Seed-transmitted *Epichloë* sp. endophyte alleviates the negative effects of head smut of grasses (*Ustilago bullata*) on *Bromus auleticus*

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Abstract

Some grasses are co-infected by “castrator” fungal pathogens, such as *Ustilago bullata*, and beneficial seed-transmitted *Epichloë* endophytes that compete for plant photosynthates and flowers. *Epichloë*-infected (E+) and *Epichloë*-free (E-) seeds of *Bromus auleticus* were inoculated (S+) or not (S-) with teliospores of *U. bullata* and an experiment under field conditions was performed to evaluate the effect of these fungal infections on different host plant traits. Head smut disease incidence was almost 0% in E+ treatment whereas in endophyte-free plants the disease incidence reached 33%, a result in concordance with the lower number of seeds produced by E-S+ plants. Neither the smut nor the endophyte affected seedling emergence or growth. Plant survival was significantly decreased by the smut in E- plants and was associated with a higher biomass production in E+ treatments. Our results provide new insights about the ecology of *Epichloë* endophytes and their protective and growth-promoting role in the interaction of its host with *U. bullata*.

Keywords

Defensive mutualism; Symbiosis, *Epichloë*; Grass-endophytes; head smut disease; *Ustilago bullata*, Forage production, Plant disease.

Introduction

Epichloë spp. (Clavicipitaceae, Hypocreales) are fungal endophytes associated with some cool-season grasses of the subfamily Pooideae. In the host plant, these endophytes grow in the apoplast of the meristems from where the fungus proliferates in most shoot and inflorescence primordia and systemically colonize, apparently by intercalary hyphal growth, developing structures of the host plant as host cells elongate (Christensen et al. 2008). This growth pattern also allows endophytes to access nutrients transported via the apoplast (Kuldau and Bacon 2008). Sexual species of *Epichloë* produce yellow-orange stromata on the leaf sheath that surround the culms, arresting the development of the inflorescences and causing total or partial sterility of the host grass (Clay and Schardl 2002; Leuchtmann et al. 2014). In the stromata, perithecia are formed bearing ascospores that are forcibly discharged and infect new plants. In contrast, asexual *Epichloë* species (formerly *Neotyphodium*) are mainly vertically transmited via the seeds of the host plant (Clay and Schardl 2002), although the conidia and mycelium produced in the phylloplane of the host can potentially horizontally transmit the endophyte to other plants (White et al. 1996; Tadych et al. 2012). In plants, the hyphae of asexual endophytes colonize the meristem of developing flowers and after pollination, when seeds develop, the endophyte colonizes the embryo and aleurone layer of the seeds so that when they germinate, the endophyte colonizes the developing seedling (Schardl et al. 2004).

The interaction between asexual *Epichloë* and their hosts is in general considered to be mutualistic. The endophyte obtains photosynthates and shelter from the plant and provides several benefits to its host. Among these benefits, the most important is protection against herbivores, due to the capability of the endophyte to produce different alkaloids, including lolines and peramine, mainly toxic to insects, and lolitrems and ergot alkaloids, primarily affecting grazing mammals (Bacon 1977; Clay and Schardl 2002; Lane et al. 2000; Latch 1993; Panaccione et al. 2006; Popay et al. 2009; Schardl and Phillips 1997; Schardl et al. 2004, 2007; Torres et al. 2008). Endophyte-infected plants may have morphological and physiological adjustments (Torres et al. 2011) that confer increased growth and tollerance to abiotic stresses (Clay 1987; Soleimani et al. 2010; Zhang et al. 2010; Nagabhyru et al. 2013), and resistance to several viruses and fungal pathogens (Gwinn and Gavin 1992; Clarke et al. 2006; Weiwióra et al. 2015; Xia et al. 2015; Wang et al. 2016).

The smut fungus *Ustilago bullata* (Ustilaginales, Basidiomycota) is, commonly known for being the causal agent of the “head smut of grasses” disease in several species of *Bromus* and *Elymus* (Meyer et al. 2016). *Ustilago bullata* is a highly polymorphic and systemic smut fungus that infects its host soon after the emergence of the coleoptile from the seed (Falloon 1979; Fischer 1940). The pathogen is dispersed by teliospores (dikaryotic spores), which in soil or in the coats of the seeds undergo karyogamy and meiosis producing basidiospores, which germinate forming a pro-mycelium or proliferate as yeast-like sporidia. When two pro-mycelia or sporidia from the opposite mating type meet, they fuse to form a dykaryotic infection hypha that penetrates the host and asymptomatically colonizes the meristem of the host and systemically grows inside the host. The presence of the fungus in its host becomes apparent at anthesis when the ovaries of infected plants are destroyed, being replaced by a dark black mass of sori with teliospores, the bullae (Falloon and Hume 1988). Rainfall causes the bullae to expand and break down so that the spores are released and most are probably dispersed by raindrops onto the soil (Meyer et al. 2016). Even though *U. bullata* can infect several genera of cool season grasses, the existence of high host specificity was determined by Fischer (1940) and different races infect different host species (Meyer et al. 2008).

