Effects of choke disease in the grass *Brachypodium phoenicoides*

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Abstract

Epichloë species (*Clavicipitaceae, Ascomycota*) are the causal agents of choke disease of grasses. This disease is characterized by the presence of cylindrical fungal stromata which wrap the immature inflorescences and inhibit the normal development of reproductive tillers. Using phenotypic and molecular characters, as well as mating compatibility tests, the fungus causing choke disease in *Brachypodium phoenicoides* (*Poaceae*) was identified as *Epichloë typhina*. A three year field experiment conducted with infected and uninfected plants of a single clone of *B. phoenicoides* showed no significant differences in biomass production during their vegetative growth stage, but the content of Ca, Mg, and Mn was greater, and that of Na was lower in infected plants compared to uninfected plants. Infected plants produced up to twice as many reproductive tillers than healthy plants, but their reproductive tissue biomass was smaller than that of healthy plants, because tiller development was arrested by choke forming stromata.

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Introduction

Epichloë species and their asexual *Neotyphodium* relatives (Fam. *Clavicipitaceae, Ascomycota*) form systemic and perennial associations with grasses, ranging from antagonistic to mutualistic. They are classified into three types according to the type of interaction (White, 1988). Type I species always form a cylindrical fungal stroma around inflorescences when host plants enter into the reproductive growth stage. As a result the panicles cannot emerge and the plant is sterilized (Figure 1). These symptoms are known as choke disease. The disease can cause economic problems in seed crops of some host grasses (Pfender & Alderman, 2006). Plants infected by Type II *Epichloë* species usually remain symptomless, flower normally, and the fungus is seed-transmitted. Occasionally, fungal stromata may develop on a few tillers of an infected plant depending on environmental conditions or genotype (Leuchtmann *et al*., 1994). All Type III species belong in *Neotyphodium*, are seed-transmitted, and never develop stromata on their hosts. Type II and III species are considered to be mutualists which provide a wide range of benefits to their host plants, including protection against herbivores by means of toxic alkaloids, and increased drought tolerance (Schardl *et al*. 2004). Most *Epichloë* species tend to have a narrow host range, being confined to one or two host genera of the same grass tribe; only *E. typhina*, a Type I species, is known to infect many different grass genera (Clay & Schardl, 2002).

Brachypodium phoenicoides (tribe *Brachypodiae*) is a perennial grass distributed in south western Europe and the Mediterranean region (Catalán & Olmstead, 2000). Choke disease has been reported in this grass (Zabalgogeazcoa *et al*., 2003), but the *Epichloë* species causing the disease remained unidentified. *Epichloë* occurs on two other *Brachypodium* species: on *B. sylvaticum* that is infected by the Type II species *Epichloë sylvatica* (Leuchtmann & Schardl, 1998; Bucheli & Leuchtmann, 1996), and on *B. pinnatum* that is infected by *E. typhina*, a Type I species causing choke disease (Craven *et al*. 2001).

While there are numerous studies on growth and physiological effects of mutualistic Type II and III *Epichloë* or *Neotyphodium* species on their host plants (Clay & Schardl, 2002; Schardl *et al*. 2004), relatively little is known about how plants are affected by interactions with Type I species, besides being sterilized (Pan & Clay, 2002). The objectives of this study were to (1) taxonomically identify the *Epichloë* species causing choke disease in *B. phoenicoides*, and (2) investigate the effects of *Epichloë* infection on the growth and nutrient content of the host plants.

Materials and Methods

Plant material

The population of *B. phoenicoides* used in this study was from a meadow in Torres del Carrizal, in the province of Zamora (Spain). Approximately 5 to 10% of the plants at this location showed symptoms of choke disease. Several plants bearing stromata were used to obtain *Epichloë* isolates.

To determine whether or not asymptomatic *Epichloë* infections occurred, apparently healthy plants with mature inflorescences were sampled at Torres del Carrizal (25 plants), and at a second location (Valcuevo, province of Salamanca, 25 plants) where no choke disease symptoms were observed. Pith scrapings from one flowering tiller of each of these plants were stained with a solution of 6.7 g l^{-1} aniline blue in 28% (v:v) aqueous lactic acid. The stained samples were mounted on a microscope slide, and observed under the compound microscope at a magnification of 200X. The presence of unbranched, blue-stained hyphae running longitudinally in the intercellular space of plant cells was interpreted as a sign of infection by *Epichloë* (Bacon & White, 1994).

