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Epichloid endophytes confer resistance to the smut *Ustilago bullata* in the wild grass *Bromus auleticus* (Trin.)

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1 ***Epichloid* endophytes confer resistance to the smut *Ustilago bullata* in the**
2 **wild grass *Bromus auleticus* (Trin.)**

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14

15 **Abstract**

16 In this work it was studied for the first time whether asexual *Epichloë* (*Neotyphodium*)
17 endophytes of *Bromus auleticus*, protect their host plants against the pathogenic fungus
18 *Ustilago bullata*.

19 Seeds of two different ecotypes of *B. auleticus*, one of them infected with the endophyte
20 *Neotyphodium pampeanum* (NpE+) and the other infected with the endophyte *N.*
21 *tembladera* (NtE+) and their respectively endophyte-free (NpE-/NtE-) counterparts were
22 used. Seeds of each ecotype and endophytic status were superficially disinfected and were
23 randomly assigned to different treatments named: S+ (smut fungus inoculated) and S-
24 (mock-inoculated). It was evaluated the effect of *Ustilago bullata* infection on plant
25 characteristics in every stage of their life cycle: seedling emergence, vegetative growth,
26 mortality and smut symptoms in the florets.

27 In NtE+ infected plants, smut disease was almost completely suppressed, whereas in their
28 endophyte-free counterparts (NpE-) the incidence of smut symptoms reached 64%. In
29 NpE+ infected plants smut incidence was significantly lower (7%) than in endophyte-free
30 plants (39%). Although *U. bullata* infection decreased the emergence rate of both
31 endophyte-infected and endophyte-free plants, neutral or protective effects of the

32 endophytes were observed in seedling development and survival. The survival during the
33 first year of NtE+ plants was higher than in their NtE- counterparts.
34 These results indicate a strong beneficial effect of vertically transmitted endophytes against
35 this pathogen.

36

37 **Keywords**

38 Defensive mutualism; Grass-endophytes; *Neotyphodium*; Smut disease; *Ustilago bullata*

39

40 **1. Introduction**

41 Some cool-season grasses (subfamily Pooideae) establish symbiotic associations with
42 endophytic fungi of the genus *Epichloë* Tul. and their asexual derivatives *Neotyphodium*
43 Glenn, Hanlin & Bacon (Clavicipitaceae, Hypocreales, Ascomycota). This association is
44 quite specific and so each endophytic species is able to colonize one or a few host species.
45 These fungi colonize the plant shoot meristems where they grow systemically in the
46 apoplast of developing leaves and culms obtaining nutrients (Kuldau and Bacon, 2008).
47 Since its growth is synchronized with the growth of the host plant and does not require the
48 degradation of cell walls of the host, no noticeable symptoms of endophytic infection are
49 produced (Christensen et al., 2008; Christensen and Voisey, 2007). *Epichloë* species
50 produce stromata with perithecia in the culms of reproductive tillers avoiding the
51 development of the flowers, causing total or partial sterility of the host plant (*choke*
52 *disease*). Ascospores produced in the perithecia are forcibly discharged and are responsible
53 for the infection of new plants. Some *Epichloë* species and most of *Neotyphodium* species
54 do not produce stromata. In these asexual species, hyphae colonize meristems of the
55 developing flowers and remain visible, in the mature seeds, between the aleurone cell layer
56 and the seed coat (Schardl et al., 2004; White, 1993). Thus, these endophytes are vertically
57 transmitted through the seeds of the host plant.

58 The associations between grasses and epichloid endophytes, mainly those established with
59 vertically transmitted endophytes, are considered in general as mutualists (Clay and
60 Schardl, 2002; Müller and Krauss, 2005; Schardl et al., 2004). The plant provides
61 photosynthates and shelter to the endophytes and they provide several benefits to the host

62 plant. Among these benefits, the most important are protection against herbivores, mediated
63 by the production of different fungal alkaloids including loline and peramine, mainly toxic
64 to insects, and lolitrems and ergot alkaloids that affect primarily cattle (Bacon, 1977; Clay
65 and Schardl, 2002; Lane et al., 2000; Latch, 1993; Panaccione et al., 2006; Popay et al.,
66 2009; Schardl et al., 2007; 2004; Schardl and Phillips, 1997; Torres et al., 2008). Increased
67 growth and drought resistance have also been attributed to these endophytes in agronomic
68 and native wild grasses (Clay, 1987; Iannone and Cabral, 2006; Novas et al., 2003).
69 However, the endophyte may be detrimental under some environmental conditions and in
70 some host species (Cheplick and Faeth, 2009; Faeth et al., 2004).

71 Endophytes seem to protect their host against some fungal pathogens (Bonos et al., 2005;
72 Clarke et al., 2006; Gwinn and Gavin, 1992; Nan and Li, 2000; Yue et al., 2000) and also
73 to modulate positively or negatively the interaction between their hosts and arbuscular
74 mycorrhizal fungi (AM) (Chu-Chou et al., 1992; Guo et al., 1992; Liu et al., 2011; Mack
75 and Rudgers, 2008; Müller, 2003; Novas et al., 2005; 2009; Omacini et al., 2006).

76 Smut fungi (Ustilaginales, Basidiomycota) are common pathogens of cereals and are
77 studied because of their impact on agriculture worldwide (Agrios, 2005; Wilcoxson et al.,
78 1996). These pathogens cause diseases and losses in crops (Martínez-Espinoza et al., 2002;
79 Wilcoxson et al., 1996) and also infect wild grasses, such as *Festuca* and *Lolium* (Durán
80 and Fischer, 1961; Vánky, 1994).