The effects of *U. bullata* have been extensively studied in the forage species *Bromus catharticus var. catharticus* in New Zealand. In this species, under field conditions, the inoculation with *U. bullata* significantly reduced seedling establishment (Falloon and Rolston 1990), biomass production (Falloon 1976, 1979; Falloon and Hume 1988; Falloon and Rolston 1990) and infected plants presented poorer persistence (Falloon and Hume 1988) than non-infected plants. In greenhouse experiments, the pathogen reduced seedling emergence rate and survival (Falloon 1976), reduced the overall size of infected plants and caused their complete sterility (García-Guzmán et al. 1996).

Ustilago bullata has also been studied as a control agent of the invasive annual species *Bromus tectorum* (Meyer et al. 2008). In this host species, depending on the pathogen genotypes, the fungus causes high levels or total sterility (Meyer et al. 2001). Disease incidence is largely affected by environmental conditions during seed germination and seedling emergence, increasing as temperature and winter precipitation increase (Boguena et al. 2007; Prevéy and Seastedt 2015).

In South America, *U. bullata* has been reported as a pathogen of several species of *Bromus* and *Hordeum* (Hirschhorn 1986). *Bromus auleticus* is a native forage grass from Argentina, Brazil and Uruguay that could be infected by *U. bullata.* Depending on the population, *B. auleticus* can be associated with *Epichloë pampeana*, *E. tembladerae* (Iannone et al. 2009) or other *Epichloë* spp. (Mc Cargo 2015). *Epichloë*-infected *B. auleticus* plants produce more biomass and seeds than *Epichloë*-free plants (Iannone and Cabral 2006; Iannone et al. 2012a). In a previous study under controlled conditions in a pot experiment, Vignale et al. (2013) demonstrated that the asexual species *E. pampeana* and *Epichloë* sp. (refered to as *E. tembalderae* in Vignale et al. 2013) reduce or suppress the development of the smut head disease in *Bromus auleticus* and are efficiently transmitted in the seeds so that both plant and endophyte are benefitted. In addition, smut-inoculated, endophyte-infected seeds produced larger seedlings that exhibited increased survival (Vignale et al. 2013). In this triple symbiosis, when plants are simultaneously infected by the smut and seed transmitted-*Epichloë,* both systemic fungi compete for photosynthates, plant resources, shoot meristems and ovaries of developing flowers. If the endophyte prevents *U. bullata* from colonizing the seedlings or the ovaries, both host and endophyte benefit.

With the aim of improving our understanding of the impact of seed-transmitted *Epichloë* endophytes, in a field experiment, we studied whether the susceptibility of *B. auleticus* to the pathogen *U. bullata* depended on the plant´s endophyte infection status. In addition, we evaluated the effect of the inoculation of teliospores in endophyte-infected and endophyte-free seeds on host traits including seedling emergence and growth, plant survival and biomass, culm and seed production.

Materials and methods

Plant and smut fungus material

Endophyte-infected (E+) and endophyte-free (E-) seeds of *B. auleticus* were harvested in December 2013 from a nursery of 20 E+ and 20 E- plants, established in 2010 in the experimental field of INTA-Concepción del Uruguay Agronomic Experimental Station, Entre Rios province, Argentina. In this nursery, E- plants were developed from E+ seeds that had lost the endophyte during storage. Two lots of seeds with different endophyte status (E+ or E-) were established by pooling the seeds collected from individual E+ or E- plants. The endophyte infection percentage of each lot was confirmed by microscopic observation (Zeiss Axioskop microscope) after softening in 10% KOH and staining with aniline blue in lactophenol (modified from Clark et al. (1983)). The percentage of *Epichloë*-infected seeds was 98% in the E+ lot and 0% in the E- lot. Teliospores of *U. bullata* were collected from E- plants of *Bromus auleticus*, obtained from a previous trial performed under greenhouse conditions by Vignale et al. (2013) to evaluate the susceptibility of *B. auleticus* to *U. bullata.* To collect teliospores, sori were ruptured by rubbing them on a mesh soil sieve. Teliospore viability (41%) was previously evaluated by germinating a suspension of teliospores on potato dextrose agar (PDA) culture medium and checking the percentage of teliospores with germ tubes under an optical microscope, after incubating for 8 h at 24 C.