Epichloë species identification

To obtain pure cultures of *Epichloë*, segments of reproductive tillers from diseased plants were surface disinfected by treatment with a 20% solution of commercial bleach (1% active chlorine), and placed on Petri dishes containing potato dextrose agar (PDA) with 200 mg l^{-1} of chloramphenicol. Mycelial fragments emerging from plant tissues onto the agar were then transferred to new PDA plates.

As an aid for taxonomic identification, nucleotide sequences of the β-tubulin gene were used. Extracts of fungal DNA were obtained using a commercial kit (Extract-N-Amp Plant PCR kit, Sigma-Aldrich). A fragment containing the first three introns of the β-tubulin gene was amplified using the primers described by Byrd *et al*. (1990). Amplification products were sequenced in an ABIPrism 377 Genetic Analyzer (Applied Biosystems).

A multiple alignment of the β-tubulin sequence of the *B. phoenicoides* isolate TC-1 and reference sequences of other *Epichloë* species (Craven *et al*., 2001) was made with ClustalX (Thompson *et al*., 1997). MEGA 3.1 software (Kumar *et al*., 2004) was used to calculate pairwise genetic distances with the Kimura 2-parameter method, and to construct a phylogenetic tree by the neighbour-joining method (Nei & Kumar, 2000). The robustness of the inferred relationships was estimated by 1000 bootstrap replicates.

Mating compatibility tests were conducted with isolate BP-14 from *B. phoenicoides* (from Torres del Carrizal) as the gametal partner applied to stromata of either *E. typhina* on its natural host *Dactylis glomerata* (from Vesancy, France) or *E. clarkii* on *Holcus lanatus* (from La Rippe, Vaud, Switzerland) using published procedures (Leuchtmann *et al*., 1994). Fertilization was achieved by transferring conidia from cultured mycelium to the receptive surface of freshly emerged stromata. Perithecia containing viable ascospores were produced after 3 to 4 weeks in successful matings, but no fertile perithecia were produced in failed pairings.

Field experiment

A set of infected and uninfected plants of identical genotype was produced to study the effects of choke disease on the vegetative growth of the plants. This set was developed from a single infected plant of *B. phoenicoides* (from Torres del Carrizal) by dividing it into several ramets, which were transplanted to 75 ml pots containing a 50:50 mixture of peat moss and perlite. One half of the ramets were treated with three doses of 400 μg of propiconazole (Tilt®, Ciba-Geigy), a systemic fungicide. The first and third doses were added in 10 ml of water to the soil in each pot, and the second dose was a foliar application sprayed in 2 ml of water onto each plant. The fungicide treatments were 10 days apart, and during this period treated and untreated plants were maintained in a growth chamber with a 12 day photoperiod and 25º C constant temperature. One month after the last fungicide treatment the infection status of each plant was determined by microscope observation of intercellular hyphae in leaf sheath samples. Epidermic strips peeled from leaf sheaths were stained and analyzed under the microscope as described above for stem pith samples (Bacon & White, 1994). In April 2001, six treated and six untreated plants of similar size were transplanted to the field at a research farm near Salamanca. A space of 60 cm was left between the randomly positioned plants. The soil was an eutric chromic cambisol with neutral pH at the surface, decreasing slightly with depth. The organic matter (1.24%), N (0.07%), and P (16.6 mg kg^{-1} in the first 30 cm, and 10.0 mg kg^{-1} at 30-60 cm depth) contents of this soil are low. The K (107 mg kg^{-1}) and Ca (984 mg kg^{-1}) contents can be considered medium. To imitate natural conditions, plants were not fertilized during the 40 months of the experiment.

Biomass of the vegetative stage of each plant was determined by cutting plants 3 cm above ground level in November 2001, April 2003, and November 2004. Leaf material was dried in a forced air oven at 60° C for 2 days before weighing. Yield in the reproductive stage was measured in harvests made in July 2002, 2003, and 2004, when the healthy plants had

mature flowering tillers. The number of reproductive tillers was counted for each plant at this time.

A few tillers of infected plants in the field experiment produced seed. Five such tillers from each of two plants were checked for the presence of intercellular hyphae of *Epichloë* in the stem pith, using the methodology described above (Bacon & White,1994).

