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Family issues: fungal endophyte protects host grass from the closely related pathogen *Claviceps purpurea*

L.I. PÉREZ[a,*], *P.E. GUNDEL*[a,b], *C.M. GHERSA*[a], *M. OMACINI*[a]

[a]*IFEVA-Facultad de Agronomía (UBA)/CONICET, Cátedra de Ecología, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina*
[b]*MTT Agrifood Research Finland, Plant Production Research, FI-31600 Jokioinen, Finland*

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ABSTRACT

Certain cool season grasses establish systemic and asymptomatic symbioses with clavicipitaceous fungi of the genus *Neotyphodium*, which affect multiple biotic interactions within host neighborhood. The presumed symbiont-mediated plant resistance to pathogens is mostly based on studies performed under laboratory and greenhouse conditions. Here we investigated, in two outdoor experiments, the relation between two fungi of the same family with opposite effects on *Lolium multiflorum* plants: the mutualist endophyte *Neotyphodium occultans*, and the pathogen *Claviceps purpurea*. Natural infection and its consequences on symbiotic and non-symbiotic plants were studied under varying conditions of stress by herbicide. In both experiments, *N. occultans* reduced significantly the infection by *C. purpurea* at population levels (70 % less). The percentage of spikes infected by *C. purpurea* was almost three times lower in endophyte-symbiotic plants than in non-symbiotic ones. However, the protective effect was not maintained under stress condition. Our results show that constitutive symbionts such as the systemic fungal endophytes mediate the interaction between host grasses and pathogens, although the effect may depend on the level of stress in the environment.

Introduction

Plants interact with myriads of microorganisms that, living inside or outside the host tissues (endophytic or epiphytic/epibiotic, respectively), may affect host population dynamics and evolution (Saikkonen et al., 2004; Thompson, 2005; Thrall et al., 2007; Sánchez Márquez et al., 2012). Symbiotic microorganisms may alter plant performance directly or indirectly by modifying the plant interaction with other organisms and the environment (Omacini et al., 2001; Hudson et al., 2006; Mitchell et al., 2006; Borer et al., 2007; Thrall et al., 2007; Rudgers et al., 2010). Depending on the net outcome for host fitness, symbiotic interactions may range from pathogenic or parasitic to mutualistic (Saikkonen et al., 2004; Thompson, 2005; Thrall et al., 2007).

Neotyphodium fungal endophytes form systemic and asymptomatic symbioses with certain cool season grasses (Clay and Schardl, 2002). Mycelium occurs in intercellular spaces of leaf sheaths, culms, and meristems, so that when the grass flowers, the fungus grows inside the developing

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[*] *Corresponding author*. Av. San Martín 4453, (C1417DSE) Ciudad de Buenos Aires, Argentina. Tel.: +54 11 4524 8000x8124; fax: +54 11 4514 8730.

E-mail address: liperez@agro.uba.ar (L.I. Pérez).

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inflorescence, colonizing the seeds (Rodriguez et al., 2009). Unlike their close relatives *Epichlöe* fungi which are known for their capability of sexual reproduction and horizontal transmission, *Neotyphodium* endophytes only reproduce asexually by growing hyphae within the developing seeds. Thus, the perpetuation of grass-*Neotyphodium* symbioses through generations relies on the vertical transmission efficiency of the fungus, a process that can be sensitive to stressful conditions (Gundel et al., 2008, 2012b; García Parisi et al., 2012). Fungal endophytes are able to produce profound changes in the physiology and ecology of the plant, impacting positively on the fitness, and thus explaining the high frequency of symbiosis in natural populations (Clay and Schardl, 2002; Rudgers et al., 2010; Gundel et al., 2011; Uchitel et al., 2011). Benefits of this symbiosis are mainly related to the host response to abiotic and biotic stress factors (Clay and Schardl, 2002; Rasmussen et al., 2008; White and Torres, 2010). Even though the underlying physiological mechanisms are not clear, symbiotic plants are usually more resistant to natural enemies. The synthesis of bioactive fungal alkaloids has been identified as the mechanism through which endophytes confer host resistance to herbivores (Bush et al., 1997; Schardl et al., 2007). However, the effectiveness of such a mechanism is usually variable depending on the partners' genotype, herbivore identity, and co-occurring stress factors (Saikkonen et al., 2010; Gundel et al., 2012b). Nonetheless, while host resistance to invertebrate and vertebrate herbivores has been intensively explored, the impact of endophytes on the relationship between host plants and pathogens has been scarcely scrutinized (Clay, 1988; Rodriguez et al., 2009).