Each seed lot was respectively divided in eight sub-lots (8 E+ and 8 E-) with 15 g of seeds each. To establish the smut inoculated treatment (S+), four sub-lots of seeds of each endophytic status were inoculated with teliospores of the smut in a dose of 0.16 mg g^{-1} seed. Seed lots assigned to no-smut treatment (S-) were mock inoculated, with a suspension of dead teliospores that were previously heat treated at 170C for 3h.

Experimental design

In April 2014 seeds of *Bromus auleticus* infected with *Epichloë* sp. (*Neotyphodium tembladerae* in Vignale et al. 2013) were sown in the field in a randomized complete block design (RCB) with 4 replicates in a subdivided plots arrangement. The main plot corresponded to the smut treatment and involved two levels: smut inoculated seeds (S+) or mock inoculated seeds (S-). The subplot was assigned to the endophytic status with two levels: endophyte-infected seeds (E+) and endophyte-free seeds (E-). Each subplot consisted of three 2m-long lines, 20 cm apart, where 15 g of seeds (5 g/line, 1000 seeds/line approximately) were sown. In October 2014 twenty-five vegetative tillers of each subplot were collected and used to microscopically check the endophytic status of each treatment. The percentage of *Epichloë*-infected tillers was 90-100% in the E+ subplots and 0% in the E- subplots.

Seedling emergence and growth

In July 2014 (3 months after sowing) the effect of the smut and the endophyte on seedling emergence was quantified by counting the number of seedlings in six 20 cm long spots (two per line) of each subplot. To evaluate the effect of the smut and the endophyte on seedling growth, the length of the longest leaf (Novas et al. 2003; Iannone and Cabral 2006) of ten randomly selected seedlings of each subplot was measured.

Plant survival and biomass production

Plants were allowed to grow for 1y and fertilizer was applied in July 2014 by adding 15g m-$^{-2}$ of diammonium phosphate ((NH_4) $2HPO_4)$) and 8gm^{-2} of urea to each plot (level of fertilization commonly applied to grasses on vertisols from Entre Rios province). In April 2015 (1y after sowing), plants were cut 7 cm above the soil, height that is usually used for this plant in field management. The aerial vegetative biomass was harvested and weighed to evaluate biomass production. Due to plant mortality, portions of the lines presented bare soil. Thus, we established the soil cover by measuring the length covered by the portion of the tillers that remain in each subplot after the cutting. To evaluate the effect of the endophyte and the smut on plant survival in the first year, the percentage of the relationship between soil cover and the total length of the lines (6 m) was established. To evaluate if the differences in biomass production during the first year in each were due to differences in plant survival among treatments, we estimated the relationship between biomass production and soil cover (biomass m^{-1}) in each subplot. Plants were then allowed to re-grow and no other cuts were performed until December 2015, to allow plants to flower.

Head smut disease incidence, vegetative biomass, culm and seed production

At the end of December 2015, 20 months after sowing, plants were cut 7 cm above the soil and harvested. Vegetative biomass was separated from the culms and weighed to measure biomass production in the second year (April 2015-December 2015). To evaluate the effects of the treatments on culm production, all the culms in each subplot were collected and counted. The number of culms that exhibited flowers with head smut symptoms was recorded and the incidence of the disease, in each subplot, was established as the proportion of symptomatic culms. Culms with seeds were allowed to dry completely for 20 days at room temperature. Seeds were separated by hand and weighed after removing unfilled seeds. The weight of 1000 seeds per subplot was also recorded. Both the weight of seeds and the weight of 1000 seeds were used to estimate the number of seeds produced by plants of each subplot (seed production).

To evaluate the effects of the smut and endophyte treatments on resources allocation, the relationship between culm production and soil cover, culm production and biomass or seed production and biomass (seed output) (Thompson and Stewart 1981) were determined.

Statistical analyses

Differences between treatments were analyzed by means of an analysis of variance for a subdivided plot arrangement in RCB ($p<0.05$) for all the parameters studied: seedling emergence and seedling growth, plant survival, biomass production in April and December 2015 (12 and 20 months after sowing, respectively), biomass m^{-1} (April 2015), smut incidence (as the percentage of symptomatic culms), culm and seed production, culm production/soil cover, culm production/biomass, 1000 seeds weight and seed output. The main factors were: Smut (two levels: S+ and S-) and Endophyte status (two levels: E+ and E-) and the interaction between Smut and Endophyte status. Differences were analyzed using the Infostat software (Di Rienzo et al. 2011). All assumptions were tested. To accomplish the model assumptions, log and square root transformations were applied to biomass production (12 months after sowing) and head smut disease incidence, respectively. All analyses were performed at 0.05 significance level. Tukey *posthoc* analyses were performed.

