

RESEARCH ARTICLE

Ecotype-specific effects of fungal endophytes on germination responses of seeds of the South American wild forage grass *Bromus auleticus*

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Abstract

Bromus auleticus is a winter wild species with promissory characteristics for domestication as forage that inhabits temperate-warm grasslands from a wide region from South America. Populations from regions with different environmental conditions are considered as ecotypes and are associated with different seedborne *Epichloë* fungal endophytes. Association with *Epichloë* provides *B. auleticus* with increased growth capacity and resistance against pathogens. The seeds mature at the end of spring and germinate in autumn, remaining dormant during wet and warm summer. As the persistence of *Epichloë* endophytes in the seed is highly sensitive to temperature and humidity, we studied how the endophytes modulate seed germination in response to these factors in two *B. auleticus* ecotypes named La Pampa (LP) and El Palmar (EP). We also studied how environmental factors during germination affect endophyte survival. In the LP ecotype, adapted to coldest temperatures in autumn, *Epichloë* improved final germination under alternating temperatures, but had no effects on germination inhibition imposed by low water availability. In the EP ecotype, adapted to warm humid summer and autumn, the endophyte promoted germination at constant 25°C and alternating 15/25°C temperatures, and prevented germination at low water potentials. At 30°C, seed germination was inhibited and whereas seeds remained viable, endophyte survival was significantly lowered. The effect of *Epichloë* on *B. auleticus* seed germination was ecotype-specific: promoting germination at alternating temperatures in La Pampa ecotype or at high temperatures in El Palmar ecotype, while inhibiting it at low water availabilities, could be an endophyte-mediated ecotypic strategy minimising endophyte mortality and increasing seedling establishment.

KEYWORDS

Bromus auleticus, endophytes, *Epichloë*, seed germination, water potential

1 | INTRODUCTION

The associations of plants with symbiotic microorganisms such as bacteria and fungi are widespread in nature. Almost all the plants in natural

ecosystems have been found to be colonised with one or more fungal or bacterial symbionts living as endophytes (Hardoim et al., 2015; Rai et al., 2014). These microbes are known to affect plant fitness, and although the term “endophyte” is commonly used for mutualistic

symbionts (Carroll, 1988), it has been reported that symbiosis outcomes are context dependent. In this sense, the effects of the endophytes be can positive, neutral or negative depending on the environmental conditions and genetic factors of the host and of the endophyte (Davitt et al., 2011; Rodriguez et al., 2009).

Fungal endophytes associated with grasses may affect different aspects of the ecology of host plants in nature. Particularly, asexual species of *Epichloë* Tul. (Clavicipitaceae, Hypocreales) (formerly *Neotyphodium* Glenn, Hanlin and Bacon) are vertically transmitted fungal endophytes in many cool-season grass species (Leuchtman et al., 2014; Semmartin et al., 2015). Asexual endophytes present an intercellular and systemic growth in aerial tissues of plants, producing no symptoms of infection, and delivering certain benefits that lead the grass-endophyte symbiosis to be considered as mutualism (Kuldau & Bacon, 2008; Rodriguez et al., 2009). This has been quite clear in domesticated cultivars of some grasses such as tall fescue (*Lolium arundinaceum*) and rye-grass *Lolium* species (Faeth & Sullivan, 2003; Saikkonen et al., 2006). In those species, for example, the endophyte infection can be associated with improved seed germination (Clay, 1987; Gundel et al., 2012; Gundel, Maseda, Ghera, & Benech-Arnold, 2006), higher biomass production and tolerance to drought (Malinowski & Belesky, 2000; Nagabhyru et al., 2013), enhanced nutrients uptake and resistance to heavy metals (Malinowski et al., 2000, 2004). In wild grasses, however, the presence of *Epichloë* fungal endophytes has shown variable effects on different aspects of host plant performance suggesting a stronger context-dependency of effects (Davitt et al., 2011; Faeth et al., 2004; Faeth & Sullivan, 2003; Sullivan & Faeth, 2008). For several indigenous grasses from Argentina (i.e., *B. auleticus*, *Bromus pictus*) it has been reported that endophytes can promote plant growth (Iannone & Cabral, 2006; Iannone, Pinget, et al., 2012; Novas et al., 2003), mycorrhizal colonisation (Novas et al., 2005, 2009, 2011; Vignale et al., 2016, 2018), and to confer resistance to pathogens in *B. auleticus* (Iannone et al., 2017; Vignale et al., 2013). Despite these overall benefits, it is unclear if endophyte mediated effects on grasses of South America can vary in different populations and contribute to plant adaptation to the local environmental conditions as reported in *Festuca arizonica* (Sullivan & Faeth, 2008) and *Poa alsodes* (Shymanovich & Faeth, 2019).

B. auleticus Trin. ex Nees is a South American native perennial grass with autumn–winter–spring growing cycle (Gutiérrez & Pensiero, 1998), distributed in the Pampean and Mesopotamian regions of Argentina, grasslands of Uruguay and south of Brazil. Plants from different regions are associated with different fungal endophyte strains/species. Populations from the Pampean region are associated with *Epichloë pampeana* (Iannone et al., 2011; Iannone, Novas, et al., 2012), while *B. auleticus* plants from other regions host different *Epichloë* species (Mc Cargo, 2015). Because of its high quality and productivity in winter (a critical season associated with forage shortage), agronomists have led the research, and initiated breeding programmes (Condón et al., 2017; Millot & Methol, 1999; Moraes & Oliveira, 1990). Besides, *B. auleticus* is also a component of vegetation communities supporting both domestic and wild grazing animals. As a highly preferred species, the abundance of *B. auleticus* tends to diminish

under intense and uninterrupted grazing (Ragonese, 1985). Seeds are fully ripe by the end of spring, spending the summer on the soil surface or buried just a few centimetres (Olmos, 1993). Thus, seeds are exposed to high temperatures and high relative humidity (RH) until the first cohort of seedling emerges by the beginning of autumn (Olmos, 1993). Although studied on other species, the combination of these environmental conditions (high temperature and high RH) has been found to be highly detrimental for the survival of fungal endophytes in seeds (Gundel et al., 2010; Tian et al., 2013). However, wild populations of *B. auleticus* exhibit high frequencies ($\approx 100\%$) of endophyte-infected plants (Iannone et al., 2011), which suggests clear fitness advantages of fungal endophytes on host plants. Antecedents from our research team have shown clear benefits of *Epichloë* endophytes on *B. auleticus* plants from different populations (Iannone & Cabral, 2006; Iannone, Novas, et al., 2012). However, it remains uncertain how fungal endophytes modulate the behaviour of seeds from different populations to maximise survival of both partners and seedling recruitment.