Regarding disease resistance, a protective role of the endophyte fungus on host plants has been suggested since the very beginning of grass-endophyte research (Siegel and Latch, 1991; Clay and Schardl, 2002; Popay and Bonos, 2008). However, the number of experimental works dealing with this issue under ecological scenarios is strikingly low (Rodriguez et al., 2009). Experiments have been mostly conducted *in vitro*, with different pathogens being exposed to plant extracts from endophyte-symbiotic and non-symbiotic plants, and pathogens growth being measured as a response variable (e.g. Siegel and Latch, 1991; Yue et al., 2000; Ren et al., 2009). These studies are important for screening and isolating compounds that inhibit the growth of other fungi, but they may not be representative of the underlying mechanisms of host resistance to diseases under real ecological conditions. In fact, *in vivo* studies have yielded puzzling results. For example, some experimental works have found negative effects of endophyte on fungal pathogens, thus protecting host plants (Gwinn and Gavin, 1992; Tunali and Marshall, 2000; Clarke et al., 2006; Tian et al., 2008), while others have found neutral (Welty et al., 1991; Burpee and Bouton, 1993; Gundel et al., 2012a) or even positive effects (Krauss et al., 2007).

The fungus *Claviceps purpurea* is a non-systemic, biotrophic pathogen that causes ergot disease on different Poaceae species listed as crops, weeds and forages (Alderman, 1993). It has been historically studied because of its negative economic consequences on crop yield and animal intoxications due to ergotamine and other fungal alkaloids (Diekman and Green, 1992; Alderman et al., 1998). Host infection is produced when a wind-borne ascospore germinates in a plant floret and grows profusely into the ovary, initially producing "honey dew", a droplet of sweet plant sap containing fungal spores called conidia, which can be dispersed by wind, insects or water, and cause new infections. The result of the infection process is the replacement of the seed by fungal biomass that eventually forms a sclerotium. There are no symptoms of necrosis during the infection process, and the growing fungal body has been associated with an increased demand of photoassimilates from the host plant (Bacon and Luttrell, 1982). Although the co-occurring colonization processes have not been described, it is striking that at the same time *C. purpurea* is infecting a floret, *Neotyphodium* is growing into ovaries in symbiotic plants (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004), thus creating the opportunity for the two fungi to interact. As shown by phylogenetic studies, both genera are closely related (family Clavicipitaceae (Ascomycota); (Moon et al., 2002)). Given that *Neotyphodium* endophytes are exclusively vertically transmitted, the result of the interaction between the host plant and these two fungi during the colonization of ovaries seems to be a critical point for their reproductive strategies and their persistence within host populations.

In this article, we explore the relationship between two phylogenetically related fungal species with apparently opposite effects on *Lolium multiflorum* host plants: *Neotyphodium occultans*: a mutualistic endophyte, and *C. purpurea*: a pathogenic epibiont. Our hypothesis is that the constitutive symbiosis of grasses with fungal endophytes inhibits the infection by the pathogenic fungus, especially because they compete for the same organ for reproduction. We predict that the endophyte-symbiotic plants will present lower incidence and severity of *C. purpurea* than non-symbiotic plants. This hypothesis was explored here, by means of two experiments where endophyte-symbiotic and non-symbiotic *L. multiflorum* plants were grown outdoors and the natural incidence and consequences of the pathogen were evaluated. Considering that susceptibility to pathogens may be higher in stressed plants and that the grass-endophyte mutualism may be broken down under stress (Katan and Eshel, 1973; Altman and Campbell, 1977; Kiers et al., 2010; Gundel et al., 2012b), we subjected the plants to a sub-lethal dose of the herbicide diclofop-methyl. The endophyte, in particular, is known to increase the tolerance to this herbicide in *L. multiflorum* seedlings (Vila-Aiub et al., 2003; Martínez-Ghersa et al., 2004; Gundel et al., 2012b), but at the same time, a recent report showed that the endophyte protective effect to herbivory is lost in survivor plants (Gundel et al., 2012b). Our study contributes to improving our understanding of the ecological and evolutionary relationship of two closely related, but antagonistic, organisms that compete in nature for the same resources.