For nutrient element analysis, vegetative stage samples harvested in April 2003 were used. Samples were dried at 60ºC in a forced air oven and ground in a Retsch ZM1 mill with a 1 mm mesh sieve. Each sample was analyzed for N, P, K, Ca, Mg, Na, Mn, Cu, and Zn concentrations as described previously (Zabalgogeazcoa *et al*., 2006).

The statistical significance of the differences between means of infected and uninfected plants in terms of biomass, tiller number, or nutrient content were tested with a Student's t-test with α=0.05.

Results

While the field experiment lasted, no stromata emerged on any of the uninfected plants developed by fungicide treatment, suggesting that these plants were not infected by *Epichloë*. Most reproductive tillers of infected plants were sterilized by *Epichloë* stromata. In July 2002, the second growing season, 94.01% (standard deviation, SD: 8.02) of the reproductive tillers had stromata, in 2003 94.28% (SD: 8.10), and in 2004 83.33% (SD: 33.37). Examination of stem pith samples showed that the few reproductive tillers that flowered normally were not colonized by intercellular *Epichloë* hyphae. In contrast, intercellular hyphae were observed in pith samples from stroma-bearing tillers. Likewise, no endophytic hyphae were observed in reproductive tillers of fifty asymptomatic plants from two natural populations of *B. phoenicoides*. These results suggest that the fungus infecting *B. phoenicoides* is a Type I species which does not asymptomatically infect reproductive tillers.

Two *Epichloë* isolates from different *B. phoenicoides* plants were sequenced, and both had identical nucleotide sequences at the first three introns of the β-tubulin gene (GenBank accession number AM4907969). In the neighbour-joining phylogenetic tree based on β-tubulin sequences of *Epichloë* species, the *B. phoenicoides* strain belonged to a clade composed of *Epichloë typhina, E. clarkii,* and *E. sylvatica* (Figure 2). Within this clade, *E. sylvatica* is the only species which can asymptomatically infect reproductive tillers (Bucheli & Leuchtmann, 1996). *E. typhina* and *E. clarkii* are obligate pathogens like the *Epichloë* sp. isolated from *B. phoenicoides*. Within this clade, the *B. phoenicoides* isolate formed a subclade together with an *E. typhina* strain isolated from *Poa nemoralis* (bootstrap value 82%).

The crosses made between an *Epichloë* isolate from *B. phoenicoides* and *E. typhina* from *Dactylis glomerata* and *E. clarkii* from *Holcus lanatus* produced viable ascospore progeny. This result indicates that the *B. phoenicoides* pathogen belongs to mating population I, an interfertility group composed of *E. typhina* from various host species and *E. clarkii* (Schardl & Leuchtmann, 1999).

There were no significant differences ($P>0.05$) in the N, P, K, Zn, or Cu content of leaves collected from infected and uninfected plants in April 2003 (Table 1). However, Ca, Mg, and Mn contents were greater ($P<0.05$) in infected than in healthy plants, and the opposite occurred with Na content ($P<0.05$).

The differences in biomass production between infected and uninfected plants in the vegetative growth stage were not statistically significant ($P>0.05$) in any of the 3 years this parameter was measured (Figure 3A).

In addition to sterilizing the plants, choke disease had a significant effect ($P<0.05$) on the number of reproductive tillers per plant (Figure 3C). In the three years that tillers were counted, the average number per plant was increased in infected plants compared to

uninfected plants. The difference was statistically significant ($P<0.01$) in 2003 and 2004 with infected plants producing 1.8 and 2.0 times more reproductive tillers than healthy plants. However, as a result of the arrested development of flowering tillers, the reproductive biomass was lower in infected than in healthy plants in all three years ($P<0.05$, Figure 3B).

Discussion

The results of this research show that (1) the fungus causing choke disease in *B. phoenicoides* is *E. typhina*, (2) differences in nutrient content exist among infected and uninfected plants during the vegetative growth phase, and (3) the number of reproductive tillers is increased in infected plants.

