Effects of Neotyphodium Fungi on Lolium multiflorum Seed Germination in Relation to Water Availability

P. E. Gundel, P. H. Maseda, M. M. Vila-Aiub, C. M. Ghersa, and R. Benech-Arnold

INTRODUCTION

Many cool-season C3 grasses establish an association with fungal endophytes (Neotyphodium spp.) (Clay and Schardl, 2002). The mutualistic nature of this plant–fungus relationship has been documented extensively: endophyte-infected (E+) individuals exhibited higher tolerance to biotic and abiotic environmental stresses than endophyte-free (E−) individuals (Saikkonen et al., 1998; Malinowski and Belesky, 2000; Vila-Aiub et al., 2003a). On the other hand, the host plant provides nutrients, protection and propagation opportunities to the fungus (Latch et al., 1987; Clay and Schardl, 2002). The differential ecological performance between E+ and E− plants has been regarded as the basis for the high levels of endophyte infection often found in grass populations (Clay and Holah, 1999; Vila-Aiub et al., 2005). However, there is consensus that the infection levels in wild and non-agronomic grass populations are more variable (Faeth and Sullivan, 2003) and relatively low (Jensen and Roulund, 2004). Recently, the general assumption that the symbiosis between grasses and Neotyphodium is a mutualism has been empirically challenged (Faeth and Sullivan, 2003; Faeth et al., 2004), suggesting that the relationship would range from antagonistic to mutualistic (Saikkonen et al., 1998; Muller and Kraus, 2005).

Endophytic infections are necessarily maternally transmitted through host seeds (i.e., vertical transmission) (Clay and Schardl, 2002). This process involves extensive hyphal growth within the plant ovary (Sugawara et al., 2004) and, as a result, the host seeds are organs with high hyphae concentration per unit of plant biomass (White and Cole, 1985). A consequence of the strict vertical endophyte transmission is the strong dependence of endophyte survival on the ecological success of the host plant (Clay and Schardl, 2002). From an evolutionary perspective, any endophyte-mediated host change that ensures successful seed germination (or its avoidance) under stressful conditions will promote Neotyphodium survival and be environmentally selected for (Vila-Aiub et al., 2003a). Hence, it is believed that endophytes may change the capacity of host seeds to sense the external environment, thus altering physiological processes such as dormancy, germination and/or seed survivorship. For instance, it has been found that endophyte infection increases seed germination in Lolium perenne (Clay, 1987) and Bromus setifolius (Novas et al., 2003). However, endophyte-infected seeds of Festuca arundinacea displayed higher germination percentages at reduced water potentials only at moderate temperatures but not at warmer temperatures (Pinkerton et al., 1990). Conversely, Neotyphodium endophyte did not affect the timing or the percentage of germination of F. arizonica seeds, either in general (Faeth et al., 2004) or under different water potentials (Neil et al., 2003). Similarly, endophyte infection did not affect seed germination in another population of F. arundinacea (Bacon, 1993). On the whole, the few
published studies suggest multiple interactions between the effect of endophyte, plant host species and environmental conditions. Additionally, no studies have identified the physiological mechanisms involved in the relationship between endophyte infection and seed germination.

Temperature and water availability are environmental variables that strongly determine the timing and proportion of seed germination (Bench-Arnold and Sánchez, 1995; Bradford, 1995). The effects of different water availabilities on germination can be modelled by the ‘hydrotime model’ (Bradford, 1995). The hydrotime model has not only proved to predict seed germination progress well, but also uses physiologically based parameters with ecological and biological significance (Bradford, 2002). Using this model, it is possible to characterize a seed population by two parameters defined in the following equation:

\[ \theta_H = \frac{|\Psi - \Psi_b(g)|}{tg} \]  

where \( \theta_H \) is the hydrotime constant (MPa h required for seed germination and assumed to be constant for all seeds in the population), \( \Psi \) is the water potential (MPa) of the germination medium, \( \Psi_b \) is the base water potential (threshold for radicle emergence) for a particular germination fraction \( g \) of the seed population and \( tg \) is the time required to germinate for the same seed fraction. This approach enables calculation of the standard deviation of base water potential values in the seed population \( (\sigma_{\psi_b}) \) (for details, see Bradford, 1995, 2002).