81 Although several smut fungi species may present differences in their life cycles, all of them
82 cause sterility in their hosts. The ovary of the infected plants is replaced by the pathogen
83 that produces masses of spores, known as teliospores, in the sori within host tissues
84 (Martínez-Espinoza et al., 2002). Teliospores are resting spores that are spread by wind and
85 remain in the soil or attached to the lemma and palea or to the cariopses coat (Agrios,
86 2005). Dikaryotic teliospores that undergo karyogamy, germinate along with the seed
87 forming a germ tube (promycelia) (Alexopoulos et al., 1996; Meyer et al., 2001). The
88 diploid nucleus migrates to the promycelium and undergoes meiosis forming four haploid
89 basidiospores. Basidiospores can either unite as compatible mating types producing the
90 infection hypha, or they can proliferate mitotically to produce sporidia. Sporidia of
91 compatible mating types may then fuse to penetrate the host as a dikaryotic hypha (Agrios,
92 2005).

93 *Ustilago bullata*, the causal organism of head smut of grasses, is a highly polymorphic and
94 systemic smut fungus that infects its host soon after the emergence of the coleoptile from
95 the seed (Falloon, 1979; Fischer, 1940). The presence of the fungus in their host becomes
96 apparent at anthesis when the glumes and ovary of infected hosts are destroyed, being
97 replaced by a dark black mass of teliospores (Falloon and Hume, 1988). The effects of *U.*
98 *bullata* on *Bromus* spp, invasive species in USA, or forage species have been extensively
99 studied by Falloon (1976; 1979); Falloon and Hume (1988); García-Guzmán et al. (1996);
100 Hirschhorn (1986); Meyer et al. (2001).

101 *Bromus auleticus* Trin., is a native perennial grass that inhabits grasslands of Argentina,
102 Uruguay and southern Brazil. In Argentina, *B. auleticus* is infected by two species of
103 endophytes with a frequency of infection higher than 95% in most of the studied
104 populations (Iannone et al., 2009). This grass has been reported as host of the smut *U.*
105 *bullata* Berk (Astiz Gassó and Molina, 2010; Traverso, 2001). Field surveys carried out in
106 Argentina indicate that, infection of *B. auleticus* by *U. bullata* has not been very commonly
107 observed in nature, but in field assays, studying endophyte-free plants, smut symptoms
108 produced by *U. bullata* are usually observed (De Battista, personal communication).

109 In grasses infected simultaneously by vertically transmitted epichloid endophytes and smut
110 fungi, both fungi compete for the colonization of the ovary and require, in a different way,
111 the flower for their reproduction and dissemination. If the endophyte is able to avoid the
112 replacement of the ovary by the smut fungus, leading to the development of a normal seed,
113 both the host plant and the endophyte will be able to reproduce and disperse.

114 The triple interaction host plant-epichloid endophyte-smut fungus represents an interesting
115 model to study the effect of endophyte on pathogenic fungi that remains to be explored.
116 Thus, the aim of this study was to establish whether vertically transmitted endophyte
117 species confer resistance to the smut fungus in the pathosystem *Ustilago bullata*-*Bromus*
118 *auleticus* Trin.

119

120 **2. Materials and methods**

121 *2.1. Plant and smut fungus material*

122 Endophyte infected (E+) and endophyte-free (E-) seeds of two different ecotypes of *B.*
123 *auleticus*, originally from Intendente Alvear, La Pampa province (LP ecotype), Argentina,
124 infected with *Neotyphodium pampeanum* Iannone & Cabral and from El Palmar (EP
125 ecotype), Entre Ríos province, Argentina associated with *Neotyphodium tembladerae*
126 Cabral & White (Iannone et al., 2009) were used. Endophyte-free seeds of each ecotype
127 were obtained in 2007 by loss of endophyte viability in long term stored seeds. Since 2007,
128 E+ and E- plants of each ecotype were grown in the field and seeds are collected every
129 year. Seeds used for all the experiments described below were collected during December
130 from the previous year to each experiment described below.

131 Teliospores of *U. bullata* were collected from infected *Bromus catharticus* plants in
132 December 2008 and 2009. Diseased florets exhibiting fully ripen sori were collected and
133 mildly ground in a mortar and a pestle to release the teliospores. The powder containing
134 teliospores and pieces of vegetal tissues was sieved in a 1mm sieve to remove plant tissues.
135 Teliospores were kept dry at 4 °C and were used during the first 12 months after the
136 collection. For the taxonomic identification of *Ustilago bullata*, ITS region was amplified
137 by PCR accordingly to White et al. 1990. PCR product was purified and sequenced in an
138 ABI 3730xl DNA Analyzer. Identification of the smut fungus was performed by means of
139 BLAST on the GenBank database and followed by phylogenetic analyses using Maximum
140 Parsimony (Winclada v0.9.9) (Nixon, 1999) and MrBayes algorithms (Mr. Bayes 3.2)
141 (Ronquist et al., 2012) (not shown).

142

143 2.2. Endophyte detection

144 The endophytic status of the seed lots and plants was established by the examination of the
145 endophyte in seeds previous to each experiment and in the seedlings or plants at the end of
146 each experiment. To confirm the presence of the endophyte in seeds, caryopses were
147 soaked for 5 h in a 10 % v/v aqueous solution of sodium hydroxide at room temperature
148 (22–24°C), and then rinsed and stained with aniline blue (0.1% aqueous) (Clark et al.,
149 1983). Endophytic mycelia were visualized in parenchymal tissues within the culm pith or
150 in the parenchyma of peeled sheaths, aniline blue stained as mentioned and observed under
151 a light microscope. Plants were considered as endophyte infected if a mass of dark blue

152 hyphae was observed between the aleurone cell layer and the seed coat or when
153 characteristic unbranched hyphae were observed in parenchymal tissues.