Results

Seedling emergence and growth

Inoculation with *U. bullata* did not affect seedling emergence (F=0.47, p=0.54) (Table 1) or seedling growth (F=0.20, p=0.69) (Table 1). Although E+ subplots presented on average slightly more and taller seedlings than E- subplots, these differences were not significant (F=0.37, p=0.56) and (F=0.12, p=0.74), respectively.

Plant survival and biomass production

In April 2015, 1y after sowing, significant differences were observed in plant survival and biomass production. Plant survival was significantly affected by the smut (F_S=12.45, p_S=0.04) and the endophyte (F_E=22.59, p_E<0.01), but the interaction between both factors was not significant (F_{SxE}=2.92, p_{SxE}=0.14). The inoculation with teliospores of *U. bullata* in the seeds, significantly reduced plant survival in E-S+ subplots in comparison with the E-S- subplots (Fig. 1 A). No differences were observed due to the smut between E+ treatments (Fig. 1 A).

Biomass production was significantly affected by the endophyte status of the plants. One year after sowing, endophyte-infected plants produced significantly more biomass than E- plants (F_E=49.78, p_E<0.001). Even though smut inoculation did not significantly affect biomass production (F_S=4.91, p_S=0.11), the biomass produced in E-S+ subplots was 26% and 50% lower than in E-S- and E+S+ subplots respectively (Fig. 1 B). The interaction, between the factors smut and endophyte status, was not significant (F_{SxE}=1.02, p_{SxE}=0.35) (Fig. 1 B). During the second year (April 2015-December 2015), vegetative biomass production (Table 2) was higher in E+ than in E- subplots but the smut did not affect this parameter (F_S=0.16, p_S=0.71), (F_E=10.7, p_E<0.02), (F_{SxE}=0.01, p_{SxE}=0.98).

Biomass production relative to soil cover (Biomass/m) during the first year (Fig. 1 C) was not significantly affected by the smut or the endophyte (F_S=7.52, p_S=0.07), (F_E=0.82, p_E=0.40), (F_{SxE}=0.95, p_{SxE}=0.36).

Effect of the smut and endophyte on disease expression

The incidence of head smut disease, measured 20 months after sowing (December 2015), was significantly higher in E-S+ than in the other treatments (F_S=15.18, p_S=0.03; F_E=94.40, p_E=0.0001; F_{SxE}=58.90, p_{SxE}=0.0003), presenting 33% symptomatic culms (Fig. 2). In E+S+ plants, only 1.5% of the culms exhibited smut symptoms and this value was not different from that observed in E+ or E- controls without smut (Fig. 2). Considering both E+ treatments, 33 culms out of 3047 presented disease symptoms and the analysis of endophyte presence indicated that 29 out of the 33 culms were endophyte-free.

Effect of U. bullata and endophyte on culm and seed production

Inoculation with *U. bullata* did not affect the production of culms in E+ or E- plants (F_S=0.29, p_S=0.63). However, in endophyte-infected subplots plants produced on average 43% more culms than in E- subplots (F_E=17.39, p_E<0.01) (F_{SxE}=0.84, p_{SxE}=0.39) (Fig. 3 A). No differences were found in culm production per soil cover (Table 2), or in culm production/biomass (Table 2).

Neither the smut nor the endophytic status affected the weight of 1000 seeds, (F_S=0.15, p_S=0.72) and (F_E=0.002, p_E=0.9), respectively (Table 2). Inoculation with *U. bullata* did not affect seed production (F_S=6.67, p_S=0.08) but in endophyte-infected subplots the plants produced more seeds than in E- subplots (F_E=21.15, p_E=0.03) (Fig. 3 B). Smut-inoculated E+ plants produced the highest number of seeds whereas plants in the E-S+ treatment produced the lowest number. Although the interaction between smut inoculation and endophytic status was not statistically significant (F_{SxE}=5.5, p_{SxE}=0.057), the inoculation with *U. bullata* seemed to affect the E+ and the E- plants differently (Fig. 3 B). Whereas E+S+ plants produced 4% more seeds than their S- counterparts, smut inoculation reduced the production of seeds by E- plants by 40%, and endophyte-infected plants in the S+ treatments produced 60% more seeds than their E- counterparts. No significant differences were observed in the number of seeds with respect to the biomass (seed output) (Table 2).