Materials and methods

Plant material

Lolium multiflorum (Italian ryegrass) is an exotic cool season grass naturalized in Pampean grasslands, Argentina, persisting as an important component in co-dominance with native perennial grasses (Soriano, 1992). Characterized as a competitive-ruderal species, its invasion success in Pampean grasslands has been associated with the presence of the endophyte *N. occultans* and to the production of a thick layer of litter that reduces the germination and establishment of seedlings of other species (Vila-Aiub et al., 2005; Gundel et al., 2009; Omacini et al., 2009).

Mature *L. multiflorum* seeds were collected from natural populations in an old-field grassland community of the Inland Pampa Region, Argentina (34°06′S, 60°25′W). A preliminary evaluation showed an endophyte association level of 95 % (based on 100 stained with Rose Bengal solution (ethanol 5 ml, Rose Bengal stain 0.5 g, distilled water 95 ml) and analyzed under the light microscope) (Bacon and White, 1994). A counterpart seed lot of non-symbiotic plants were generated by treating half of the seeds with the systemic fungicide Triadimenol (150 g a.i. kg^{-1}; dose: 5 mg per gram of seed). Both seed lots, non-treated (E+) and fungicide-treated (E−), were grown in monoculture in 1 m^2 adjacent plots at the experimental field of the Agronomy Faculty, University of Buenos Aires. These two populations were cultivated for more than one generation to diminish any phytotoxic effect of the fungicide, and plants were allowed to exchange pollen during flowering to avoid genetic divergence (Gundel et al., 2012b). 100 seeds of each population were stained with Rose Bengal solution (ethanol 5 ml, Rose Bengal stain 0.5 g, distilled water 95 ml) and observed under alight microscope to evaluate endophyte symbiotic level (Bacon and White, 1994). This procedure was repeated for each experiment. The endophyte symbiotic level was over 90 % and below 15 % for symbiotic and non-symbiotic plants (E+ and E− plants), respectively. Seeds of these populations were used for the two experiments that were carried out in the same experimental field in successive years.

Experiment 1

The experiment was conducted outdoors to evaluate the effect of the symbiosis with the fungal endophyte *N. occultans* on the natural incidence of the pathogen *C. purpurea* on *L. multiflorum* plants. Fourteen plots (1 m^2 size) were sown with E+ or E− *L. multiflorum* seeds. Seeds were sown in autumn (March) and grown during the normal growing season (until December) for the species. The plots were arranged in a grid with between-plots paths of 0.5 m, and their symbiotic level was assigned at random (seven replicates each). Eight grams of E+ or E− seeds (≈4 000 seeds) were directly sown in plowed soil and covered with a thin layer of soil to improve seed germination and seedling establishment. The effective sowing rate was ≈500 plants m^{-2} for both biotypes. Weeding and watering were applied regularly throughout the experiment. At plant maturity, two wire rings (15 cm radius) were placed at random over the soil surface of each plot to subsample the aboveground plant biomass. All the harvested plant material was dried at 70 °C for 7 d. In each sample, we separated leaves and pseudostems as aboveground vegetative biomass, and seeds and spikes as reproductive biomass. The number of spikes per plant was also counted. Natural incidence of *C. purpurea* fungus was estimated as the proportion of spikes presenting at least one sclerotium, while infection level (severity) was estimated as the proportion of seeds per plot replaced by fungal sclerotia (Fisher et al., 2005).