In the grass-endophyte symbiosis, the seed is not only the means for multiplying and disseminating the host species but also the associated endophyte symbionts. Thus, the seed appears as a crucial life history trait under high natural selection pressure from biotic and abiotic factors on both partners (Gundel et al., 2011, 2017). In species experiencing great seasonal variability, mechanisms of dormancy allow seeds to sense the environment and germinate in time and place, thus increasing the probability for successful establishment (Donohue, 2005; Finch-Savage & Leubner-Metzger, 2006; Willis et al., 2014). Mostly governed by the dynamics of temperature, seasonal patterns of seed dormancy can be very complex and involve effects of multiple factors occurring simultaneously such as water availability, nitrate concentration and light quality (Benech-Arnold et al., 2000). The complete dormancy release usually matches the optimal environmental conditions for emergence and establishment (Donohue, 2005; Finch-Savage & Leubner-Metzger, 2006; Willis et al., 2014), with germination rate as a vigour trait associated with (future) successful competition for resources (Bewley, 1997; Fenner & Thompson, 2005). While medium water potential may control the proportion of germinated seeds, the germination rate is positively correlated with the temperature—within the suboptimal range (Bradford, 1995, 2002; Finch-Savage & Leubner-Metzger, 2006). Seed responses to temperature and water availability can depend on the species but also on the population origin arising as ecotypical characteristics of adaptation to the local environment (Donohue, 2005; Finch-Savage & Leubner-Metzger, 2006). Despite the importance for the symbiosis persistence, effects of seedborne endophytes on the response of seeds to temperature and water availability have been (relatively) poorly studied (Hamilton & Faeth, 2005; Iannone & Cabral, 2006; Novas et al., 2003; Zhang et al., 2012). In the cosmopolitan grass species *Lolium multiflorum*, the infection with endophytes was found to modulate the germination response of seeds to temperature and water potentials and also suggested a regulation over the dormancy level (Gundel, Maseda, Ghera, & Benech-Arnold, 2006; Gundel, Maseda, Vila-Aiub, et al., 2006). Nonetheless, considering that endophyte survival in the

seed is highly sensitive to high temperatures and moisture contents (Bylin et al., 2016; Gundel et al., 2009, 2010; Rolston et al., 1986; Tian et al., 2013; Welty et al., 1987), it is unclear how the fungal endophytes modulate the host seed germination without compromising its survival.

In the present work, we studied the influence of ecotype-specific fungal endophytes on the response of *B. auleticus* seeds to different thermal treatments and water availability. We manipulated the endophyte symbiotic status by removing the fungus from naturally infected plants from two ecotypes of *B. auleticus*, La Pampa (LP) and El Palmar (EP). We hypothesised that each fungal endophyte differentially modulates the response of seeds to different temperatures and water availabilities, increasing the performance of host populations under the environmental conditions of the collection site. As a result, *Epichloë* fungal endophytes would improve the host ecotypic adaptation to the local prevailing environmental conditions.

2 | MATERIALS AND METHODS

2.1 | Seed material

B. auleticus Trinius ex Nees is a C3 perennial grass, native to Rio de la Plata grasslands, including central east of Argentina, Uruguay and south of Brazil (Gutiérrez & Pensiero, 1998). Phenotypic characterisation of plants from different accessions suggests the existence of ecotypes with specific adaptations to local environmental conditions (Condón et al., 2017; Gutiérrez et al., 2006, 2015). In this article, we worked with two Argentinean ecotypes that in accordance with the collection site, we named La Pampa (LP) and El Palmar (EP). Plants of the LP ecotype proceed from a population in Intendente Alvear county, Province of La Pampa, in the Pampean region (Argentina) (35°15'S, 63°39'W), while those of EP ecotype were collected in the "El Palmar" National Park (31°51'30.9"S 58°19'21.5"W), Province of Entre Ríos in the Mesopotamian region (Argentina). The geographic distance between populations is 600 km. The climate of both sites is considered warm and humid (World Weather Online, 2020a, 2020b; Weather Spark, 2020a, 2020b); however, each site shows its own climatic characteristics (Table 1) which make EP warmer and more humid than LP.

The original plant material was collected in 2007. In each site, seeds were taken from at least 25 plants separated for more than 5 m and were later pooled. An analysis of seeds ($n = 100$ /ecotype), showed that 100% presented *Epichloë* fungal endophytes (see below for technical details regarding the determination of endophyte infection in seed). In previous research, it was determined that plants from LP were associated with *E. pampeana* and plants from EP were associated with *E. tembladerae* (Iannone et al., 2009). Ongoing molecular studies indicate that the endophyte from EP is a different species (Mc Cargo et al., 2014). Therefore, since there is not published a formal description of the associated endophyte to the EP plants, we name it as *Epichloë* sp.