Tolerance to water stress is probably the most documented ecological advantage in Neotyphodium-infected grasses (reviewed by Malinowski and Belesky, 2000; but see Cheplick, 2004). Avoidance, tolerance and recovery from drought are the described adaptations mediated by morphological and physiological mechanisms that enable endophyte-infected individuals to display higher growth rates and dry matter accumulation than endophyte-free plants under water stress exposure (Malinowski and Belesky, 2000; Hesse et al., 2003). However, few studies have considered the possible effects of water stress during germination–seedling establishment (Pinkerton et al., 1990; Neil et al., 2003), and none of these has identified the physiological parameters involved in the relationship between endophyte-infected seed germination and water availability.

In this study, the hydrotime model was used to characterize the effect of Neotyphodium endophyte on Lolium multiflorum Lam. seed germination at a range of water availability. We tested the prediction of enhanced germination for endophyte-infected (E+) seeds compared with endophyte-free (E-) seeds under water stress conditions. In addition, the effects of endophyte infection and water potential on seed survival were evaluated.

**MATERIALS AND METHODS**

**Seed material**

*Lolium multiflorum* is an exotic and invasive species widely naturalized in the Pampa region, Argentina (Soriano et al., 1992) that has shown a high infection level with *Neotyphodium* endophyte (Vila-Aiub et al., 2003a; De Battista, 2005). In comparative studies, infected plants produced more roots and twice as much reproductive biomass (Vila-Aiub et al., 2005), were attacked less by leaf mining insects (Omacini et al., 2001) and were more tolerant to a herbicide (Vila-Aiub et al., 2003a). *Lolium multiflorum* seeds were collected from an old-field community lot (>2 ha) of the Inland Pampa region (Carlos Casares, Province of Buenos Aires, Argentina, 34°06'S, 60°25'W) and showed >85% frequency of *Neotyphodium* endophyte infection (Vila Aiub et al., 2005). This old-field community’s lot had been closed to grazing and uncultivated for >20 years. Half of these seeds were treated with triadimenol [β-(4-chlorophenoxy)-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol] fungicide (Baytan, Bayer, F 150 g kg⁻¹) to obtain endophyte-free seeds. Treated and untreated seeds were sown in plots, and seedlings of treated seeds were sprayed with another fungicide: benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] (Benlate, Du Pont, WP 500 g kg⁻¹) at the 2–3 leaf stage. Seeds were collected from mature plants. E+ and E- biotypes were sown in contiguous 1 m² plots that allowed cross-pollination among plants of both biotypes, which were located in the Campus of the Agronomy Faculty, Buenos Aires University (34°35'S, 58°35'W). Plants were grown at a density of approx. 1000 plants m⁻² over four generations. Endophyte infection was evaluated annually using a seed staining method for detection of endophyte through a light microscope (Latch et al., 1987). The endophyte infection level is expressed in percentage units based on 100 examined seeds per biotype (E+ and E-).

**Germination experiments**

Two experiments were carried out to evaluate the effect of water availability on seed germination of E+ and E- *L. multiflorum* biotypes. Seeds produced in 2001 (lot A) (85% E+, 9% E-) and 2003 (lot B) (90% E+, 10% E-) were used. After harvest, seeds of each biotype were stored for 4 months (lot A) and 5 months (lot B) in dark glass jars at 10 ± 2°C. In both experiments, we used seed samples obtained by mixing all the seed harvested from a plot with a particular biotype (E+ or E-).