Discussion

Most of the knowledge about the symbiosis between grasses and *Epichloë* species proceed from agronomically selected forage grasses as *Festuca arundinacea*, *Lolium multiflorum* and *Lolium perenne*. In general, defense against herbivores and environmental stress are considered the main driving selective forces behind mutualism between grasses and *Epichloë* endophytes (Clay 2009; Saikkonen et al. 2010). In forage grasses, growth promotion has also been associated with seed-transmitted *Epichloë*. However, in some wild grasses, such as *Festuca arizonica*, the endophyte seems to be detrimental for the host plant, reducing plant growth and survival (Faeth and Sullivan 2003; Faeth et al. 2004) or negatively affecting seed germination (Hamilton and Faeth 2005). Despite this discrepancy between wild and domesticated grass-*Epichoë* associations, an increasing number of experiments demonstrate that protection against fungal pathogens may play an important role in the evolution and maintenance of these associations (Clarke et al. 2006; Kuldau and Bacon 2008; Vignale et al. 2013; Xia et al. 2016). In this work, we have evaluated how two fungal symbionts, a seed-borne pathogen and a mutualistic seed-transmitted endophyte, both of which systemically colonize grasses, affect survival, growth and reproduction of their host grass. Our results show a beneficial role of asexual *Epichloë* on resistance to the pathogen, *Ustilago bullata.*

Smut fungi are considered to be castrator pathogens (Burdon 1993) because seeds are replaced by "the bullae", sori with teliospores. In *Bromus auleticus* the association with *Epichloë* suppressed the development of head smut disease, whereas in E- treatments the incidence of the disease reached 33%. This result is consistent with a previous study performed under controlled conditions by Vignale et al. (2013), who, working with the same *U. bullata-Epichloë-B. auleticus* combination, reported that plants associated with *E. tembladerae* (*Epichloë* sp. in this work) were more resistant to the ‘‘head smut of grasses" disease caused by *U. bullata* than endophyte-free plants. The incidence of disease (although low) observed in the E+S+ treatment could be due to the absence of *Epichloë* in those culms, since the endophyte was not detected in the microscopic examination of the symptomatic E+ culms. This could be explained by the fact that 98% of endophyte-infected seeds were used to establish the E+ seed lots and E- tillers can be produced by E+ plants, with the smut being able to colonize E- plants or tillers.

In the experiments performed under controlled conditions by Vignale et al. (2013) the disease incidence reached 60% while we obtained 33% of head smut incidence. This difference may be due to differences in environmental conditions during seed and teliospore germination. Vignale et al. (2013) conducted germination at 24°C whereas in our work, during seed germination, temperature varied between 10 and 25ºC. Soil temperature at the time of seed and teliospore germination has been shown to be a crucial factor on disease incidence in *Bromus catharticus* and *B. tectorum* (Boguena et al. 2007). Given these differences, more experiments should be conductedto establish the effect of the endophyte and temperature at the time when the infection of *B. auleticus* by *U. bullata* occurs.

As a systemic pathogen, *U. bullata* may also alter other plant traits such as seedling emergence and growth (Falloon 1976; García-Guzmán et al. 1996), but in our study smut inoculation did not seem to affect these traits. These results differ from those observed by Vignale et al. (2013) where the inoculation with teliospores diminished the percentages of seedling emergence and produced shorter seedlings regardless of the endophytic status. However, the higher percentage of bare soil observed in our work in smut inoculated E- subplots, 12 months after sowing, indicates that the pathogen increased plant mortality of endophyte-free plants during the first year of growth. This result is in accordance with that observed by Vignale et al. (2013) working with the same seed and smut genotypes, having reported that in a pot experiment, 85% of the plants died during the first year. Plant mortality has been associated with *U. bullata* in *B. catharticus* (Falloon 1976), but Fischer and Holton (1957) and Falloon and Hume (1988) reported that *U. bullata*-infected prairie grass plants were more susceptible to root rot pathogens than non-infected plants. Thus, plant mortality observed in our experiment could be attributed to higher sensitivity to other soil-borne pathogens due to the infection with *U. bullata* in E- plants, and not directly to *U. bullata*.

Endophyte-infected subplots produced more biomass than E- ones inoculated with the smut, and smut inoculated E- subplots produced less biomass than the S- controls. Negative effects of *U. bullata* on biomass production were also observedin field studies with *B. catharticus* (Falloon 1976, 1979; Falloon and Hume 1988; Falloon and Rolston 1990). In our experiment, the higher amount of biomass produced during the first year by plants in E+ or E-S- treatments was due to the higher soil cover and not to the size of the plants, since the ratio between biomass production and soil cover in E+ subplots was not significantly higher than in the E- subplots (Fig. 1 C).