154

155 2.3. Treatments

156 For all the experiments discussed below, seeds of each ecotype and endophytic status (*N.*
157 *pampeanum*-infected (NpE+); *N. pampeanum*-free (NpE-); *N. tembladera*-infected (NtE+)
158 and *N. tembladera*-free (NtE-)) were superficially disinfected by consecutive washes as
159 follows: ethanol 50%, 1 minute; sodium hypochlorite 2%, 5 minutes and ethanol 50%, 1
160 minute. E+ and E- seeds of each ecotype were randomly assigned to the different
161 treatments named: S+ (smut fungus inoculated) and S- (mock-inoculated). To achieve this,
162 seeds assigned to S+ treatments were placed in Petri dishes and a powder of teliospores
163 (0.15 mg teliospores.seed⁻¹) was poured on them. For control treatments, a mock-
164 inoculation with heat inactivated teliospores (180°C for 4 hours) was done. The Petri dishes
165 were closed and gently shaken for 5 minutes to obtain a homogeneous spore distribution on
166 the seeds. In this way, 4 treatments were established for each ecotype named as follows:
167 NpE+S+; NpE+S-; NpE-S+; NpE-S-; NtE+S+; NtE+S-; NtE-S+ and NtE-S-. Before the
168 inoculation, the viability of the teliospores was evaluated by preparing a suspension of
169 teliospores in water (1.5×10⁸ spores.ml⁻¹). Fifty µl of the solution were spread in Petri
170 dishes with water agar 2% and incubated 6 hours in darkness at 24°C. Spores able to
171 germinate (producing a germinating tube) were considered as viable and the percentage of
172 germination was registered. Teliospores viability ranged between 30 and 60%, and in those
173 inactivated for the S- treatments the percentage of germination was zero (even when the
174 inactivated teliospores were re-checked after 72 hours of incubation).

175

176 2.4. Effect of *Ustilago bullata* on seedling emergence and plant development

177 In order to determine the effect of infection by *U. bullata* on *B. auleticus* seedling
178 emergence and development, 150 seeds of each treatment and ecotype were sown in ten
179 Petri dishes (ten replicates with fifteen seeds/dish in each treatment) filled with sterilized
180 sand and incubated in a growing chamber at 22 °C under 12 hours photoperiod. The
181 percentage of seed germination was recorded and shoot length was measured after 15 days

182 from the sowing. Results were analyzed by a two way ANOVA ($p < 0.05$) for each ecotype
183 where the inoculation with the smut fungus and the endophytic status were the main
184 factors. All data analyses were performed using the Infostat software (Di Rienzo et al.,
185 2011).

186

187 *2.5. Evaluation of plant survival and smut symptoms development*

188 One hundred and fifty seeds of each ecotype and endophytic status were inoculated with
189 teliospores as described above. Seeds of each treatment were germinated in trays filled with
190 sterilized sand in a growth chamber at 22 °C under 12 hours photoperiod. Two-month-old
191 seedlings were transplanted individually to 25 cm deep x 15 cm in diameter pots, filled with
192 commercial garden soil: sand: perlite 3:1:1 and transferred outdoors to the experimental
193 field of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires where
194 they were allowed to grow and produce flowers. During this period, the survival of the two-
195 month-old seedlings (before being transplanted to pots in the field), plant survival before
196 flowering and the incidence of the disease in NpE+/NtE+ or NpE-/NtE- plants were
197 evaluated. The incidence of the disease in each treatment was evaluated as the number of
198 plants with symptoms (flowers with sori/number of flowered plants). For each ecotype, the
199 differences among treatments in seedling and plant survival and disease incidence were
200 compared by means of a *Chi-square* test of homogeneity of proportions and the Marascuilo
201 procedure was used to make comparisons between all pairs of groups (Marascuilo and
202 McSweeney, 1977).

203

204 *2.6. Vertical transmission of the endophyte via seeds*

205 In those plants that produced seeds, the transmission of the endophyte was evaluated by
206 checking the presence of endophyte in the seeds, as previously described.

207

208 **3. Results**

209 *3.1. Seedling emergence*

210 The inoculation with teliospores of *U. bullata* in seeds decreased the overall percentage of
211 emergence of *B. auleticus* (in LP ecotype $F_{1,36}=63.46$ $P<0.0001$ and in EP $F_{1,36}=5.28$
212 $P=0.0275$). The presence of the endophyte did not affect seedling emergence (LP:
213 $F_{1,36}=0.40$ $P=0.5293$; EP: $F_{1,36}=2.98$ $P=0.0930$) (Fig.1).

214 In LP ecotype the seedling emergence in NpE+S+ treatment was 49% lower than in
215 NpE+S- treatment, whereas in NpE-S+ treatment was 35% lower than in NpE-S- treatment,
216 but the difference in the emergence between NpE+S+ and NpE-S- treatment was not
217 statistically significant (Fig. 1A). In EP ecotype seedling emergence decreased 16% in
218 NtE+ seeds while in NtE- seeds the germination was 23% lower than in the control (Fig.
219 1B).

221 3.2. Seedling growth

222 No significant differences were observed in the shoot length between E+ and E- plants of
223 each ecotype (LP: $F_{1,36}=1.31$, $P=0.2592$; EP: $F_{1,36}=2.43$, $P=0.1278$) (Fig. 2). However, in
224 both ecotypes, seedlings were negatively affected by the presence of the smut fungus (LP:
225 $F_{1,36}=138.14$, $P<0.0001$; EP: $F_{1,36}=39.46$, $P<0.0001$). *Ustilago bullata* effects were more
226 evident in LP ecotype where NpE+S+ and NpE-S+ plants were 46% and 43% smaller
227 respectively than their S- counterparts (Fig. 2A). In EP ecotype, NtE-S+ seedlings were
228 39% smaller than the NtE-S- ones, whereas NtE+S+ seedlings were only 29% smaller than
229 their NtE+S- counterparts (Fig. 2B), but this difference was not statistically significant.