Thirty seeds of each biotype were sown on Petri dishes containing two blotter papers moistened with 6 ml of distilled water (0 MPa) or different polyethylene glycol (PEG 6000) solutions (−0.3, −0.8, −1.0 and −1.5 MPa), and incubated in a growth chamber at 20°C (General Electric, Precision Scientific, Incubator model 805). PEG solutions were prepared using the non-linear model proposed by Steuter et al. (1981) and were verified using a vapour pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA) and psychrometer chambers (C-52, Wescor Inc.). Each treatment had five replicates. Seeds were incubated on PEG solutions for 24 h (imbibition phase) and then transferred to fresh solutions to maintain desired water potential levels in the germination medium (Ni and Bradford, 1992). The number of germinated seeds (radicle appearance) was counted periodically (once and twice a day
for seed lot A and lot B, respectively), until no further germination was recorded (approx. 11 d). Seed germination was evaluated under laboratory fluorescent light conditions, and germinated seeds were removed from Petri dishes. When no further seed germination was observed (i.e. germination reached a plateau), the remaining ungerminated seeds were transferred to distilled water (0 MPa) and incubated at 10°C until germination was completed. This approach enabled identification of dead seeds, which showed a soft consistency and symptoms of disease infection. Germination was calculated as a percentage of total living seeds. Potential maternal effects of endophyte infection on various plant traits with importance for seed germination were considered by assessing seed morphology (length, width and height), dry weight and water content at the beginning of the experiment (seed lot B). Seed morphology was measured on 50 seeds per biotype (E⁺ and E⁻). Seed dry weight and water content were measured in three sub-samples of 25 (0.05 g each) seeds from each biotype, weighing and drying seeds for 1 h at 130°C (ISTA, 1996). The water content was calculated gravimetrically and expressed as percentage of dry seed mass.

Data analysis

Analysis of variance (ANOVA) for repeated measures was performed on each seed lot to assess the effect of endophyte infection (E⁺ and E⁻), water potential and incubation time on seed germination. To evaluate the effect of endophyte infection on the final percentage of germinated seeds within each water potential treatment, a post hoc procedure was used (l.s.d. Fisher test, \( P = 0.05 \)). Percentage germination was arcsine transformed to comply with the ANOVA assumptions. The hydrotimel model (Bradford, 1995) was applied to characterize seed germination of E⁺ and E⁻ biotypes in relation to water availability. Experimental evidence indicates that seeds could differentially adjust their germination to high and low water potential (Ni and Bradford, 1992). For this reason, the analyses were performed using two approaches: one in which a single hydrotimel model was adjusted disregarding the water potential levels, and another in which two hydrotimel models are fitted separately to data from low (0 and −0.3 MPa) or high (−0.8, −1.0 and −1.5 MPa) water potentials. The accuracy of each approach was tested for E⁺ and E⁻ seeds within each lot (A and B) by means of the Levene’s test for variance homogeneity. Endophyte infection and water availability effects on seed survival were analysed using a generalized linear model, assuming a binomial response variable and a logit link function (only on lot B). To test the effect of endophyte on survival within each water availability treatment, least squares means for multiple comparisons were used (LSMEANS; PROC GENMOD, SAS Institute, 1990). The effect of endophyte infection on seed morphology (length, width and height), dry weight and water content was evaluated performing a separate Student t-test. Statistical analyses were conducted using InfoStat/Profesional, Version 1.1.

### RESULTS

The length, width and height of E⁺ and E⁻ L. multiflorum seeds did not differ (\( P = 0.96, P = 0.33 \) and \( P = 0.74 \), respectively) (Table 1). No differences in dry weight (\( P = 0.26 \)) and water content (\( P = 0.71 \)) of E⁺ and E⁻ L. multiflorum seeds were observed (Table 1). The rate and final percentage of germination were affected by endophyte infection. However, ANOVA showed a triple interaction between endophyte infection, water potential and incubation time (\( P < 0.05 \)). In both seed lots A and B, germination of E⁺ seeds was significantly more inhibited by negative water potentials than was germination of E⁻ seeds (Fig. 1). The E⁻ biotype displayed approx. 90 and 75% germination at −0.8 and −1.0 MPa, respectively, whereas the E⁺ biotype showed 25% less germination at the same water potentials (Fig. 1, lot A). In seed lot B, E⁺ seeds showed a lower germination rate and total proportion of germination at −0.3, −0.8 and −1.0 MPa compared with seeds of the E⁻ biotype (Fig. 1). Germination was completely prevented in both E⁺ and E⁻ seed lots with a water potential of −1.5 MPa, except for E⁻ seeds of lot B, which showed 4% germination (Fig. 1). In addition, final germination of the E⁺ biotype at 0 MPa never reached 100% (Fig. 1).

The accuracy of hydrotimel models to describe germination was enhanced when their parameters were fitted for low (0 and −0.3 MPa) and high (−0.8, −1.0 and −1.5 MPa) water potential separately. The differences between actual and modelled data were lower in every case analysed. The Levene’s test \( P \)-values were \( P = 0.039 \) and \( P = 0.014 \), respectively, for E⁻ and E⁺ biotypes for seed lot A, and \( P = 0.037 \) and \( P < 0.001 \), respectively, for seed lot B. However, in spite of the high precision, the accuracy of the model diminished when water potential treatment was more negative (Fig. 1).