The fungus infecting *B. phoenicoides* has characteristics of a Type I species: infected plants had more than 90% of their tillers sterilized by stromata (Figure 1), and the fungus does not appear to colonize flowering tillers asymptomatically. The β tubulin nucleotide sequence placed the *B. phoenicoides* strain in a clade formed by *E. typhina*, *E. clarkii*, and *E. sylvatica* (Figure 2). *E. clarkii* is a Type I species that only infects *Holcus lanatus* and is interfertile with *E. typhina*. *E. sylvatica* infects another *Brachypodium* species, but it is a Type II species and belongs to a different mating population than *E. typhina* and *E. clarkii* (Schardl & Leuchtmann, 1999). Therefore, based on the fertility of the *B. phoenicoides* strain with *E. typhina* demonstrated in this study, and its molecular affinity to the above mentioned clade, the *Epichloë* species causing choke disease in *B. phoenicoides* is *E. typhina* as currently defined.

E. typhina is the only *Epichloë* species which is able to infect a wide range of hosts belonging to different genera and tribes in the *Poaceae*. All other *Epichloë* species are restricted to hosts of one or two genera belonging to the same tribe (Clay & Schardl, 2002). The *E. typhina* isolates from different hosts show different β-tubulin sequences (Figure 2),

and distinct β-tubulin genotypes appear to be conserved in different host populations of *E. typhina* suggesting that the *E. typhina* complex may represent a group of cryptic species isolated by host specialization (Schardl *et al*., 2007). The *E. typhina* strains from *B. phoenicoides* could represent such a cryptic species, either alone or together with *Poa nemoralis* strains, that were placed in the same subclade of the phylogram (Figure 2).

Differences in concentration of Ca, Mg, Mn, and Na were observed among infected and uninfected plants in the vegetative stage (Table 1). This suggests that *E. typhina* infection may have an effect on the tissue chemistry of its host, or the fungus itself may accumulate those nutrients which are more abundant in infected plants (Ca, Mg, Mn). According to reports from other grasses, the plant Ca and Mn contents were adequate for optimal growth (4.0 – 1.2 g kg^{-1} for Ca, 30-100 ppm for Mn). This was not the case for Mg, whose content for optimal growth is in the range of 1.5-3.5 g kg^{-1} (Marschner, 1995). Thus, Mg may have been limiting in our experiment and a slight increase mediated by endophyte infection might favourably affect the persistence of infected plants.

The effects of *Epichloë* infection on nutrient content of *B. phoenicoides* plants could depend on particular soil conditions. For instance, Malinowski *et al*. (1998) reported that the concentrations of P, Mg, and Ca were higher in plants of *Festuca arundinacea* infected by *Neotyphodium coenophialum,* but only when plants were grown in a soil with a low P level (17 mg kg^{-1}). The soil where our *B. phoenicoides* field experiment was done had a similar P content (16.6 mg kg^{-1}). Therefore, it is possible that endophyte effects observed in our experiment are due to the low nutrient status of the soil. In addition, endophyte effects on nutrient content may not be permanent. While the P content of *Festuca rubra* was consistently higher in plants infected by the Type II endophyte *E. festucae*, differences in Ca, Mg, and Mn between infected and uninfected plants only occurred in some years of that experiment (Zabalgogeazcoa *et al*., 2006). Nevertheless, differences in nutrient content, as shown here,

could influence competitive abilities and the long term population dynamics of infected and uninfected ramets of *B. phoenicoides*.

The total biomass of plants in the vegetative stage did not differ for infected and uninfected plants in any of the three years that this parameter was measured (Figure 3). A similar observation was made in plants of *Glyceria striata* infected by the Type I pathogen *E. glyceriae* (Pan & Clay, 2002). However, in our experiment the average number of tillers per plant was greater in infected than in uninfected plants (Figure 3). Since only one stroma of similar size is formed per tiller, the increased tillering results in an increased number of stromata with greater potential to produce progeny, which should be advantageous for contagious spread of the fungus. We have observed a similar increase in reproductive tiller number in plants of annual ryegrass (*Lolium rigidum*) inoculated with an *E. typhina* isolate from *Lolium perenne* (unpublished).

Increased production of tillers can occur in response to clipping in some grasses (Skinner & Nelson, 1994; Hamilton *et al.*, 1998). Therefore, the increased tillering observed in plants with choke disease might be a response of the plant to the arrest of tiller development caused by the stromata (similar to clipping), and not an effect of fungal biochemical signalling. On the other hand, changes in patterns of vegetative growth observed in plants of *Glyceria striata* infected by *E. glyceriae* (Pan & Clay, 2002) suggest that a mechanism different from a mechanical stimulus could be involved in increased tillering. For instance, growth regulating compounds, produced directly by *E. typhina*, or by the host in response to infection, could play a role in the increased tillering.