231 3.3. Plant survival

232 The inoculation of seeds with teliospores of *U. bullata* decreased the seedlings survival of
233 both ecotypes, during the first 60 days of growth (LP: $\chi^2_{0.95,3}=32.02$; $P<0.0001$ and EP:
234 $\chi^2_{0.95,3}=61.04$; $P<0.0001$) (Fig. 3A and B). However, while in LP ecotype no differences
235 were observed due to the endophytic status among the smut inoculated plants, in NtE+S+
236 seedlings was significantly higher than in the NtE- ones.
237 Among the plants grown to evaluate the development of the disease at the flowering time,
238 the percentage of survival during the first year of growth in the field was significantly

239 higher in plants grown from S- seeds (Fig. 3C and D) (LP: $\chi^2_{0.95;3}=74.67$; $P<0.0001$ and
240 EP: $\chi^2_{0.95;3}=78.32$; $P<0.0001$).

241 In LP ecotype, even though only the 15% of the NpE-S+ plants survived, this value was not
242 significantly different from the 31% of survival presented by the NpE+S+ ones (Fig. 3C).

243 On the other hand in EP ecotype the 65.8 % of survival presented by the NtE+S+ plants
244 was significantly higher than that observed in the NtE-S+, where only the 3% of the plants
245 survived (Fig. 3D).

246

247 3.4. Development of smut symptoms in field

248

249 The presence of smut disease symptoms in the florets was evaluated in one or two year old
250 plants grown in pots at field conditions. Disease incidence was almost totally suppressed or
251 significantly diminished in E+ plants of both ecotypes (LP: $\chi^2_{0.95;1}=12.67$; $P=0.0004$ and
252 EP: $\chi^2_{0.95;1}=78.21$; $P<0.0001$) (Fig. 4). None of the control plants (mock-inoculated)
253 presented symptoms of disease (not shown in figure 4). In the plants that presented smut
254 symptoms all the flowers were destroyed by the pathogen.

255

256 3.5. Vertical transmission of the endophyte

257 None of the NpE+ or NpE- smut-symptomless plants (from LP ecotype) produce fully ripen
258 seeds. In EP ecotype 11 plants produced fully ripen seeds, but only two to five seeds were
259 produced by each plant. The analysis of the presence of the endophytes in the seeds showed
260 that all the seeds were endophyte infected; indicating that in EP ecotype, the inoculation
261 with *Ustilago bullata* did not affected the transmission of the endophyte to the seeds.

262

263 4. Discussion

264 The present work, to our knowledge, is the first report of protective effect of *Neotyphodium*
265 endophytes against a systemic pathogen like *U. bullata* that produces castration of the

266 plants. Our findings suggest that plants of *Bromus auleticus* associated with *Neotyphodium*
267 *templaderae* or *N. pampeanum* were more resistant to the “head smut” of grasses produced
268 by *Ustilago bullata* than endophyte-free plants.

269 In this work we found that, whereas in endophyte-free plants the incidence of the disease
270 reached 39 to 64%, in endophyte-infected plants disease incidence ranged from 1 to 7%. In
271 those plants that presented smut symptoms seed production was totally suppressed
272 producing sterility in the affected plants. Thus, our results show that the endophytes prevent
273 castration of the host plant, ensuring sexual reproduction of the host. Although the amount
274 of fully ripen seeds produced by control or symptomless plants, in the S+ treatment, and
275 checked for endophyte infection, was not enough to evaluate accurately the efficiency of
276 the transmission of the endophyte through the seeds; our results also showed that the
277 vertical transmission of the endophyte is not affected by the inoculation of the smut fungus.
278 *In vitro* assays, performed in our laboratory, showed that teliospore germination is inhibited
279 by *N. pampeanum* and *N. templaderae* (Iannone et al., 2012b). Protective effects of
280 epichloid endophytes against plant fungal pathogens such as *Laetisaria fuciformis* (Bonos
281 et al., 2005), *Alternaria alternata*, *Fusarium* (Nan and Li, 2000), *Cercospora*,
282 *Cryphonectria parasitica (in vitro)* (Yue et al., 2000), *Sclerotinia homeocarpa* (Clarke et
283 al., 2006), *Rhizoctonia zaeae* (Gwinn and Gavin, 1992) have been also reported. All
284 together, these results are in agreement with the hypothesis of the defensive mutualism
285 suggested for the grass-endophyte associations (Clay, 1988; 1989; Saikkonen et al., 2010).

286 In spite of the beneficial effects observed in E+ plants with respect to prevention of smut
287 disease development, the endophytes had neutral effects on seedling emergence and
288 growth, since these variables were similarly (negatively) affected by the presence of the
289 smut fungus both in the E+ as in the E- treatments. Considering that *U. bullata* requires
290 flower production for its dissemination, negative effects on plant survival and development
291 should not be expected. However, these kind of effects produced by this pathogen on its
292 host plants were also reported in *Bromus catharticus* (Falloon, 1976; García-Guzmán et al.,
293 1996). In addition, we consider that the amount of teliospores used in each experiment was
294 significantly higher than that expected to be found in nature since after the inoculation the
295 seeds remained totally covered by a black coat of spores. Thus, detrimental effects of the

296 smut fungus could have been enhanced and some of the protective effects of the endophyte
297 could have been masked in our experiments. Protective effects of the endophyte could be
298 even more important in natural conditions where the charge (inoculum) of teliospores is
299 expected to be lower.

