three harvest times ($F = 41.56$, $P < 0.0001$) (Table 2). The greatest proportional difference (55.2%) occurred at week 14 being 41.43 cm for E+ and 18.58 cm for E– (Table 3). In this case, there was no interaction between the different times and populations. For dry weight, there was an interaction between times and populations ($F = 38.16$, $P < 0.0001$). The effects of populations ($F = 124.85$, $P < 0.0001$) and time ($F = 83.51$, $P < 0.0001$) on this variables were both significant. Differences in dry weight were not significant at week 6, but plants of P1 (E+) were heavier at weeks 10 and 14 (Table 2). At week 14, plants of P1 were 81.7% heavier than those of P2 (E–).

Table 1. Emergence and mortality percentages of the populations P1 (E+) and P2 (E–) in the first assay and in the populations P3 (E+) and P4 (E–) for the second assay.

	Emergence percentage	Mortality percentage
1st assay		
Infected (P1)	87% a	2% a
Uninfected (P2)	62% b	38.7% b
2nd assay		
Infected (P3)	75% a	24% a
Uninfected (P4)	57% b	17% a

Values followed by different letters are significantly different ($P < 0.05$)

Second assay: There was a significant interaction between harvest time and endophyte status for leaf length ($F = 32.25$, $P < 0.0001$). The leaf length of plants from P3 (E+) was significantly greater than that from P4 (E–) ones ($F = 223.49$, $P < 0.0001$) at all three harvests ($F = 88.52$, $P < 0.0001$) (Table 2). Leaves from infected plants were 33.3%, 45.18% and 58.6% longer than uninfected plants at week 6, 10 and 14 respectively (Table 3). Not significant differences for dry weight were detected between the two populations ($F = 1.08$, $P = 0.3023$) each time. Significant differences for dry weight were detected only as a consequence of harvest times ($F = 79.95$, $P < 0.0001$). There was no significant interaction between harvest time and endophyte status for this character ($F = 0.53$, $P = 0.594$) (Table 2).

The presence of *Neotyphodium* endophyte in the seedlings was analysed when the assays were completed. Frequency of infection was the same as the one determined at the beginning of the study.

Table 2. Two-way analysis of variance for time of harvest and infection status in *Bromus setifolius* seedlings of the populations P1 (E+) and P2 (E–) in the first assay and in the populations P3 (E+) and P4 (E–) for the second assay.

Character	1st assay			2nd assay		
	Time harvest	Infection	TH × I	Time harvest	Infection	TH × I
Length of longest leaf	*	*	NS	*	*	*
Dry weight	*	*	*	*	NS	NS

NS: not significant; $^*P < 0.0001$

Table 3. Length of longest leaf and dry weight of infected and uninfected *Bromus setifolius* populations: P1 (E+) and P2 (E–) in the first assay and in the populations P3 (E+) and P4 (E–) for the second assay.

	WEEK 6		WEEK 10		WEEK 14	
	Length of longest leaf	Dry weight	Length of longest leaf	Dry weight	Length of longest leaf	Dry weight
1st assay						
Infected (P1)	27.77a	28.1a	33.42a	79.2a	41.43a	258.2a
Uninfected (P2)	8.93b	4.3b	12.27b	8.7b	18.58b	47.1b
2nd assay						
Infected (P3)	21.07a	18.2a	37.7a	80.3a	53.06a	367.6a
Uninfected (P4)	14.06b	11.1a	20.67b	73.4a	21.97b	312.1a

Within a measurement period, values followed by different letters are significantly different ($P < 0.05$). Length in cm and weight in mg.

Mortality

In the first assay, population P1 (E+) showed a significant lower rate of mortality than P2 (E–), 2.3% and 38.7% respectively ($\chi_c^2 = 38.236$, $\chi^2_{1,0.05} = 3.841$). Mortality rate in the second assay was 24% and 17% for populations P3 (E+) and P4 (E–), respectively ($\chi_c^2 = 1.104$, $\chi^2_{1,0.05} = 3.841$). This difference was not significant (Table 1).