The number of tillers bearing stromata in infected plants decreased with plant age. In the third year, only 83% of the tillers were choked in infected plants compared to 94% on the first year. Tillers of larger and more vigorous plants appear to escape infection more often, which may counteract the sterilizing effect of the fungus. Alternatively, the apparent change

in the number of choked tillers may be due to normal variation in response to the differences in weather conditions between years. The relative growth of meristematic tissues and fungal hyphae may determine which tiller buds become infected (Kirby 1961) and this would undoubtedly be influenced by the weather, particularly temperature and rainfall. Additional long term studies are necessary to verify the origin of observed disease dynamics in *Epichloë* infected populations of *B. phoenicoides*.

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Table 1. Average nutrient content (± standard deviation; measurements taken from six plants) of leaves of infected and uninfected plants in vegetative stage harvested in 2003. Means of each nutrient were compared by means of a Student´s t-test. Statistically significant difference exists between means whose P value is marked by an asterisk (*: P<0.05; **: P<0.01).

Element	**Infected**	**Healthy**	**t-test P value**
N (g kg^{-1})	25.57 ± 1.20	25.62 ±1.36	0.94742
P (g kg^{-1})	2.07 ± 0.44	1.92 ± 0.07	0.43084
K (g kg^{-1})	11.45 ± 2.05	12.90 ± 1.90	0.23188
Ca (g kg^{-1})	2.85 ± 0.10	2.42 ± 0.07	0.00009**
Mg (g kg^{-1})	0.73 ± 0.05	0.63 ± 0.05	0.00731**
Na (g kg^{-1})	0.07 ± 0.03	0.12 ± 0.03	0.03488*
Mn (ppm)	60.54 ± 4.81	52.25 ± 6.06	0.02535*
Zn (ppm)	12.58 ± 2.00	12.21 ± 1.09	0.69492
Cu (ppm)	2.46 ± 0.53	2.83 ± 1.22	0.50646

Figure 1. Two reproductive tillers of *Brachypodium phoenicoides* "choked" by orange stromata *of Epichloë typhina.* A healthy reproductive tiller whose panicle is normally developed is shown at the right side.

Figure 2. Neighbor-joining phylogram of *Epichloë* species with midpoint rooting based on Kimura two-parameter distance model and derived from nucleotide sequences of introns 1-3 and exons 1-2 of the β-tubulin gene. Members of each *Epichloë* species are indicated by their GenBank accession number. Bootstrap values are based on 1000 replications.