300 Different behaviors were observed between plants of different ecotypes, whereas smut
301 development was almost totally suppressed in NtE+ plants (EP ecotype; *N. tembladerae*-
302 infected), in NpE+ plants (LP ecotype; *N. pampeanum*-infected) the incidence of the
303 disease was diminished but not so drastically as in NtE+ plants. In the presence of the
304 pathogen, survival of NtE+ plants was higher than in the NtE- ones, but no differences were
305 observed between NpE+ and NpE- plants. These differences observed in plant survival and
306 disease incidence between the E+ plants of each ecotype seem to indicate that the
307 protective effects of *N. tembladerae* against this pathogen are stronger than those conferred
308 by *N. pampeanum*. However, we cannot discard that the observed differences could be due
309 to differences in the susceptibility of each plant ecotype. Supporting our hypothesis of a
310 higher protective capacity of *N. tembladerae*, there are *in vitro* studies that showed that *N.*
311 *tembladerae* presented the highest inhibitory capacity against several fungal plant
312 pathogens with respect to other *Epichloë/Neotyphodium* species (Yue et al., 2000) and
313 against *U. bullata* (Iannone et al., 2012b).

314 The protective effects shown in the E+ plants against the head smut of grasses disease
315 could be due to, 1) the endophytes preventing the infection by *U. bullata* at seedling stage
316 or 2) the endophytes preventing the colonization of the ovary by the pathogen. The
317 detrimental effects of smut fungus inoculation observed on seedling emergence, seedling
318 survival and development in E+ and E- treatments would support hypothesis 2, indicating
319 that the smut fungus is able to infect the seedlings of *B. auleticus* irrespectively of their
320 endophytic status.

321 The higher survival and resistance of endophyte-infected plants to *U. bullata*, in addition to
322 other beneficial properties observed in endophyte infected plants (Iannone et al., 2012a)
323 could explain the higher incidence of endophytes in populations (smut-symptomless) of this
324 host in nature. Endophyte infected plants produced more seeds than E- ones (Iannone et al.,
325 2012a) and seed production was suppressed in E- plants when infected with *U. bullata*.

326 However, considering that *B. auleticus* is a highly perennial plant, more long term studies

327 are necessary in order to evaluate the importance of *Ustilago bullata* and the effects of both
328 symbionts on the dynamics of the populations of this host.

329 Although more research should be done in order to establish the mechanism through which
330 both fungal symbionts interact in the host plant so that the incidence of the disease is lower
331 in E+ plants; our findings are relevant for a better understanding of the biology of the grass-
332 endophyte symbiosis and could be also applied in grass breeding programs. Currently,
333 studies are being conducted in our laboratory in order to evaluate the effect of the
334 endophyte-smut fungus interactions in plant competition, and seed production in field.
335 Finally, O'Hanlon et al. (2012) stated that more attention should be paid to dissecting the
336 potential of fungal endophytes as biological control agents against cereal pathogens. In this
337 sense, our studies and results on smut resistance should be expanded to other endophyte-
338 infected grasses, mainly wild barley species as *Hordeum bogdanii*, *H. brevisubulatum* and
339 *H.comosum*.

340

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346

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496

497 **Fig. 1.** Effect of *Ustilago bullata* and *Neotyphodium pampeanum* (Np) (A) or *N. tembladerae* (Nt)
498 (B) endophytic status on seedling emergence of *Bromus auleticus*. Endophyte infected
499 (NpE+/NtE+) and endophyte free (NpE-/NtE-) seeds, inoculated (S+) or mock-inoculated (S-) with
500 *Ustilago bullata*. Different letters indicate significant differences ($P<0.05$).

501

502 **Fig. 2.** Effect of *Ustilago bullata* and *Neotyphodium pampeanum* (Np) (A) or *N. tembladerae* (Nt)
503 (B) on *Bromus auleticus* seedlings shoot length (cm). Endophyte infected (NpE+/NtE+) and
504 endophyte free (NpE-/NtE-) seeds, inoculated (S+) or mock-inoculated (S-) with *Ustilago bullata*.
505 Data are means; SE. Different letters indicate significant differences ($P<0.05$).

506

507 **Fig. 3.** Effect of *Ustilago bullata* and *Neotyphodium pampeanum* (Np) (A, C) or *N. tembladerae*
508 (Nt) (B, D) on *Bromus auleticus* seedlings survival during the first two months of growth (A, B)
509 and plants survival during the first year of growth under field conditions (C, D). Endophyte infected
510 (NpE+/NtE+) and endophyte free (NpE-/NtE-) seeds, inoculated (S+) or mock-inoculated (S-) with
511 *Ustilago bullata*. Different letters indicate significant differences ($P<0.05$).

512

513 **Fig. 4.** Percentage of *Bromus auleticus* plants inoculated with *Ustilago bullata* with smut symptoms
514 in the florets. NpE+: *Neotyphodium pampeanum* infected; NpE-: *N. pampeanum* free; NtE+: *N.*
515 *tembladerae* infected and NtE-: *N. tembladerae* free. Different letters indicate significant
516 differences within each ecotype ($P<0.05$). The plants in those treatments inoculated with inactive
517 teliospores (S-) did not present smut disease symptoms (not shown in the figure).