In general, \( \theta_1 \) and \( \sigma_{\theta_1} \) values for high water potentials show a decrease when they are fitted for low water potentials. Conversely, \( \Psi_0(50) \) shows an increase (less negative) at low water potentials in relation to high water potentials (Table 2). Differences in hydrotimel requirements (\( \theta_1 \)) were more related to seed lots than to E⁺ and E⁻ biotypes: lot B showed significantly higher values than lot A for both high

### Table 1. Morphological dimensions (length, width and height), dry weight and water content of Lolium multiflorum seeds from seed lot B infected (E⁺) and non-infected (E⁻) with Neotyphodium endophyte

<table>
<thead>
<tr>
<th>Biotypes</th>
<th>E⁺</th>
<th>E⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>5.08 (0.07)</td>
<td>5.18 (0.08)</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>1.50 (0.23)</td>
<td>1.29 (0.01)</td>
</tr>
<tr>
<td>Height (mm)</td>
<td>0.76 (0.01)</td>
<td>0.78 (0.01)</td>
</tr>
<tr>
<td>Dry weight (mg)</td>
<td>1.85 (0.18)</td>
<td>2.09 (0.04)</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>9.60 (0.17)</td>
<td>9.53 (0.09)</td>
</tr>
</tbody>
</table>

Morphological variable values are the mean±s.e. of 50 seeds, while dry weight and water content values are the mean±s.e. of three replicates of 25 seeds.
and low $\theta_H$ parameters. Similarly, $\sigma_{\psi_b}$ values were different between seed lots, although no important differences were observed between $E^+$ and $E^-$ seeds (Table 2). However, the base water potential $[\Psi_{b}(50)]$ displayed by $E^-$ seeds for both lots A and B was consistently lower (more negative) than those estimated for $E^+$ seeds (Table 2).

Differential seed mortality after exposure to water stress was also observed between the $E^+$ and $E^-$ biotypes. The generalized linear model analysis revealed that only the endophyte status ($P = 0.0002$) and not the water potential treatment ($P = 0.19$) accounted for differences in dead seed fractions. In spite of the lack of interaction ($P = 0.24$), the difference between $E^+$ and $E^-$ seed survival after exposure to water treatment was higher at extremely low water potential (i.e. $-1.0$ and $-1.5$ MPa) (Fig. 2).

**DISCUSSION**

The present study has revealed that $E^+$ and $E^-$ *Lolium multiflorum* biotypes that share a common genetic background and were collected from the Inland Pampa grasslands exhibit significant differences in their ability to germinate under limiting water conditions. The working prediction was partially supported by the results. $E^+$ seeds showed a reduced
rate of germination and proportion of germinated seeds under increasing levels of water stress compared with E− seeds (Fig. 1), while E+ seeds showed a higher survival percentage than E− seeds at severe water stress conditions (i.e. fewer dead seeds, Fig. 2).

The underlying physiological mechanism for the differential germination responses is unknown. Evaluation of various seed traits (seed size, dry weight and water content) did not account for the difference between the E+ and E− biotypes (Table 1). However, the hydrotime model indicated that the higher germination rate and proportion of germinated seeds displayed by the E− biotype under different water conditions compared with the E+ biotype may be partially explained by differences in their median base water potentials [Ψ(50)] (i.e. lower for the E− biotype) (Table 2; Bradford, 1995). Although the E+ and E− Ψ(50) values overlapped when considering their variances [σΨ(50)], the hydrotime model still denoted at the population level higher frequencies of E− seeds with lower base water potentials (data not shown).

E+ seeds also displayed a slower germination rate than E− seeds when water availability was high (i.e. 0 MPa). This result agrees with a previous report in which the rate of germination in E− seeds was faster than that of E+ seeds at different temperature treatments under non-limiting water conditions (Vila-Aiub et al., 2005). However, Medvescigh et al. (2004) demonstrated that Neotyphodium endophyte enhances L. multiflorum seed germination in water or at slightly reduced water potentials. As was shown in the latter study, seed dormancy may have interfered with germination, as the final proportion of germinated seeds was relatively low at 0 MPa (i.e. E+ 73 % and E− 60 %). When comparing different seed populations, it is important to account for after-ripening and dormancy status of the seed lots (Benech-Arnold et al., 2000), as well as the germination temperature under which the experiments were performed (Larsen et al., 2004). Moreover, species and even provenances or varieties within a species may differ in the ability to germinate at reduced water potential (Bradford, 1995). Therefore, the lack of consensus on how endophyte infection may affect germination responses in L. multiflorum could be attributed to a difference either in endophyte strains and plant phenotypes or in the interactions between them as promoted by environmental differences.