Upon collection, the seeds were stored under room conditions. Fungal endophytes lose viability at a higher rate than seeds unless they are stored in cold and dry conditions (Bylin et al., 2016; Gundel

TABLE 1 Climatic characteristics of the two original collection sites (La Pampa [LP] and El Palmar [EP]) of the two populations of *Bromus auleticus* (World Weather Online, 2020a, 2020b; Weather Spark, 2020a, 2020b)

Climatic variable	Populations of <i>B. auleticus</i>	
	LP	EP
Mean annual precipitation	679 mm	1,213 mm
Mean monthly precipitation (rainiest trimester)	January–March 110 mm	February–April 150 mm
Mean monthly precipitation (driest trimester)	June–August 19 mm	June–August 70 mm
Mean minimum temperatures (March)	13–17°C	24–27°C
Mean maximum temperatures (March)	10–13°C	20–24°C
Mean minimum temperatures (April)	6–9°C	16–20°C
Mean maximum temperatures (April)	16–19°C	27–30°C
Mean minimum temperatures (May)	13–16°C	23–26°C
Mean maximum temperatures (May)	9–13°C	18–22°C

et al., 2009, 2010; Rolston et al., 1986; Tian et al., 2013; Welty et al., 1987). After 1 year of storage, we confirmed that the seeds generated endophyte-infected (E+) and endophyte-free (E-) individual plants, respectively (Iannone, Pinget, et al., 2012). Since then, E+ and E- plants of each ecotype were established in two common gardens (one per ecotype) at the INTA Experimental Field Station, Concepción del Uruguay, Province of Entre Ríos, Argentina. In each garden, 40 plants per ecotype (LP and EP) and symbiotic status (E+ and E-), were arranged in a grid with positions assigned at random and distance between plants was 60 cm. This allowed us to multiply and have every year, El Palmar ecotype endophyte-infected seeds (EP E+), El Palmar ecotype endophyte-free seeds (EP E-), La Pampa ecotype endophyte-infected seeds (LP E+) and La Pampa endophyte-free seeds (LP E-) for the experiments.

In December 2014, seeds produced by all plants from the common garden were harvested and pooled according to the ecotype and symbiotic status. The endophytic infection level in each bulk was checked by inspecting seeds under microscope ($\times 100$) and searching for the conspicuous fungal hyphae in the aleurone layer. A subsample of 100 caryopses per bulk, was soaked for 5 h in aqueous KOH 10% (v/v), then rinsed in tap water, stained with aniline blue 0.1%, and each seed was pressed between slide and coverslip (Clark et al., 1983). We confirmed that all the seeds from E+ plants from both ecotypes were 100% infected and all the seeds from E- plants were all endophyte free. The viability of the seeds was checked before the beginning of the experiments by means of the tetrazolium chloride technique (Porter et al., 1947). One hundred seeds per bulk were checked and in all of them, viability was 100%.

2.2 | Germination experiments

2.2.1 | Germination responses to constant and alternating temperatures

We carried out two types of treatments simultaneously, to evaluate the influence of the fungal endophyte on seed germination responses to both constant and alternating temperatures. The gradient of constant temperatures was 15, 20, 25 and 30°C, while we compared 15/25°C against the constant mean 20°C for the alternating treatment. In the alternating treatment, temperature changed every 12 h (15/25°C, 12/12 h). The light condition was not manipulated. For each ecotype-by-endophyte combination and temperature treatment, we established five replicates. Twenty seeds were shown on every of the five Petri dishes (9 cm diameter) with two filter papers moistened with 2 ml of deionised water. To avoid changes in water availability because of evaporation, each dish was initially weighed, and the missing water was replenished. On every Petri dish, germination (2 mm of emerged radicle) was recorded daily for a period of 45 days until the accumulated dynamics reached a plateau or germination had ceased. For each replicate, final germination was expressed as percentage of germinated seeds over the total seeds. Germination rate was estimated according to Bewley and Black (1994). Briefly, the seed population germination rate is calculated as:

$$\Sigma(t \cdot n) / \Sigma n \quad (1)$$

where t is time (days), from $t_0 \rightarrow t_n$ (the time when germination no longer occurs), while n is the number of germinated seeds at day t (Bewley & Black, 1994).

We intended to describe the response of seed populations to constant temperature by adjusting the thermal time model. For non-dormant seeds, this model assumes a linear relationship of the germination rate and the increasing constant temperature, between a base and an optimal temperature (Bradford, 2002; Finch-Savage & Leubner-Metzger, 2006; Garcia-Huidobro et al., 1982). However, we observed great variation in the final germination among treatments, which evidenced seed dormancy and prevented us from adjusting the mentioned model. In addition, seeds incubated at 30°C were excluded from the experiment (and the associated statistical analysis) since after being in the chamber for 3 weeks, no germination occurred. Nonetheless, all the Petri dishes placed in the 30°C chamber were moved to the chamber set at 20°C, to test for the seed viability and fungal endophyte survival (see below).

2.2.2 | Germination responses to different water availabilities

We carried out an experiment to evaluate the influence of the fungal endophyte on seed germination responses to water availability. Seeds from the two ecotypes of *B. auleticus* and endophyte infection status, were put to germinate at four medium water potentials: 0.0, -0.5, -1.0 and -1.5 MPa. Twenty-five seeds were sown directly over the two filter papers placed at the bottom of each Petri dish (five per

ecotype-by-endophyte combination). Prior to sowing, each dish was moistened with 2 ml of deionised water (0 MPa) or with the solutions with different concentrations of polyethylene glycol PEG 6000 (polyethylene glycol 6000) to achieve the nominal water potentials according to Michel (1983). All the Petri dishes were incubated at 20°C in a chamber; no specific light condition was manipulated. Germination was daily recorded until no more germination was observed. Since the great absorption of water by the seeds during the first hours of imbibition changes the medium water potential (Ni & Bradford, 1992), each Petri dish was refilled with water to complete the weight (as it was done in the temperature experiments). In the same way as in the temperature experiment, we estimated final germination and germination rate for each replicate.