Experiment 2

The experiment was conducted to evaluate the effect of the symbiosis with the fungal endophyte *N. occultans* on the incidence and individual infection level of the pathogen *C. purpurea* on *L. multiflorum* plants under natural conditions and subjected to abiotic stress caused by herbicide. Twelve plastic pots (25 l) were filled with a mixture of organic black soil, sand and peatmoss (50/25/25), and half of them were sown with endophyte-symbiotic seeds (E+) and the other half with non-symbiotic seeds (E−) at a sowing rate of nine plants per pot. When seedlings had two or three expanded leaves, half of the pots belonging to each symbiotic status, were subjected to stress by spraying a sub-lethal dose of diclofop-methyl herbicide (i.e., 70 g ai ha^{-1}). The herbicide was applied with a 1 l-constant-pressure hand-sprayer (Commercial formulation 284 g ai l^{-1}, Iloxan; Hoechst-Aventis, Strasbourg, France; recommended dose 1.12 kg ai ha^{-1}). The mechanisms of action for this herbicide in target plants are inhibition of lipid biosynthesis and oxidative stress (Vila-Aiub et al., 2003; Martínez-Ghersa et al., 2004; Gundel et al., 2012b). The experiment was maintained outdoors throughout the whole growing season, and the pots were watered on-demand. At plant maturity, all the spikes from every plant were individually harvested. We recorded plant survival, spike number, spikelet number per spike, seed number per plant and seed weight, and seeds replaced by *C. purpurea*. In each pot, incidence of *C. purpurea* was estimated as the proportion of plants presenting at least one sclerotium, while infection at individual level was estimated as the average percentage of affected spikes per plant with sclerotia.

Statistical analysis

All variables measured in experiment 1 were analyzed by one-way ANOVA with symbiotic level as factor. Variables measured in experiment 2 were analyzed by a two-way ANOVA with symbiotic level and herbicide as factors. Infection level values were Log_{10} transformed to achieve homoscedasticity of residuals. Plant survival values were arcsin(sqroot(*p*)) transformed to reach homoscedasticity of residuals. Seed number values were LN transformed to reach homoscedasticity of residuals.

Results

Experiment 1

Aboveground vegetative biomass was higher in endophyte symbiotic than in non-symbiotic plants ($F_{1,12} = 5.10$; $p = 0.043$), while there was no difference between treatments in terms of reproductive biomass (Fig 1). Ergot incidence in *L. multiflorum* plants was significantly reduced by the symbiotic association with the endophyte *N. occultans*. Spike number was one third greater in symbiotic than in non-symbiotic plants ($F_{1,12} = 28.49$; $p < 0.001$, Fig 2), however, the percentage of spikes with *C. purpurea* sclerotia was three times lower in E+ plots than in

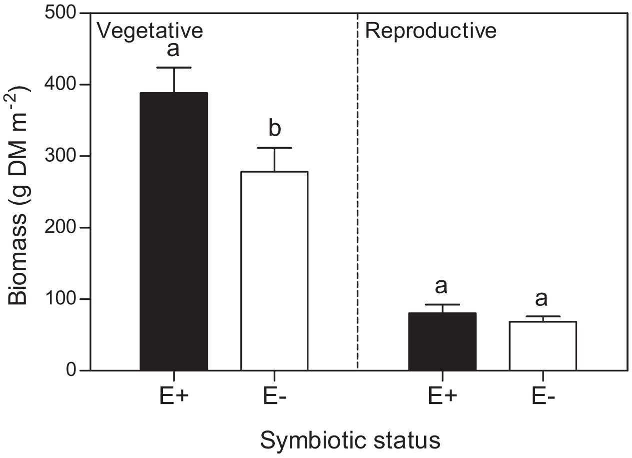

Fig 1 – Vegetative and reproductive biomass of *Lolium multiflorum* plants symbiotic (E+: black bars) and non-symbiotic (E−: white bars) with the endophyte fungus *Neotyphodium occultans* from experiment 1. Values are means and SE ($n = 7$). Different letters indicate significant differences ($p < 0.05$) between treatments for each variable.

E− plots ($F_{1,12} = 21.52$; $p < 0.001$; Fig 3A). In the same way, infection was also modified by endophyte association at spike level with values almost four times lower in symbiotic plants than in non-symbiotic ones ($F_{1,12} = 18.40$; $p = 0.001$; Fig 3B).