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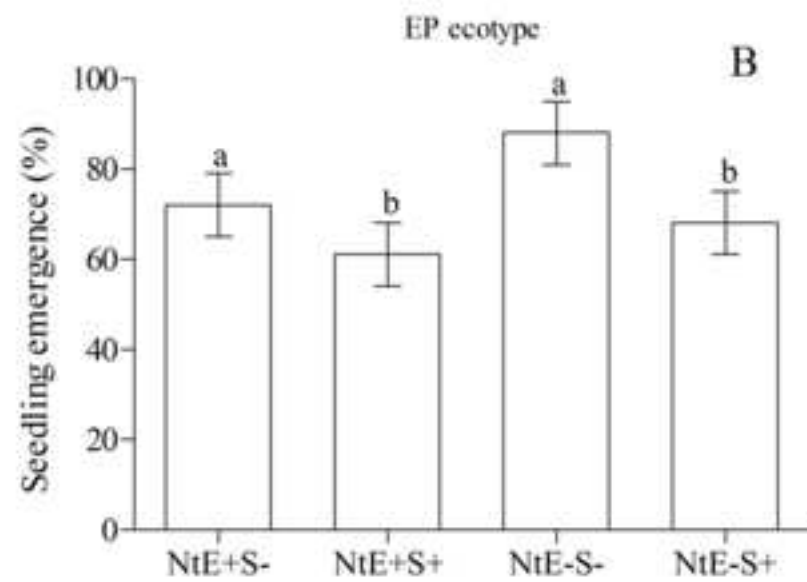
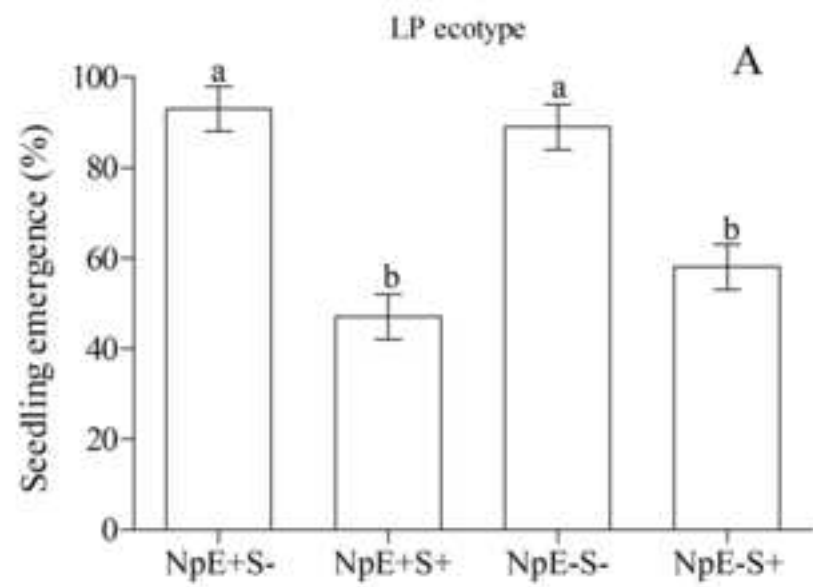
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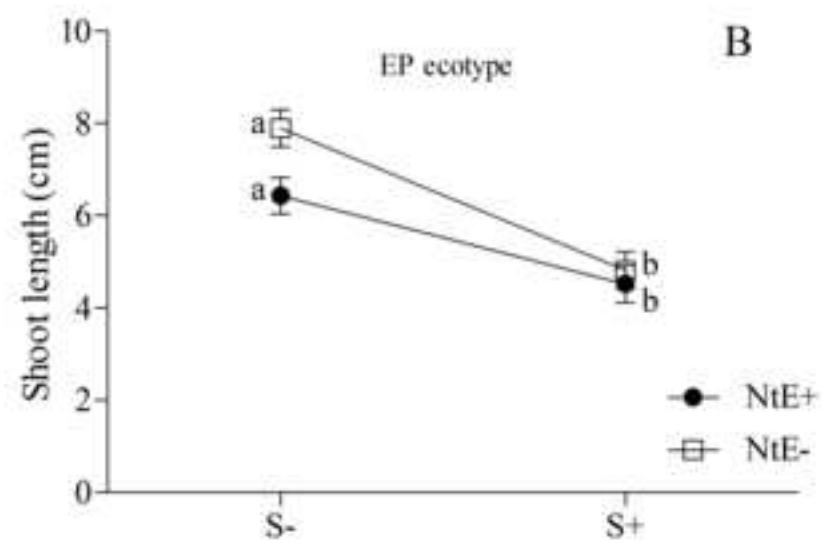
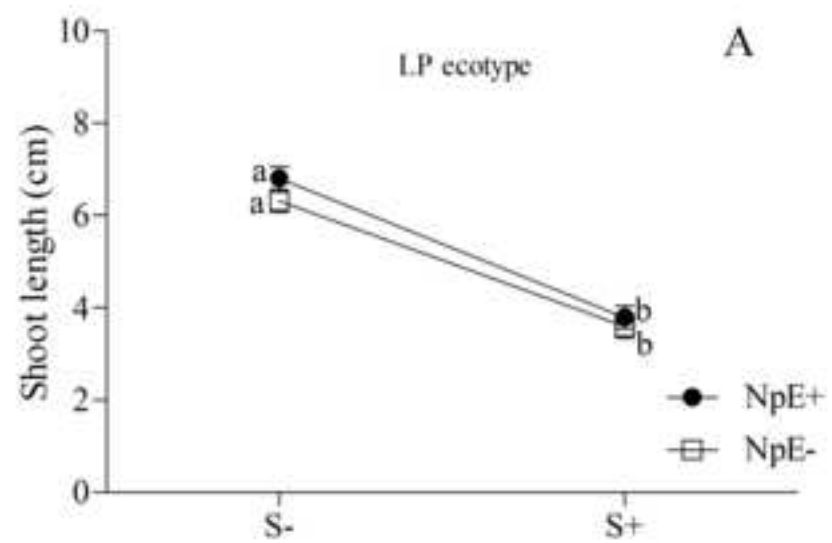
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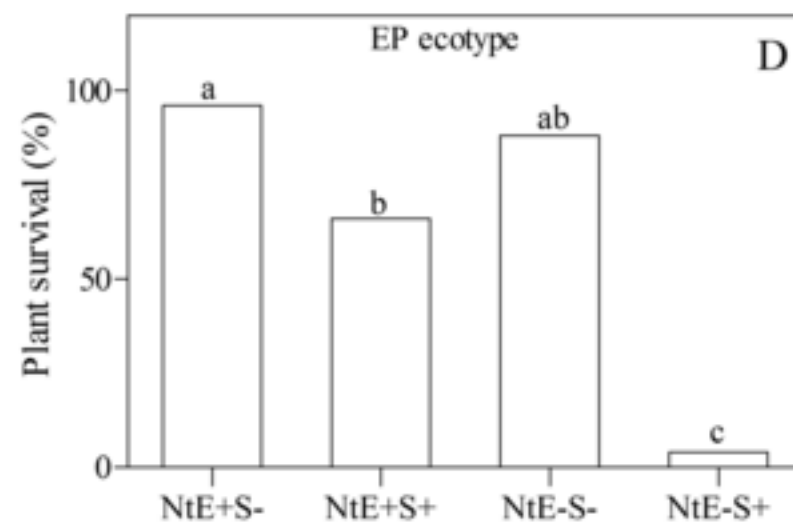
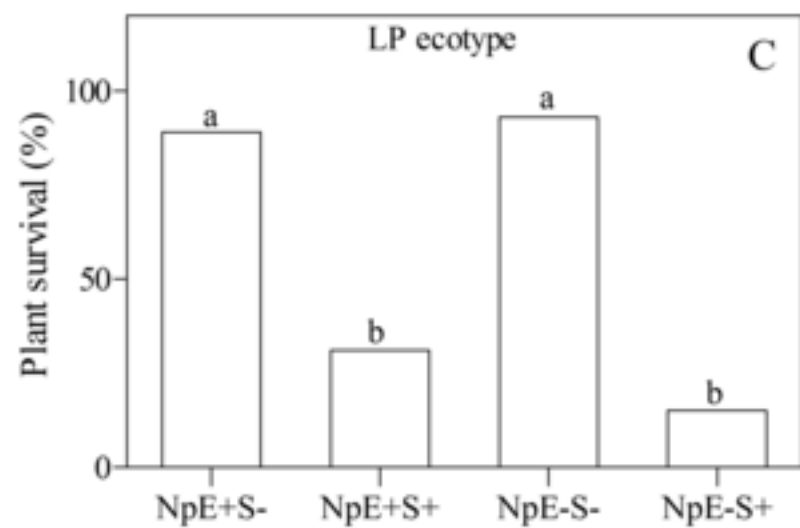