As an additional analysis, we adjusted the Hydrotime Model to characterise the response of seeds from each ecotype and endophyte infection status to water availability. The model allows determining physiological parameters of seed populations and provide insight on the potential performance under certain ecological conditions (Allen et al., 2000; Bradford, 1995, 2002). In this model, biological time can be calculated by the amount in which Ψ_w exceeds the base water potential, below which germination will not reach completion (Finch-Savage & Leubner-Metzger, 2006). When using this model, a seed population can be characterised by two parameters defined by the following equation:

$$\theta_H = [\Psi - \Psi_{b(g)}] \cdot t_g \quad (2)$$

where θ_H represents the hydrotime (MPa h required for seed germination and assumed to be constant for all the individual seeds), Ψ is the medium water potential (in MPa), Ψ_b is the base water potential for a particular germination fraction (g) of the seed population and t_g is the time required to germinate the same seed fraction. This enables calculation of the standard deviation of base water potential values in the seed population (for details, see Bradford, 1995, 2002 and references therein).

2.2.3 | Effect of the temperature and Ψ_w during seed germination on endophyte survival

Since fungal endophytes have shown to be more sensitive to environmental storage conditions than seeds (Gundel et al., 2009; Welty et al., 1987), the anticipated death of fungi generates the emergence of E- seedlings from E+ seeds (Gundel et al., 2011). Therefore, by checking the endophyte presence in the seedlings emerged from the germinated seeds in the previous experiments, we were able to evaluate the effect of incubation conditions (temperature and water potential) on the endophyte viability. Two days after germination, the emerged seedlings from E+ seeds (only) were transferred to a greenhouse and transplanted to plastic pots (300 cm³) filled with a mixture of non-sterile sand and soil (1:2). The seedlings were watered on demand to avoid water stress. When seedlings had at least two tillers (2–3 leaves each), hyphae from the endophyte fungus were searched for in leaf sheaths under microscope (Clark et al., 1983). For each replicate, the response variable was infection frequency of seedlings

calculated as E+ seedlings over the total evaluated seedlings. We maintained the association of every evaluated seedling to the original replicate ($n = 5$) per treatment and experiment.

2.3 | Statistical analysis

For the temperature experiments, the manipulated factors were temperature (three and two levels for the constant and alternating temperature experiments), the *B. auleticus* ecotype with two levels (LP and EP), and the fungal endophytic status (E+ and E-). Besides the ecotype and the endophyte, the manipulated factor in the water availability experiments was the water potential of the germination medium with four levels.

Final germination was analysed with generalised linear models (GLM) due to the binomial distribution of the variable. Over- and sub-dispersion assumptions were checked for final germination. Differences in germination rates were analysed with a three-way analysis of variance, owing to the continuous character of this parameter. Homoscedasticity and normality were checked. The effect of the temperature and water availability during seed germination on endophyte transmission was analysed with Bernoulli distribution. Over and sub-dispersion assumptions, or homoscedasticity and normality were checked and corrected. When the effects of treatments were significant, the least significant difference (LSD) value ($p = 0.05$) was used to compare means. The software used for the analysis was R Studio (Racine, 2012) version 3.4.2, packages *lme4*, *Matrix*, *Mass* and *agricolae*.

3 | RESULTS

3.1 | Effects of temperature, ecotype and endophytic status on seed germination

Under the thermal constant treatments (15, 20 and 25°C), the final germination of *B. auleticus* seeds ranged between 20% and 70%

(Figure 1). However, final germination was highly inhibited at constant 30°C with only four EP E+ seeds able to germinate.

The relative effect of fungal endophytes and host ecotypic origin was found dependent on the incubation temperature of seeds. Overall, the ecotype was the factor that most affected germination of *B. auleticus* seeds. The effect of temperature on final germination percentages depended on the ecotype and on the endophytic status, resulting in a significant interaction among temperature \times ecotype \times endophytic status ($p < 0.01$) (Figure 1a). The highest final germination was reached by LP E+ seeds at 15°C, although no significant differences were found among temperatures ($p = 0.469$) or between E+ and E- seeds at any of the studied temperatures ($p > 0.05$). In EP ecotype, the final germination of E+ seeds reached 65% at 25°C and was not affected by temperature ($p = 0.2422$), but final germination of EP E- seeds diminished significantly at 25°C ($p < 0.001$) (Figure 1a). Germination rates of seeds from LP ecotype were significantly higher than in seeds from EP ($p < 0.001$), regardless of the temperature ($p > 0.05$) or the endophytic status ($p > 0.05$).

Despite having been imbibed and incubated for 3 weeks at 30°C, seed viability remained unaffected ($p = 0.968$). With no effects assigned to the ecotype or the endophyte symbiotic status, seeds transferred from 30 to 20°C showed the same level of germination ($\approx 49.96\%$) as those incubated at constant 20°C ($p = 0.15$) (Figure 1a).

When comparing the effect of the alternating (15–25°C) temperature regime with constant 20°C temperature on the final germination of *B. auleticus* seeds, the results depended on the ecotype, the endophytic status and on the temperature regime (significant interaction temperature \times ecotype ($p = 0.015$), temperature \times endophytic status ($p = 0.009$) (Figure 2a). In EP, the alternating temperature tended to reduce the final germination, but this reduction was only significant for EP E- seeds at 25°C ($p < 0.05$). In LP the alternate regime tended to reduce the final germination of LP E- seeds and to increase the germination in those LP E+, in such a way that under the alternating temperature regime, final germination of LP E+ seeds was