The inoculation with *U. bullata* did not affect the number of sexual reproductive tillers (culm production), and did not have distinctive effects on E+ or E- plants. The higher number of culms observed in E+

subplots was due to the higher number of plants, since no significant differences were observed in the number of culms relative to soil cover or vegetative biomass (Table 2) among the treatments.

In accordance with the differences observed between E+ and E- subplots regarding the incidence of the disease, smut inoculation had different effects on seed production by E+ and E- plants. Whereas in *Epichloë*-free plants, seed production was reduced by the smut, in E+ plants the endophyte prevented the infection of flowers by the smut, maintaining seed production and so ensuring successful dispersal of the host and the endophyte.

Considering that *B. auleticus* is a perennial forage grass and that *U. bullata* infection persists as an endophyte (latent-pathogen) during vegetative growth periods, it is worth considering the effect of co-infection by the latent pathogen and *Epichloë*-endophytes on vegetative biomass production with time. The effect of the smut on biomass production depended on the endophytic status of the plant. *Epichloë*-infected subplots produced more biomass over the 20 months period than E- subplots and the smut did not affect this parameter. In addition, when infected with *U. bullata,* E- subplots produced on average half the biomass as their E+S+ counterparts. The greater biomass produced by E+ subplots or the E- without smut was not due to the bigger size of the plants, since no significant differences were noticed in the biomass production/soil cover ratio among the treatments. In smut-inoculated E- subplots, biomass production in the first year of growth was significantly reduced with respect to E- controls but these differences disappeared during the second year. As we used the plant cover as an estimator of plant survival, this result indicates that the low amount of biomass produced in E-S+ subplots was associated with a lower number of plants due to mortality induced by the smut during the first year. During the second year, these differences were mitigated since the smut did not cause mortality in established E- plants. This result is in agreement with Iannone et al. (2012 a) where E+ individual plants of *B. auleticus* produced more biomass than E- plants and it highlights the importance of the endophyte on plant survival during the first months of development with respect to biomass production.

We cannot establish if the antagonism between *Epichloë* and *U. bullata* occurs in the spermosphere, preventing E+ seedlings to be infected by the smut or if the endophyte prevents the colonization of floral meristems by the smut. *In vitro* experiments showed that in dual cultures the endophyte inhibited teliospore germination (Iannone et al. 2012b), but in preliminary experiments performed in moist chambers at 24ºC (not shown) we observed that teliospores are able to germinate on E+ seeds and produce sporidia 12 h after being inoculated. Future studies will allow us to understand the mechanism by which endophyte-infected plants of *B. auleticus* become resistant to *head smut seed of grasses* disease and if this protective effect also occurs in other *Epichloë*-host associations.

Finally, the results of this work increase our current understanding of the impact of a seed-transmitted endophyte on the host susceptibility to the systemic seed pathogen *U. bullata* and the host growth traits under field conditions. We not only show the importance of the endophyte in protecting their hosts from a systemic seed pathogen and preventing the negative effects of *U. bullata* on seedling establishment and biomass production, but also show the positive effect of the endophyte on forage production.

Acknowledgments

This paper is dedicated to the memory of J. P. De Battista and we want to thank him for his support and his significant contribution to our research. This research was supported by the University of Buenos Aires (grant UBACyT 20020150200075BA), Consejo Nacional de Investigaciones Científicas y Técnicas (grant PIP 2015-2017 11220150100956CO) and Agencia Nacional de Promoción Científica y Tecnológica (grant PICT 2014-3315). INMIBO UBA-CONICET: Publication n° 214.

Conflict of interest: the authors declare that they have no conflict of interest.