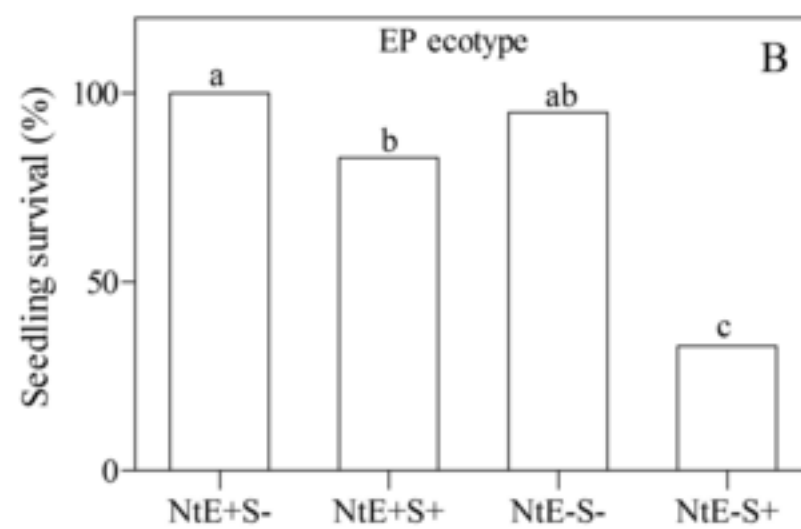
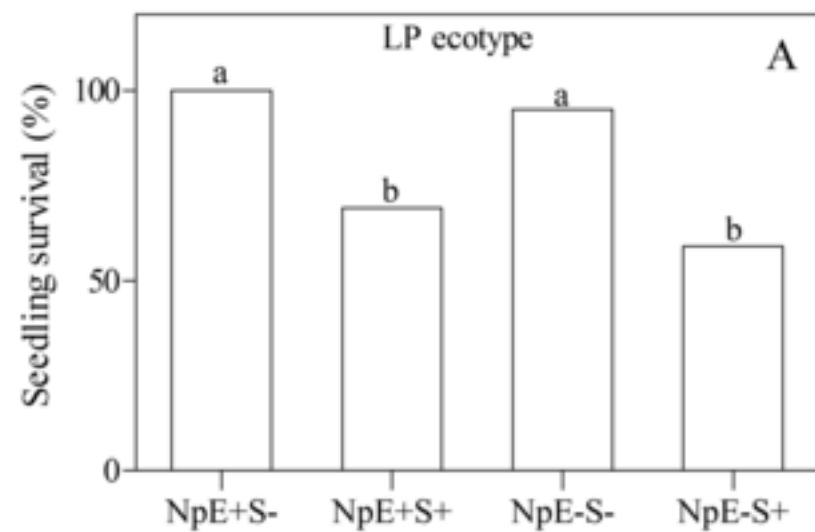
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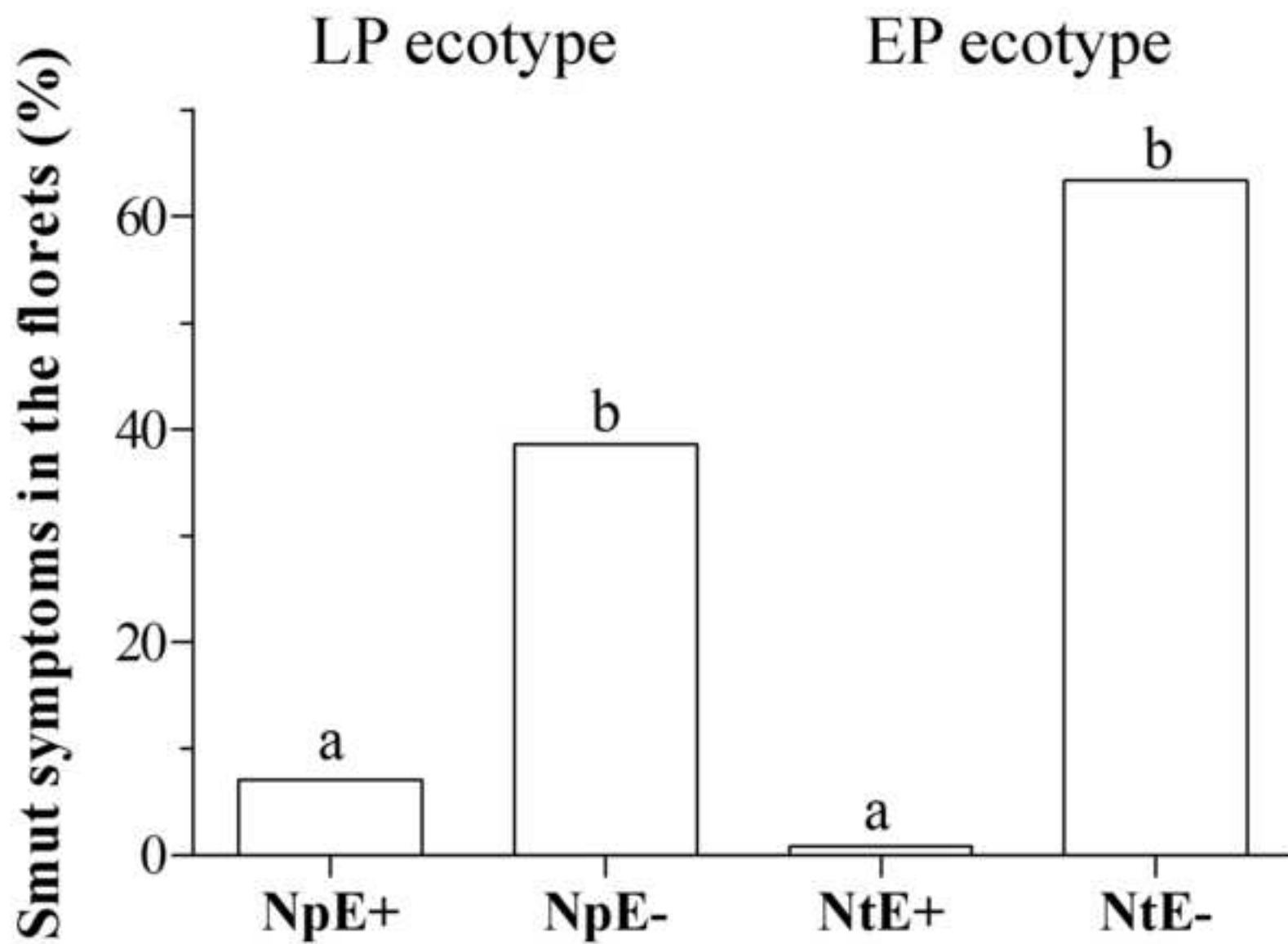
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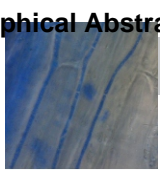
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Endophyte-infected
Bromus auleticus
(NpE+/NtE+)