Experiment 2

Irrespective of the endophyte symbiotic level, plant survival was 50 % lower under herbicide than in control condition ($F_{1,8} = 150$; $p < 0.001$) (Fig 4). There was an increase in the number of spikes and seed number in herbicide treated plants for both symbiotic statuses, while no significant differences were detected in spikelet number per spike and mean seed weight (Table 1). Symbiotic plants treated with herbicide had double the number of seeds produced by non-herbicide treated plants despite their symbiotic status ($F_{1,8} = 5.86$; $p = 0.042$; Table 1). The incidence of *C. purpurea* in the pots with symbiotic plants was one third of that in the pots with non-symbiotic plants ($F_{1,8} = 9.69$; $p = 0.014$; Fig 5A). This difference in pathogen infection associated to the grass-endophyte symbiosis was erased by the herbicide ($F_{1,8} = 9.69$; $p = 0.014$; Fig 5A). At the individual plant level, no significant effect of the endophyte against pathogen infection (severity) was observed in any treatment, while the lowest infection level was seen on the spikes of symbiotic plants in the control treatments (Fig 5B).

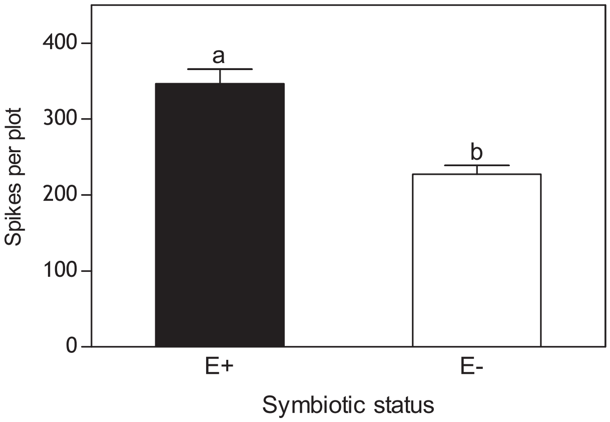

Fig 2 – Number of spikes of *Lolium multiflorum* plants symbiotic (E+: black bars) and non-symbiotic (E−: white bars) with the endophyte fungus *Neotyphodium occultans* from experiment 1. Values are means and SE ($n = 7$). Different letters indicate significant differences ($p < 0.05$) between treatments for each variable.