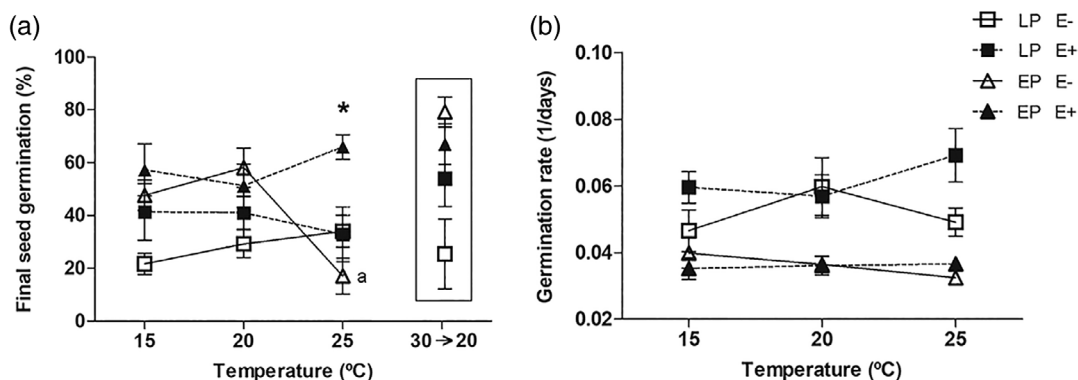


FIGURE 1 Final germination (%) (panel a) and mean germination rate (1/days) (panel b) of endophyte-free (E-) and endophyte-infected (E+) seeds from two ecotypes of *Bromus auleticus*, La Pampa (LP) and El Palmar (EP), at different constant temperatures (15, 20, and 25°C) and final germination at 20°C of seeds that did not germinate during incubation by 3 weeks at 30°C. Values are means \pm SE ($n = 5$). Asterisks above the symbols show differences due to endophytic status in EP ecotype. Letters next to the symbols show differences due to temperature in an Ecotype \times Endophyte combination

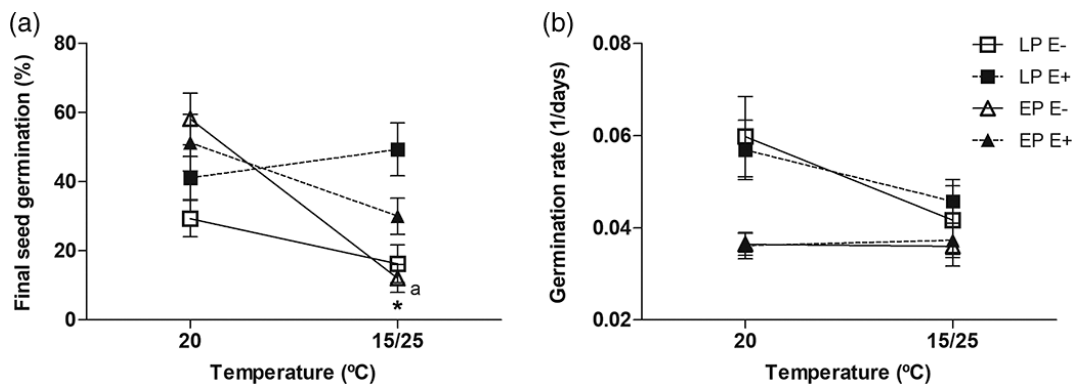


FIGURE 2 Final germination (%) (panel a) and mean germination rate (1/days) (panel b) of endophyte-free (E⁻) and endophyte-infected (E⁺) seeds from two ecotypes of *Bromus auleticus*, La Pampa (LP) and El Palmar (EP), at constant (20°C [panel a]) and alternating (15/25°C [panel b]) temperatures. Values are mean \pm SE ($n = 5$). Asterisks below the symbols show differences (LSD, $p < 0.05$) due to endophytic status in LP ecotype. Letters next to the symbols show differences (LSD, $p < 0.05$) due to temperature in an Ecotype \times Endophyte combination

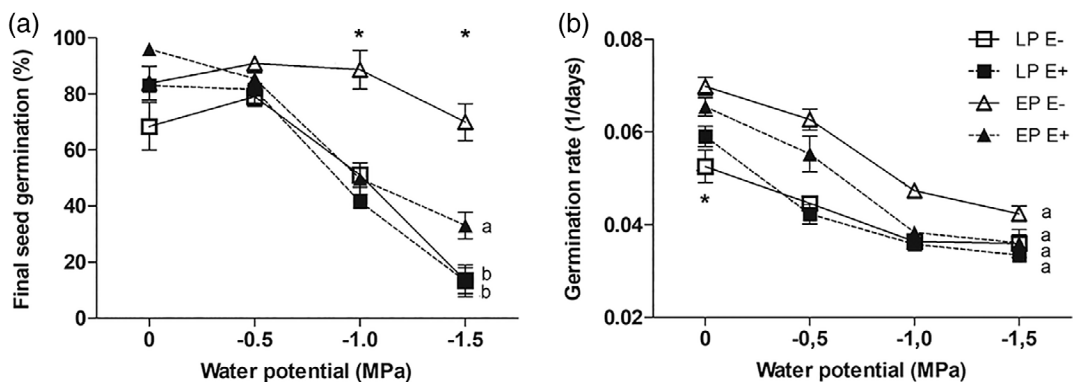


FIGURE 3 Final germination (%) (panel a) and mean germination rate (1/days) (panel b) for endophyte-free (E⁻) and endophyte-infected (E⁺) seeds from two ecotypes of *Bromus auleticus*, La Pampa (LP) and El Palmar (EP), in relation to the medium water potential (MPa). Values are means \pm SE ($n = 5$). The asterisks above the symbols show differences (LSD, $p < 0.05$) due to endophytic status in EP ecotype. The asterisks below the symbols show differences due to endophytic status in LP ecotype. Letters next to the symbols show differences (LSD, $p < 0.05$) with the control (0 MPa) in an Ecotype \times Endophyte combination

significantly higher than in LP E⁻ ones ($p < 0.05$). Germination rates were not affected by the alternating temperatures ($p > 0.05$) (Figure 1b) and seeds of LP germinated at a higher rate than EP ones ($p = 0.017$), (Figure 2b).