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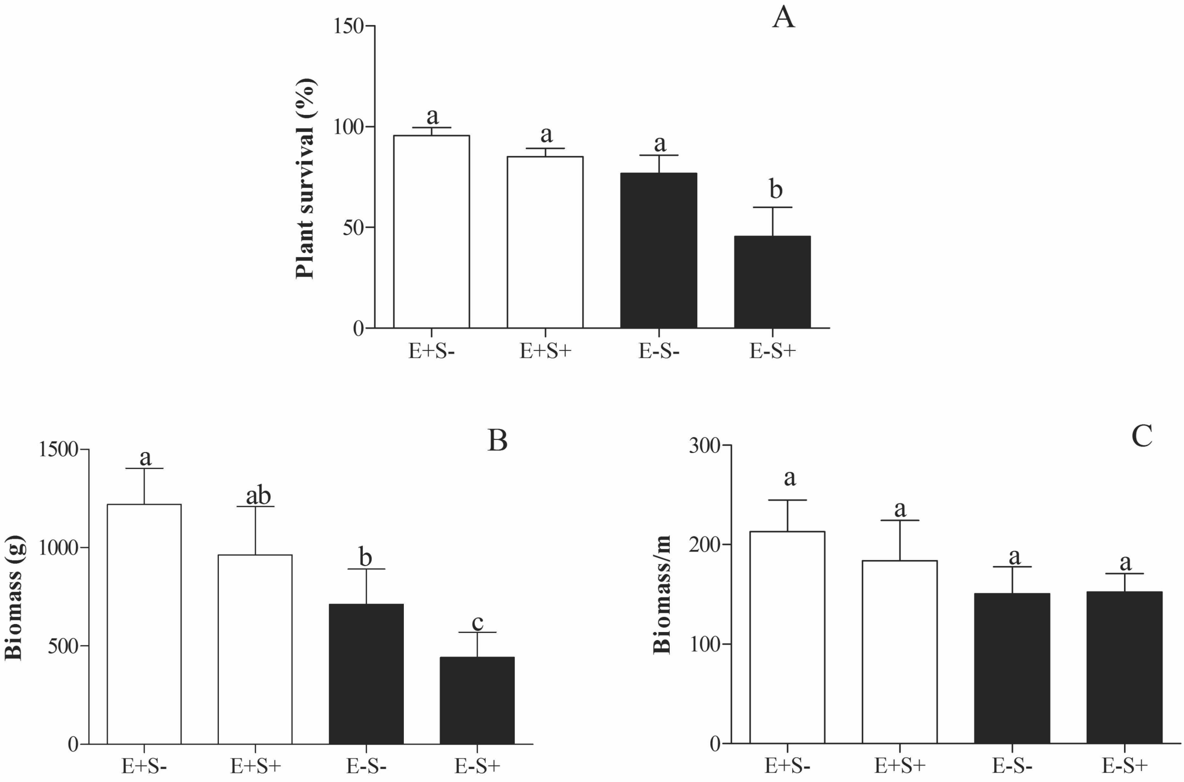
Fig. 1 Effect of *U. bullata* (S+/S-) and *Epichloë* endophyte status (E+/E-) on *B. auleticus* plants. **A.** Plant survival, estimated as the percentage of the relationship between soil cover and the total length of the lines, **B.** Aerial vegetative biomass production (g), and **C.** Aerial biomass relative to soil cover. Measurements were made after the first year of growth. Error bars represent standard error. Different letters indicate significant differences among treatments (p<0.05)

Fig. 2 Effect of *U. bullata* (S+/S-) and *Epichloë* endophyte status (E+/E-) on head smut of grasses disease incidence, estimated as the percentage of culms that exhibited head smut disease symptoms. Error bars represent standard error. Different letters indicate significant differences between treatments (p<0.05)

Fig. 3 Effect of *U. bullata* and *Epichloë* on reproductive traits of *Bromus auleticus.* **A.** Culm production, **B.** Seed production in endophyte-infected (E+) and endophyte-free (E-) subplots. Measurements were made in December 2015, 20 months after sowing. Error bars represent standard error. Different letters indicate significant differences among treatments (p<0.05)


A
Plant survival (%)
150
100
50
0
a
a
a
b
E+S-
E+S+
E-S-
E-S+
B
Biomass (g)
1500
1000
500
0
a
ab
b
c
E+S-
E+S+
E-S-
E-S+
C
Biomass/m
300
200
100
0
a
a
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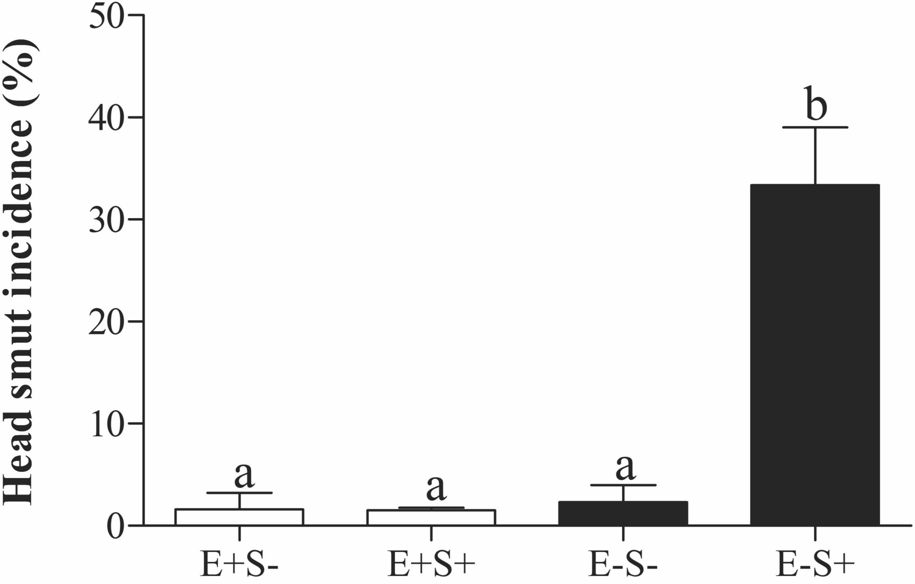
E+S+
E-S-
E-S+