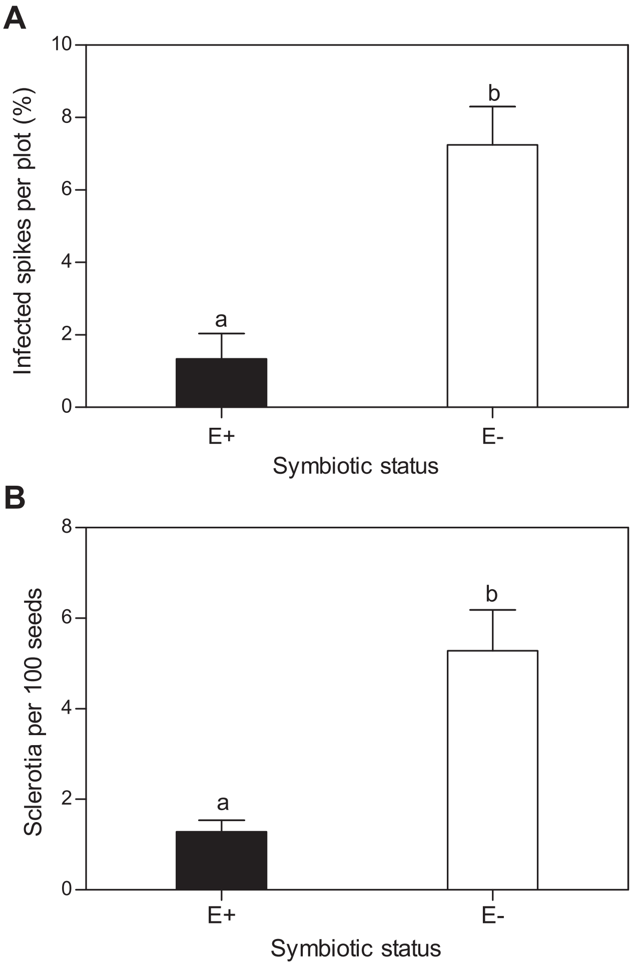

Fig 3 – Percentage of spikes (A) and seeds (B) affected by *Claviceps purpurea* in *Lolium multiflorum* plants symbiotic (E+: black bars) and non-symbiotic (E−: white bars) with the endophyte fungus *Neotyphodium occultans* from experiment 1. Values are means and SE ($n = 7$). Different letters indicate significant differences ($p < 0.05$) between treatments for each variable.

Discussion

Our work shows that constitutive vertically transmitted symbionts of grasses mediate the interaction between host

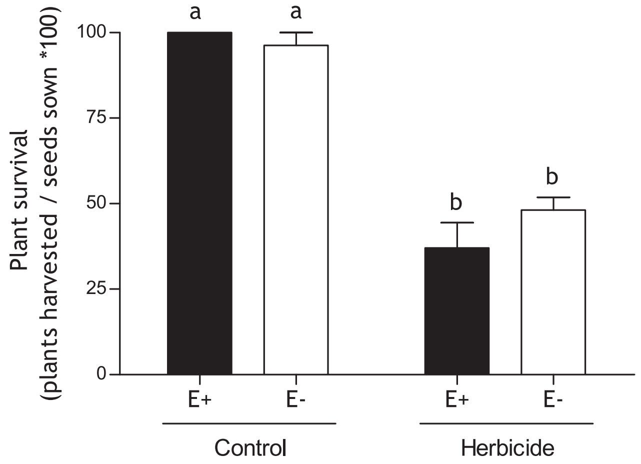

Fig 4 – Plant survival of *Lolium multiflorum* plants symbiotic (E+: black bars) and non-symbiotic (E−: white bars) with the endophyte fungus *Neotyphodium occultans* under two levels of stress [No herbicide (control) and Herbicide (70 g ai ha^{-1})] from experiment 2. Values are means and SE ($n = 3$). Different letters indicate significant differences between treatments for each variable (Tukey test, $p < 0.05$).

plants and pathogenic fungi. Particularly in our tripartite system, symbiosis with *N. occultans* endophyte was associated with a lower incidence of the pathogen *C. purpurea* by reducing infection at population levels in *L. multiflorum*. Despite the clearly higher resistance to the pathogen in the symbiotic plants compared to the non-symbiotic ones, there were no evident effects of both fungi on plant seed production. These results suggest that non-symbiotic plants were more tolerant, compensating for the losses due to the pathogen infection through the production of more flowers, than symbiotic plants. Therefore, this may be part of contrasting strategies for symbiotic and non-symbiotic plants in response to pathogens resembling the evolutionary trade-off in life history traits between resistance and tolerance (Karban and Baldwin, 1997; Partida-Martinez and Heil, 2011). For example, these contrasting strategies between symbiotic and non-symbiotic plants have been previously described in response to defoliation by herbivores (Saari et al., 2010; Partida-Martinez and Heil, 2011). Furthermore, we found that host resistance to *C. purpurea* mediated by endophyte symbiosis was highly dependent on the stress caused by the herbicide,