Discussion

In the present study we found significant differences in most of the growth parameters between E+ and E– populations of *B. setifolius* from Santa Cruz. Other studies have also shown that endophyte presence tends to increase shoot mass, tillering, and diaspore production in tall fescue (West 1994).

We have worked with native populations. Therefore, possible genotypic differences between them could have some influence upon the results we are presented here. However, we believe that the diversity found in native populations will reflect the entire genotype range of each population. In addition, the populations used in this study are only a few kilometres distant from each other and without geographic barriers between them, enabling gene flow between them. It has been proven that although the wind-vectored pollen occurs near the pollen source, low levels of dispersion and crossed fertilisation can extend over substantial distances (Levitt & Kerster 1974). So genetic variability nevertheless should not obscure the differences between the populations differing in the endophyte presence.

Diaspore weights of E+ populations were significantly greater than those of E– ones. Comparisons of means showed that diaspore weight was positively associated with percentage of endophyte infection. The most interesting result was that populations with percentages of endophyte infection between 0% and 100% showed an intermediate diaspore weight. In addition, these populations were sampled in localities with similar ecological characteristics of those of P2 and P4 (both E–), therefore the ecological factor has no influence on the diaspore weight.

The ability for a plant to colonise appears to be associated with the amount of food reserve, which the diaspore contains, and large diaspores have an initial advantage under competitive conditions (Werker 1997). On the other hand, in plants where diaspores are windspread, the light weight becomes an advantage. Considering that strong winds, characteristically found in Patagonia, are responsible for the diaspore dispersal, the greater diaspore weight in E+ populations could account for a greater reserve content and a better survival under extreme environmental conditions without affecting the dispersion. These results are totally different from those found in populations of *B. auleticus* collected from several fields in the Argentine pampas (Ianonne 2003). In this study the E– populations have heavier diaspores than the E+, however, E+ populations showed higher rates of germination and a higher biomass of seedling as in our assays. This region does not present the strong winds found in Patagonia, so in this case the diaspore's light weight would become an advantage.

The percentage of emergence was significantly different between E+ and E– populations in both assays. This is consistent with the statement of Clay (1990) who said that *F. arundinacea* and perennial ryegrass diaspores infected with endophytes germinate quicker and in a greater proportion than uninfected ones. It seems that the presence of the endophyte could increase host fitness compared with uninfected plants, since heavier diaspores and faster emergence increase survival in the harsh environmental conditions of Patagonia which are characterised by poor and eroded soil.

Our results showed that in the first assay E+ plants of *B. setifolius* are heavier and with longer leaves than E– plants at all harvest times. Previous work showed that some endophyte infected grasses generally exhibited increased growth under controlled environment conditions where soil moisture and nutrients were supplied in abundance (Latch et al. 1985; Clay 1987; Stovall & Clay 1988). Differences observed in both parameters for E+ and E– populations support the idea of Clay (1988), that the endophytes of grasses increase plant growth and dry weight. The mortality rate was also significantly different between populations in this assay.

In the second assay, the differences of growth parameters were not as clear as those in the first assay. Although differences observed in leaf length were significant, differences in dry weight and mortality were not significant as expected, but principally they seem to follow the same trend as the first assay. It has been suggested that endophyte could increase host competitive power, and an enlargement of the leaf length could represent an advantage under these conditions (Kelley & Clay 1987).

Many factors could be responsible for the observed variation among the assays. Recent molecular analysis revealed the existence of endophytic diversity between populations of *B. setifolius* from Santa Cruz province, Argentina (Gentile et al., unpublished), so we could expect a diverse response among the populations. Saikkonen et al. (1999) suggested that the endophytes protect their host or increase their fitness in a different way depending on the population, the endophytic diversity and/or the environmental condition where the association takes place.

In areas with extremes of low precipitation or poor soils, as those mentioned for Patagonia, studies of native grasses in association with endophytes are of particular interest for the improvement of agriculture and cattle grazing. These preliminary results will allow future improvement of this species as a widespread forage grass in Southern Argentina.

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