3.1.1 | Effect of water availability, ecotype and endophytic status on seed germination

Seed germination was overall reduced as water availability diminished, but the response of each ecotype depended on the endophytic status ($p = 0.011$) (Figure 3a). At 0 and -0.5 MPa, final germination ranged between (68 ± 8 and $96 \pm 2\%$), and no differences were observed among the seed characteristics (either ecotype or endophyte) ($p = 0.98$). Interestingly, the germination of the EP E⁻ seeds was not affected by the medium water potential ($p = 0.930$). However, the final germination of EP E⁺ decreased significantly at -1.0 MPa and -1.5 MPa ($49 \pm 5\%$

and $33 \pm 5\%$, respectively); these reductions were about half the final percentage of germination of the EP E⁻ seeds for the same treatments (-1.0 MPa: $88 \pm 7\%$, and -1.5 MPa: $70 \pm 7\%$). In LP ecotype, the percentage of seed germination at -1.0 and -1.5 MPa decreased significantly, regardless of the endophytic status of the seeds (Figure 3). At -1.0 MPa, the final germination was $51 \pm 4\%$ for LP E⁻ and $42 \pm 2\%$ for LP E⁺, while at -1.5 MPa it was $13 \pm 5\%$ for LP E⁻ and $13 \pm 6\%$ for LP E⁺.

Germination rates diminished as water availability diminished ($p > 0.05$), but EP seeds exhibited a higher germination rate than LP ones ($p > 0.05$) and particularly, at -1.0 MPa germination rate of EP E⁻ seeds was higher than in their EP E⁺ counterparts (Figure 3b).

The parameters derived from the adjusted *hydrotime* model (Table 2) indicated that the capability of EP E⁻ seeds to maintain high levels of seed germination and speed, were clearly associated with a very low base water potential. Although the *hydrotime* constant in EP E⁻ seeds was higher than in EP E⁺ seeds (29 vs 16 MPa h), the lower

base water potential in the EP E⁻ seed population allowed displaying a high germination level even at very low water potential in the medium solution (−1.0 and −1.5 MPa). In LP ecotype, the differences between E⁺ and E⁻ seeds were only found at the hydrotime constant (θ_H), the base Ψ_w for the germination of 50% of the seeds ($\Psi_{b(50)}$) and the standard deviation of base water potential values (σ_{Ψ_b}) of LP E⁺ and LP E⁻ seeds were very similar (Table 2). In contrast with LP, in EP seeds, higher differences between E⁺ and E⁻ seed were observed for all the parameters. The value of θ_H for EP E⁻ seeds were twice that of the θ_H of the EP E⁺ ones. Also, $\Psi_{b(50)}$ of EP E⁻ seeds was 1.13 MPa lower than for E⁺ seeds, and the σ_{Ψ_b} of the E⁻ seeds almost twice the σ_{Ψ_b} for E⁺ seeds.

3.1.2 | Seedling endophyte infection frequency: Consequences of seed incubation at different temperatures and water potential

Endophyte infection frequency of the seedlings emerged from the seeds from the previous experiments was, on average, high ($\approx 84.26\%$) (Figure 4). This was the same for all the seedlings coming from germinated seeds

TABLE 2 Parameters derived from the *hydrotime* model describing germination dynamics of endophyte-free (E⁻) and endophyte-infected (E⁺) seeds from two ecotypes of *Bromus auleticus*, La Pampa (LP) and El Palmar (EP), at different medium water potentials (0, −0.5, −1.0, and −1.5 MPa)

Parameters	La Pampa		El Palmar	
	E ⁺ LP	E ⁻ LP	E ⁺ EP	E ⁻ EP
θ_H (MPa h)	17.00	24.00	16.00	29.00
$\Psi_{b(50)}$ (MPa)	−1.48	−1.33	−1.43	−2.56
σ_{Ψ_b}	0.61	0.80	0.60	1.18

Note: The parameters are the hydrotime constant (θ_H), the base water potential (Ψ_b) for the germination 50% of the seed population, and the standard deviation of base water potential (σ_{Ψ_b}).

of both ecotypes at most of the thermal treatments ($p > 0.05$), except for those incubated at constant 30°C. The endophyte infection frequency of the seedlings developed from seeds that remained dormant during incubation at 30°C fell to 25.6% (59% lower) ($p = 0.001$) without differences between ecotypes ($p = 0.892$) (Figure 4).

The water potential of the solutions where seeds germinated (at 20°C) had no effects on the endophyte infection frequencies of seedlings ($p = 0.773$). Overall, it was also very high (83.85%) and there were no differences between ecotypes ($p = 0.653$) (Figure 4).

4 | DISCUSSION

The study of germination responses to temperature and water can provide an insight to understand the adaptive value of seeds traits to a given ecological setting (Baskin & Baskin, 1998; Saatkamp et al., 2019). Despite the importance endophytic microorganisms can have for determining distribution range and abundance of plant species (e.g., Afkhami et al., 2014; Kazenel et al., 2015), seedborne microorganisms are seldom considered in the agenda (Gundel et al., 2017; Nelson, 2018). The presence of *Epichloë* fungal endophytes in seeds has been found to affect the germination response of host seeds as well as the level of dormancy (Bao et al., 2019; Gundel et al., 2006,b). Here we studied endophyte-driven functional traits in seeds that can give insights on the adaptation of *B. auleticus* populations to local environmental conditions. Our results show that the presence of fungal endophytes affected germination parameters of *B. auleticus* seeds in response to temperature and water availability but more importantly, these effects depended on the population origin and seemed to be related with the environmental characteristics of collection site. Considering that endophyte-specific effects may result from partners' co-adaptation to the prevailing environmental conditions (Shymanovich & Faeth, 2019), our results suggest that these differential effects associated to population origin and site of collection are the expression of an endophyte-mediated ecotypic response.