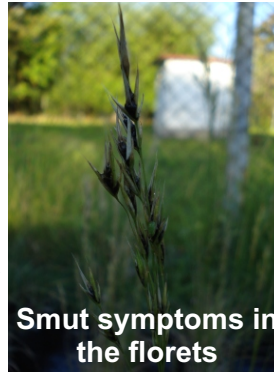


Healthy plant

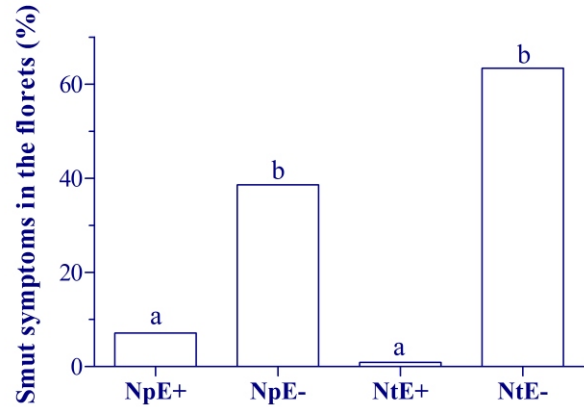


Ustilago bullata
(smut fungus)

Endophyte-free
Bromus auleticus
(NpE-/NtE-)



Smut symptoms in
the florets



NpE+ : *Neotyphodium pampeanum*-infected
NpE- : *Neotyphodium pampeanum*-free
NtE+ : *Neotyphodium tembladerae*-infected
NtE- : *Neotyphodium tembladerae*-free

525 Highlights

526 Some grasses are usually co-infected by smut fungi and mutualist epichloid endophytes.

527 Endophytes are transmitted via seeds and smut fungi replace the seeds with teliospores.

528 The endophyte and the smut fungus compete in a race for the colonization of the ovary.

529 The effect of *Neotyphodium* spp. against head smut of grasses was evaluated.

530 Disease incidence was diminished in endophyte-infected *Bromus auleticus* plants.

531

ACCEPTED MANUSCRIPT