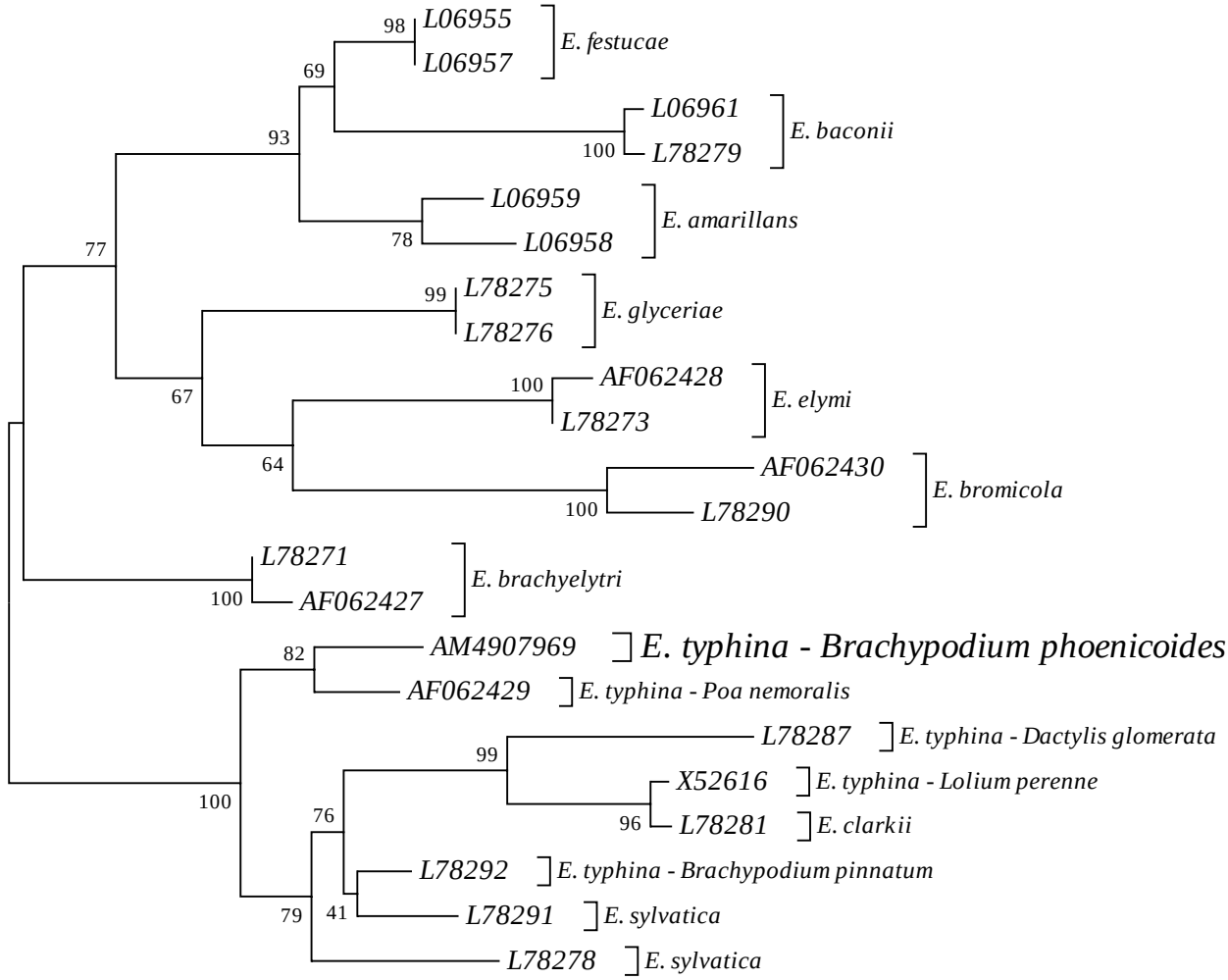
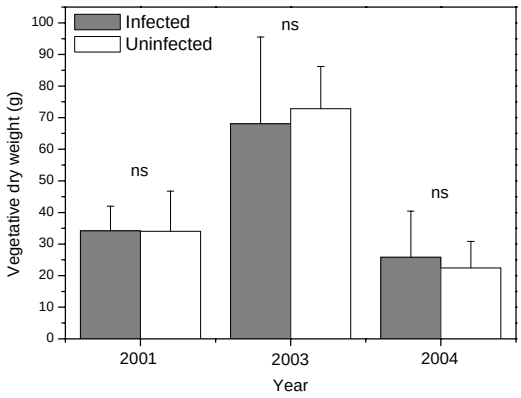
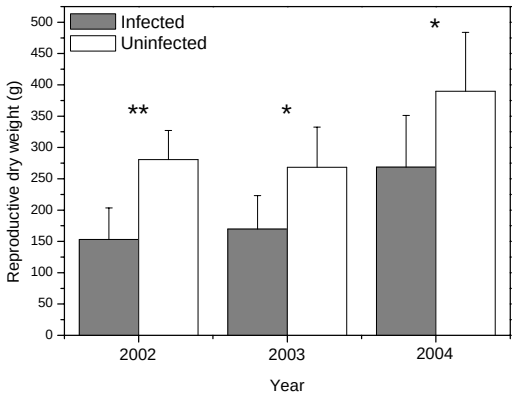


Figure 3. Some growth parameters of *Epichloë*-infected and healthy plants growing under field conditions in Salamanca (Spain) over a three year period. Means and standard deviations (bars) of (A) dry weight of infected and uninfected plants in vegetative and (B) reproductive stage, and (C) number of reproductive tillers produced. Means of infected and healthy plants each year were compared with a Student's t-test. Statistical significance of the difference between pairs of means is indicated for each pair of columns (ns: not significant; *: P<0.05; **: P<0.01).

A



B



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