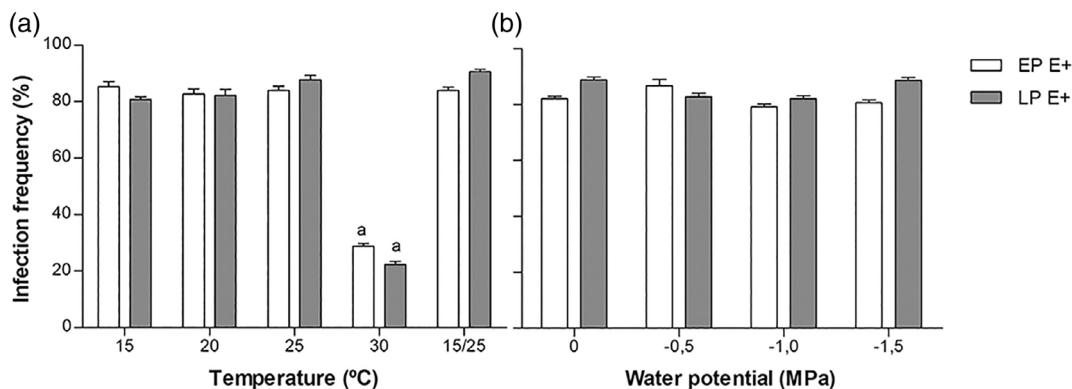


FIGURE 4 *Epichloë* transmission from seed-to-seedling in two endophyte-infected (E⁺) ecotypes of *Bromus auleticus*, La Pampa (LP) and El Palmar (EP). Endophyte presence was determined in 2-month old seedlings originated by the seeds that germinated at different temperatures [(a) constant (15, 20, 25, 30°C) and alternating (15/25°C) temperatures] and medium water potentials [(b) 0, −0.5, −1.0, and −1.5 MPa]. Values are means \pm SE ($n = 5$). Letters indicate significant different means (LSD, $p < 0.05$)

Numerous studies have shown that seeds from different *B. auleticus* accessions present different degrees of dormancy (Gonzalez & Condón, 2020; Kindiger, 2019; Ruiz et al., 2006). However, none have previously considered the role of fungal endophytes on *B. auleticus* seed germination. To achieve our comparative study, we used seeds from plants developed from seeds that lost or kept the endophytes 7 years ago. Thus, it should be taken into account that if some genotypes tended to lose endophyte more easily than others, the step of curing seed of the endophyte may have selected for two sets of plants with some systematic genetic difference between them. However, as in each population, E+ and E− plants are free pollinated, these differences should be partially alleviated. Previous reports indicate that natural germination can occur when temperatures range between 10 and 25°C (Ruiz et al., 2006). In our work, we recorded germination between 15 and 25°C, and a strong inhibition at 30°C. Since seeds of *B. auleticus* in the field germinate in autumn (Olmos, 1993), when mean lowest and mean highest temperatures range between 12 and 25°C (Table 1), sensitivity to high temperatures (>30°C) may be a mechanism to prevent germination in unfavourable conditions. However, even within the range of suboptimal thermal conditions (15–25°C), final germination values in our experiment (<70%) were lower than those previously reported for *B. auleticus* (Ruiz et al., 2006; Iannone, Pinget, et al., 2012; Gonzalez & Condón, 2020, but not in Kindiger, 2019). Considering that seed viability was 100%, low germination could be attributed to a high proportion of dormant seeds in the populations or induction of secondary dormancy. The seeds used here were stored in a refrigerator (cold and dry conditions), hence temperature was too low during storage to release seed dormancy (Benech-Arnold et al., 2000; Finch-Savage & Leubner-Metzger, 2006) as it occurs in summer in the field (Table 1). Dormancy can be also reinforced by the occurrence of high temperature as a security mechanism preventing seed from germinating at an inadequate time (Benech-Arnold et al., 2000). Although a throughout experiment is further needed to understand the after ripening dynamics of seed dormancy, we observed that seeds of LP seeds germinated at a higher rate than seeds of EP ecotype, which could be associated with genotypic differences attributed to the geographic origin of the ecotypes. In each ecotype, the effect of the endophyte became significant under different thermal regimes, but in general, E+ seeds presented higher germination percentages and/or rates than E− seeds.

Alternating temperatures may promote germination as, besides their effect on germination rate of non-dormant seeds, they release seeds from dormancy (Baskin & Baskin, 1998; Benech-Arnold et al., 2000). However, we did not observe any stimulating effect of alternating temperature on either final germination or rate of germination. This somehow unexpected result can be because the highest extreme in the alternating temperature treatment (i.e., 25°C) was very close to the highest optimal peak for seed germination of this species. Nonetheless, the response of *B. auleticus* seeds to alternating temperature (20°C vs 15/25°C) depended on the host ecotype and on the endophytic status. In the LP ecotype, E+ seeds displayed a significantly higher final germination than E− seeds when subjected to the 15/25°C regime, while no differences in germination rate were observed between LP E+ and LP E− seeds (although germination rate

was lower than at 15/25°C). In EP, germination of E− seeds was inhibited in the 15/25°C regime as well as at constant 25°C, which together would suggest a high sensitivity to increasing environmental temperatures. The different seed response to endophyte presence between E+ and E− seeds of each ecotype, may reflect adaptive advantages of the association with *Epichloë* to the prevailing environmental conditions in each locality. These kinds of effects were also observed in other endophyte-infected grasses like *Achnatherum inebrians* (Bao et al., 2019).

With a few articles focusing on seed germination and seedling growth/survival, the relationship between fungal endophyte presence and host tolerance to water deficit has been, by far, one of the most studied topics (Gundel et al., 2016; Malinowski & Belesky, 2000). *B. auleticus* is broadly considered a species tolerant to drought (Puebla et al., 1997), although our study suggests ecotypic variation and endophyte dependent effects. As expected for this kind of experiment (Bradford, 2002; Finch-Savage & Leubner-Metzger, 2006), germination is inhibited as medium water potential is more negative. Germination rate was more sensitive to Ψ_w than final germination, decreasing almost linearly with Ψ_w , and germination rate of LP E+ seeds showed differences even between pure water and −0.5 MPa. Interestingly, as Ψ_w became more restrictive for germination, seeds from EP showed higher germination than those from LP. In LP, *E. pampeana* did not affect germination at the different Ψ_w , as reflected by final germination, germination rate, and the hydrotime parameters. Similar results were observed in *F. arizonica* under similar water availability conditions (Neil et al., 2003). In EP ecotype, E+ seeds showed the same response pattern as LP ecotype seeds, but strikingly, final germination of EP E− seeds was unaffected by water deficit. The response of EP E− seeds corresponded to a very low Ψ_b (50) that, in spite of a higher θ_H , can explain the superior germination rate displayed at all medium water potentials. The high germination inhibition of E+ seed by low water potentials has been previously observed in *L. multiflorum* and *Festuca rubra*, but at the same time, the presence of the endophyte increased the survival of ungerminated seeds (Gundel et al., 2012; Gundel, Maseda, Vila-Aiub, et al., 2006). It can be speculated that endophytes benefit the host by preventing seed germination under growth restrictive water levels; but if water shortage is followed by benign conditions for growth, E− seedlings will have a competitive advantage over E+ individuals. Although these speculations support the idea that endophytes are advantageous for plants under stress (Gundel et al., 2016; Malinowski & Belesky, 2000; Rodríguez et al., 2009), this must be proven in the field.