No important changes in hydrotime requirements (θH) were associated with endophyte infection. However, there was an important difference in the quantitative magnitude of θH between the seed lots A and B (seeds produced in 2001 and 2003, respectively). As θH is related to the lag phase of germination (Bradford, 1995), this parameter is likely to account for the differences in lag phase of seed germination between the different seed lots for both high and low water potentials (Fig. 1). The values of hydrotime model parameters derived from the analysis using high water potentials were very different from those derived from analysis using low water potentials. The θH and σθH values were always higher at high water potentials than those derived from analysis using low water potentials. Conversely, Ψ(50) values were lower when they were derived from the same Ψ level, except for E+ lot B. Our results are different from those obtained by Ni and Bradford (1992) in tomato (Lycopersicon esculentum). These authors found that the two model approaches (i.e. using high and low water potentials for the analysis) could be related to an acclimation mechanism, by means of which tomato seeds are able to reduce Ψ(50) and increase θH when exposed to low water potentials. We do not have evidence to consider if this acclimation mechanism is taking place in L. multiflorum seeds. However, our results show that, despite the fact that the models derived from analysis at high and lower water potential separately fitted the observed data better than using parameters derived from analysis of pooled data for high and low water potential, the Ψ(50) increased instead of decreasing with the reduction in water potential. In our system, the reduction in θH is compensated by an increase in Ψ(50) and, as a result of this, a significant reduction in the germination rate as well as a significant increase in the time needed to reach the final germination at low water potentials can be seen.

Studies conducted at the whole plant level have shown that endophyte-mediated plant changes under water stress involve adjustments in plant stomatal conductance driven by the production of osmotically active substances, hormones and alkaloids (Malinowski and Belesky, 2000). Neotyphodium endophyte has also been shown to modify the nutrient balance at the whole plant level by changing the resource allocation pattern (Omacini et al., 2005), affecting the total nitrogen content in green tissue, and presumably in seeds. Furthermore, differential plasma membrane functionality associated with H+ transport across cell membranes has been distinctly characterized in E+ and E− intact seeds (Vila-Aiub et al., 2003b). Whether these endophyte effects, operating either on the mother plants or directly in host seeds, are responsible for the reduced germination under limiting water conditions requires further investigation. Additional experiments that integrate
the biochemistry, physiology and ecology of fungal endophytic–host seed interaction are needed to address these specific questions.

Seed germination and seedling emergence are important developmental stages contributing to plant fitness especially in species with an annual life cycle (Grime, 1979). In field conditions, water evaporation can generate dramatic decreases in water content in the upper centimetres of the soil (Bradford, 1995), having a major effect on the timing of seed germination and seedling emergence (Benech-Arnold and Sánchez, 1995). Seeding establishment in environments with wet and dry periods depends on both the rate of germination in the wet periods and survival of ungerminated (i.e. dormant) seeds during the dry periods. Despite the lower proportion of germinated E+ seeds under water stress conditions compared with E− seeds (Fig. 1), the rate of mortality in those ungerminated seeds under the inhibitory effect of low water potentials was also lower in the E+ biotype (Fig. 2). The effects of Neotyphodium endophyte on seed germination of L. multiflorum from the Pampa grasslands could be interpreted as generating more limited and conservative conditions for germination: the environmental limit which determines a dry condition is moved towards higher levels of water availability. The resulting reduction in seed germination when water is scarce (E+ biotype) may represent an advantage to avoid seed loss and/or subsequent seedling mortality if water stress increases over time. Conversely, an enhanced germination under limiting water conditions (E− biotype) may ensure a more rapid seedling establishment if later water conditions do not restrict plant growth. Further experiments are necessary to determine the ecological significance of each of these two seed germination strategies.

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LITERATURE CITED