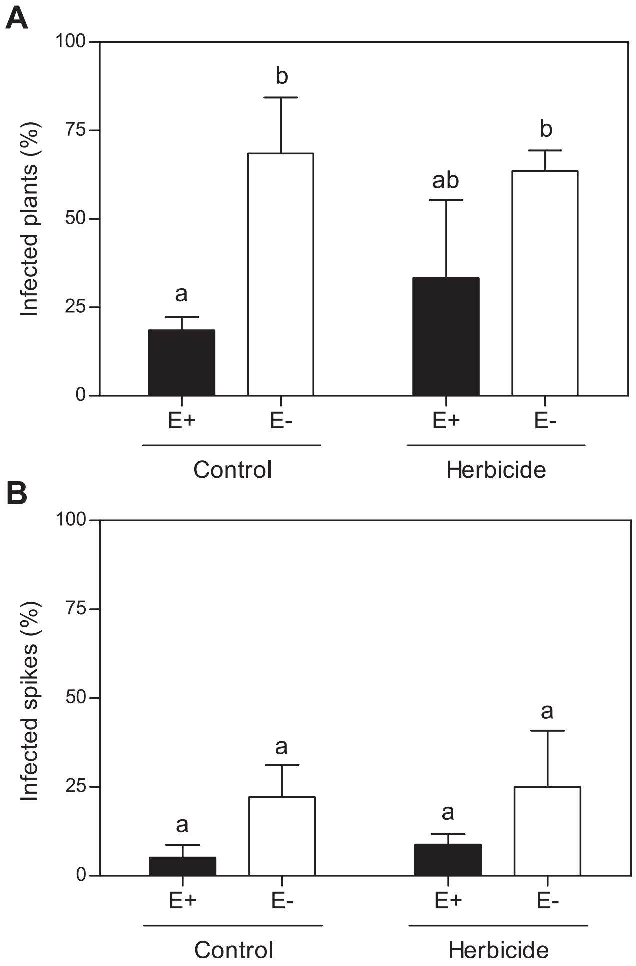

Fig 5 – Percentage of plants (A) and spikes (B) affected by *Claviceps purpurea* in *Lolium multiflorum* plants symbiotic (E+: black bars) and non-symbiotic (E−: white bars) with the endophyte fungus *Neotyphodium occultans* under two levels of stress [No herbicide (control) and Herbicide (70 g ai ha^{-1})] from the experiment 2. Values are means and SE ($n = 3$). Different letters indicate significant differences between treatments for each variable (Tukey test, $p < 0.05$).

Table 1 – Number of spikes, spikelets per spike, seeds, and mean seed weight of *Lolium multiflorum* plants symbiotic (E+) and non symbiotic (E−) with the endophyte fungus *Neotyphodium occultans* under two levels of stress condition [No herbicide (control) and Herbicide (70 g ai ha^{-1})]. Values are means ($n = 3$) and SE from experiment 2

Endophyte	Herbicide	*n*	Spikes per plant		Spikelets per spike		Seeds per plant		Seed weight (g)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
E−	0	3	12.5	2.37a	18.89	0.388a	915.4	285.3a	0.0020	0.0001a
	70	3	43	3.18b	20	0.444a	2 537	199.4ab	0.0022	0.0002a
E+	0	3	10.3	0.541a	19.84	0.692a	620.3	115.2a	0.0024	0.0001a
	70	3	48.6	5.78b	25.64	2.909a	3 920	1 140b	0.0025	0.0001a

Different letters indicates significant differences between treatments for each variable (Tukey test, $p < 0.05$).

which is concordant with the general idea that mutualism could break-down under stressful or restrictive growth conditions (Hahn et al., 2008; Gundel et al., 2012b).

Our results are in agreement with the hypothesis that postulates a protective role of the endophyte on host plants suggesting that, besides the alkaloid-based mechanism against herbivory (Bush et al., 1997; Clay and Schardl, 2002), a protection against diseases is also possible. This is firmly supported by the results that show antifungal activities of endophyte-symbiotic plant extracts on fungal pathogen growth in culture media (Siegel and Latch, 1991; Clay and Schardl, 2002; Popay and Bonos, 2008; Rodriguez et al., 2009). Another explanation could be an endophyte protective effect to stresses based on an enhanced production of antioxidants (White and Torres, 2010; Hamilton et al., 2012). The same mechanism could explain the high tolerance of symbiotic plants to stresses, in general, and to the diclofop-methyl herbicide, in particular (Martínez-Ghersa et al., 2004; Gundel et al., 2012b). However, the balance between alternative mechanisms or the crossed effects of the same mechanism may be broken-down as it has been shown, for instance, for drought and herbicide, affecting alkaloid production and the effectiveness of the anti-herbivory mechanism (Hahn et al., 2008; Gundel et al., 2012b). With elements from the evolutionary origin of the symbiosis and the presumption that the endophyte is no longer a threat for the host plants, it has been proposed that endophyte could be attenuating the plant defence systems against pathogens (Naffaa et al., 1999; Zhang et al., 2011). However, as it has been shown for the effectiveness of the endophyte-mediated antiherbivory mechanism compared to the hormone-mediated defences of plants (Simons et al., 2008), our results suggest that the protective effect of endophytes against other fungi must be greater in magnitude than the plant's own defence mechanisms.