4.1 | Playing at the limit: How to modulate seed germination, increasing seedling survival, and not die while trying?

During the quiescent state of the seed, the fungal endophytes must remain alive for being transmitted to the seedling (Gundel et al., 2011). As most winter species (Baskin & Baskin, 1998; Donohue, 2005), *B. auleticus* produces seeds in spring, forms transient soil

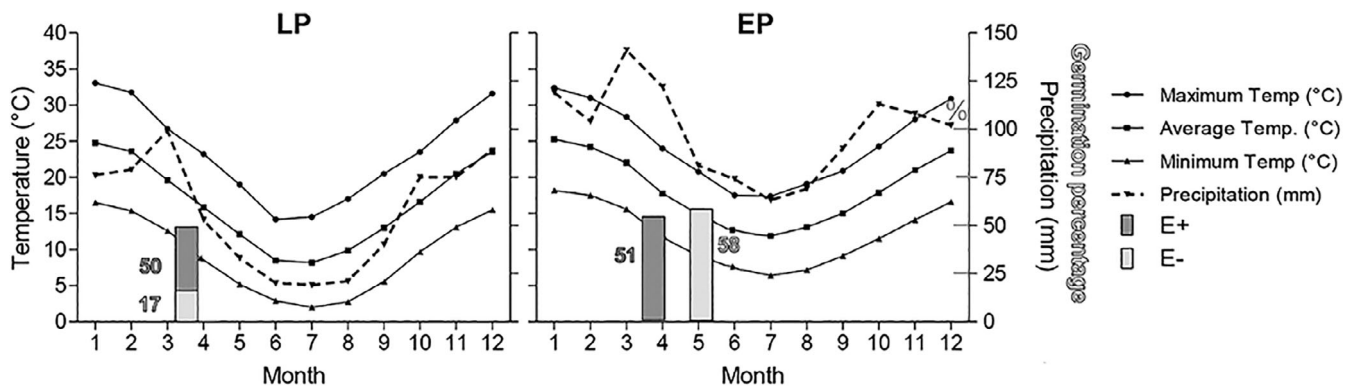


FIGURE 5 Environmental conditions (mean precipitation [mm] and temperature range [°C]) throughout the year in the collection sites of the seeds. Germination of *Bromus auleticus* occurs when temperature is under 30°C. The position and the height of the bars indicates the time and the percentage of germination of endophyte-infected (E+) and endophyte-free (E-) seeds under such environmental conditions, accordingly to our results

seed banks during summer, and the first cohort normally germinates in autumn (Gutiérrez & Pensiero, 1998; Olmos, 1993). By checking the endophyte presence in seedlings, we could trace-back for failures in transmission from seed-to-seedling due to the seed incubation treatments. We observed that the endophyte infection frequency was very high in seedlings of both ecotypes (>80% and <90%), irrespectively of (most) thermal treatments and water conditions. However, we did observe a significant reduction in the infection frequency of seedlings coming from seeds that were incubated at constant 30°C. These seeds remained full-imbibed but ungerminated for 3 weeks at 30°C and germinated normally once transferred to constant 20°C; they produced normal but E- seedlings. The very well-known sensitivity of fungal endophytes to temperature and ambient relative humidity has been studied under storage conditions (Bylin et al., 2016; Gundel et al., 2009, 2010; Rolston et al., 1986; Tian et al., 2013; Welty et al., 1987). Here, we found that high temperature killed the fungal endophyte and also inhibited seed germination.

High summer temperatures tend to release seed dormancy in winter species and can be detected as a relaxation of the range of temperatures that permit germination if temperature and water availability are adequate (Benech-Arnold et al., 2000; Finch-Savage & Leubner-Metzger, 2006). The complete inhibition of *B. auleticus* seed germination by constant 30°C may be a mechanism to prevent seedling emergence under conditions that are still characterised by high atmospheric demands, drought, and active growth of competitive summer species. High peak of temperature can impose dormancy that coupled with high water availability, as it occurs in both localities in summer (Table 1), becomes a risky condition for endophyte survival. However, *B. auleticus* populations show high levels ($\approx 100\%$) of endophyte infection in the field (Iannone et al., 2009, 2012,b). The differential effects of the endophyte in each ecotype could reflect an endophyte–host integrated response to each environment (Figure 5). In La Pampa, where temperatures between 15 and 25°C are common in March–April (beginning of autumn), LP E+ seeds would germinate at a higher proportion than LP E- seeds. In El Palmar, EP E+ seeds

would start germinating at the beginning of autumn (April) when average maximum daily temperatures are near 25°C but with high probability of precipitation. Alternatively, EP E- seeds would start germinating 1 month later (May–June) when maximum temperatures are lower than 25°C (Figure 5), facing the driest period of the year. Enhancing germination at high temperature or preventing it from occurring in a wrong place and time may result from species specific adaptive seed physiological traits, and to be endophyte-modulated seed fitness trait that also minimises endophyte loss. These effects on seed germination altogether with the sum of other clear benefits would make *B. auleticus* E+ plants prevail over E- plants (Iannone et al., 2017; Iannone, Pinget, et al., 2012; Vignale et al., 2013) and reinforce the importance of the symbiosis with *Epichloë* in the management of this wild forage species.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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