Head smut incidence (%)
50
40
30
20
10
0
a
a
a
b
E+S-
E+S+
E-S-
E-S+

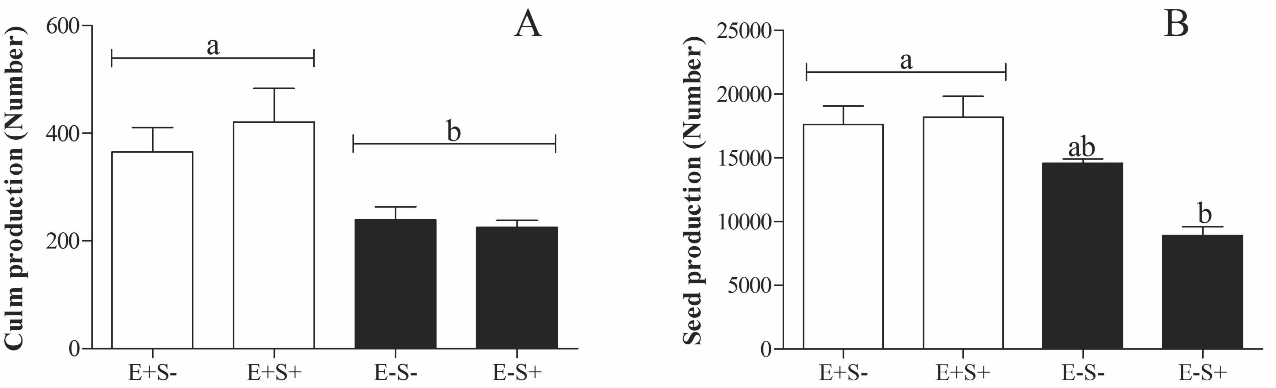

A
Culm production (Number)
600
400
200
0
a
b
E+S-
E+S+
E-S-
E-S+
B
Seed production (Number)
25000
20000
15000
10000
5000
0
a
ab
b
E+S-
E+S+
E-S-
E-S+

Table 1 Effect of the smut fungus *Ustilago bullata* (S) on *Epichloë*-infected (E+) and *Epichloë*-free (E-) *Bromus auleticus*: **A.** seedling emergence, measured as the number of seedlings/20 cm, **B.** seedling growth, measured as the length of the longest leaf. Measurements were taken 3 months after sowing. Values are means of each treatment. Error bars represent standard error. Different letters indicate significant differences among treatments (p<0.05)

Treatment	**Seedling emergence (plants/20 cm)**		**Seedling growth (cm)**	
E+S-	11.42 ± 2.89	a	9.58 ± 0.34	a
E+S+	8.25 ± 0.58	a	10.73 ± 0.38	a
E-S-	7.75 ± 0.48	a	10.10 ± 0.69	a
E-S+	8.75 ± 1.79	a	9.42 ± 1.17	a

Table 2 Effect of the inoculation of *U. bullata* (S+/S-) in *Bromus auleticus* seeds infected or not with the asexual *Epichloë* sp. (E+/E-) on vegetative biomass production during the second year, culm production relative to soil cover, culm production relative to biomass, weight of 1000 seeds of *Bromus auleticus* and seed output. For each variable the average ± standard error is shown. Letters indicate significant differences $p<0.05$

Treatment	**Biomass**		**Culm production/soil cover**		**Culm production/Biomass**		**1000 seeds weight**		**Seed output**	
E+S-	712.5 ± 55.43	a	54.95 ± 9.90	a	0.55 ± 0.06	a	4.49 ± 0.10	a	26.51 ± 3.45	a
E+S+	687.5 ± 104.8	a	83.76 ± 15.22	a	0.64 ± 0.08	a	4.55 ± 0.09	a	28.70 ± 5.01	a
E-S-	512.5 ± 55.43	b	63.15 ± 10.89	a	0.47 ± 0.06	a	4.49 ± 0.06	a	28.49 ± 2.84	a
E-S+	462.5 ± 62.50	b	102.36 ± 30.55	a	0.48 ± 0.07	a	4.55 ± 0.13	a	18.78 ± 2.51	a