Considering the ecological attributes of *C. purpurea*, its infection could be also modulated by endophyte-induced changes in host phenotypic traits such as number of florets, pollen production, spike number and size, and phenology. All these attributes could ultimately affect the incidence and severity of a pathogen attack on a host population (Thakur and Williams, 1980). For example, pollen charge in relation to flowering dynamics may exert some control on pathogen severity by competing for host ovaries. *Neotyphodium* endophytes colonize the ovary from before stigma fertilization, and continue growing during seed development (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004). Alternatively, *C. purpurea* spores germinate on the stigma and start growing from the top to the ovule (Bacon and Luttrell, 1982). Therefore, the flower appears to be the arena for the interaction (e.g. competition) between the two relatives – with opposite effect on the host plant. In this instance, the endophyte-mediated fungistatic effect on the growth of other pathogens that has been found *in vitro* may be playing a role in this interaction (Siegel and Latch, 1991; Yue et al., 2000; Ren et al., 2009). In addition, changes in biomass allocation and partitioning have been reported with the endophyte symbiosis associated with a higher number of light tillers compared to non-symbiotic counterparts (Vila-Aiub et al., 2005; Gundel et al., 2012a, 2013). In accordance with this latter pattern, it would be interesting to see how these differences in number and size of tillers affects the canopy structure and behavior of non-specific insects (mainly true bugs and moths) carrying spores of *C. purpure*a (Prom et al., 2003, 2005; Prom and Lopez, 2004). Finally, other ecological aspects of the symbioses with *Neotyphodium* endophytes, as the insects' deterrence effect, could also be associated with a specific emission profile in volatile organic compounds that could repel the pathogen's vectors from symbiotic plants (see Steinebrunner et al., 2008a,b).

Variability observed in our results can be explained by the genetic diversity of the *L. multiflorum* populations studied. Recent works suggest that effects of host plant genotype are usually more important than those due to the endophyte. In an experiment carried out in Switzerland, the natural incidence of *C. purpurea* on *Lolium perenne* (perennial ryegrass) plants was higher in endophyte-symbiotic individuals than in non-symbiotic ones, but it was also dependent on the cultivar (Krauss et al., 2007). In an experiment carried out in Finland, the incidence of the same pathogen species on *Schedonorus phoenix* (tall fescue) plants was also found to be dependent on the host population and the symbiosis with endophyte (Gundel et al., 2012a). None of the experiments carried out to date, including ours, has manipulated or controlled the pathogen as treatment. Considering the potential effect of pre-existent co-evolutionary processes shaping the triple-interaction between host plant population, future experimental designs should include the pathogen spore pressure and the strain origin as treatments.

In summary, our results showed a critical role for *Neotyphodium* endophyte controlling the epidemiology of the pathogen *C. purpurea* in host plant populations of *L. multiflorum* under ecological settings. The role of *Neotyphodium* endophytes on host grass relationships with pathogenic fungi has been mostly explored *in vitro* suggesting that fungistasis is the main underlying mechanism. However, this kind of trial may underestimate the emergent properties of complex ecological interactions. Given that the pattern of response changed under stress, future experiments should be addressed to explore the cross-link between different endophyte-mediated mechanisms of host protection to abiotic and biotic stress factors. This is an important issue in light of the predicted scenarios of current global changes where positive interactions could disappear or turn into negative ones (Thompson, 2005; Kiers et al., 2010). Special attention should also be paid to phylogenetic relationship and life history traits of both partners and the abiotic context, as factors that could significantly alter the outcome of the grass–endophyte–pathogen interaction.

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