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Germination response of endophytic *Festuca rubra* seeds in the presence of arsenic

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Abstract

Epichloë fungal endophytes colonize the intercellular space of aerial organs of their plant hosts without causing symptoms. These symbionts are known to improve the performance of their host grasses in some situations of biotic and abiotic stress, leading to the suggestion that they can be used to improve grass tolerance to contaminants. The grass *Festuca rubra* is a host of the endophyte *Epichloë festucae*. We used two half-sib lines of *F. rubra*, each line composed of infected (E+) or endophyte-free (E−) seeds, to study the effect of varying levels of arsenic (6, 12, 25, and 50 mg L^{-1}), and of the endophyte in seed germination and radicle growth. The results showed that seed germination was not significantly affected by arsenic (As) levels lower than 25 mg L^{-1}, indicating that this grass has a relatively high tolerance of As at the germination stage. The decrease in germination observed at 25 and 50 mg L^{-1} was due to increased seed mortality and to the reversible inhibition of the germination of viable seeds caused by As. The presence of the endophyte did not change the germination response to arsenic of one line, but affected negatively the germination of the other line. In contrast to the process of germination, radicles of E+ seeds of both lines were longer than those of endophyte-free seeds. The results of this work indicate that *Epichloë* endophytes can affect the performance of some *F. rubra* genotypes when As is present in the soil.

Keywords: red fescue, endophytes, *Epichloë*, mutualism, root growth, soil contaminants, symbiosis

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Introduction

In the current context of global change, higher incidence of contamination processes is expected as a side effect of anthropogenic activities on biological systems at all levels of organization (Solomon *et al.*, 2007; Godfray *et al.*, 2010). To deal with these environmental changes, plants are endowed with different strategies such as adaptive evolution, plasticity, migration and demographic changes (Parmesan, 2006; Cleland *et al.*, 2007). It has been recently emphasized that through the association with viruses, bacteria and/or fungi, plants have another, perhaps more important, strategy for facing the consequences of global change (Kiers *et al.*, 2010; Redman *et al.*, 2011; Singh *et al.*, 2011). In this work, we explored the effect of the symbiosis with systemic fungal endophytes on the host seed germination and seedling growth in the presence of arsenic. Arsenic is a metalloid ubiquitous in soils at trace quantities. However, soil levels can increase highly as a result of industrial activities such as mining, metal smelting, coal combustion and glass manufacturing, as well as by the present use of arsenic compounds such as fertilizers, pesticides, desiccants and growth promoters for poultry and pigs (Christen, 2001; Mahimairaja *et al.*, 2005).

Festuca rubra L. (red fescue) is a perennial grass which grows in very diverse habitats in Europe (Markgraff-Dannenberg, 1980). Its fine foliage and diversity of growth characteristics make it an important turfgrass species and is used alone or in mixtures with other grasses in ornamental and sports lawns. It is also appreciated as forage, being abundant in natural grasslands in mixtures with other plant species. Grass mixtures containing *F. rubra* have shown good tolerance to high concentrations of As in soils (Zacarias *et al.*, 2012). This grass is a host of the fungal endophyte *Epichloë festucae* Leuchtmann, Schardl & Siegel, an ascomycete that colonizes systemically the leaves and reproductive stems of host plants, and it is vertically transmitted to most seeds

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produced by infected plants (Leuchtmann *et al.*, 1994; Schardl *et al.*, 2004). Infected individuals are usually symptomless, although a few plants might show fungal reproductive structures (stromata) that prevent inflorescence development in some stems. In natural populations of *F. rubra* across Europe, plants are often infected by *E. festucae* (Bazely *et al.*, 1997; Zabalgogeazcoa *et al.*, 1999, 2006b; Granath *et al.*, 2007; Wäli *et al.*, 2007). Some evidence indicates that the symbiosis between *F. rubra* and *E. festucae* is mutualistic. Infected plants of this species contain toxic alkaloids produced by the fungus that protect them against vertebrate and invertebrate herbivores (Bazely *et al.*, 1997; Wilkinson *et al.*, 2000; Vázquez de Aldana *et al.*, 2010). Furthermore, symbiotic plants inhibit the root growth of competing legume species, probably by means of an allelopathic mechanism (Vázquez de Aldana *et al.*, 2013) and have shown increased resistance to some fungal pathogens (Clarke *et al.*, 2006). In addition, some particular plant–fungus combinations are more tolerant to aluminium in soils than non-symbiotic plants of the same genotype (Zaurov *et al.*, 2001). Because of the ease of endophyte incorporation to *F. rubra* cultivars by means of seed transmission and the beneficial effects observed in symbiotic plants, endophytes are used for the improvement of *F. rubra* turfgrass cultivars, and at the present time, symbiotic cultivars are commercialized in several countries (Brilman, 2005).

Experiments of tolerance to heavy metals have shown promising results to support the role of *Epichloë* endophytes in phytoremediation (Monnet *et al.*, 2001; Zaurov *et al.*, 2001; Soleimani *et al.*, 2010). However, the role of endophytes in tolerance to toxic metals has not been explored in seed and seedling stages of the host grass. Seed germination is a critical plant stage impacting on the successful establishment of new individuals and population dynamics recruitment (Harper, 1977). Accordingly, the endophyte effect on seed germination has received attention from an eco-physiological perspective. For instance, the epichloid endophyte *Neotyphodium occultans* was found responsible for changes in seed water dynamics in *Lolium multiflorum* Lam., affecting germination under different water availabilities and seed longevity under storage and field conditions (Gundel *et al.*, 2006a, 2010). The endophyte *E. festucae* affected seed germination in *F. rubra*, depending on the plant genotype and environmental condition, and it was also associated with higher seed and seedling survival (Wäli *et al.*, 2009; Gundel *et al.*, 2011).

Arsenic toxicity has multiple effects in plants and is expressed in multiple organs and developmental stages; some of the most obvious symptoms are a reduction in germination and root growth (Abedin and Meharg, 2002; Smith *et al.*, 2010). One or few plant genes are involved in the tolerance of some grasses to arsenate; these genes suppress high affinity phosphate–arsenate transporters in roots, decreasing arsenate uptake in plants. As a result, intraspecific variation to arsenate tolerance occurs in several grass species (Watkins and MacNair, 1991; González Chavez *et al.*, 2002). In addition to plant genes, arbuscular mycorrhizae present in the roots of plants in contaminated soils increased plant tolerance to As by decreasing accumulation of the contaminant in tissue (González Chavez *et al.*, 2002; Smith *et al.*, 2010). Considering these antecedents together with the well-known beneficial effect of systemic fungal endophytes on host plants to biotic and abiotic stress factors (Malinowski and Belesky, 2000; Schardl *et al.*, 2004), there is a potential for this symbiosis to affect plant tolerance to As.

Our objective was to study the response *Festuca rubra* seeds to arsenic in the presence of the symbiotic fungal endophyte *Epichloë festucae*. For this purpose, germination and seedling radicle growth of infected and non-infected seeds of *F. rubra* were controlled under varying concentrations of As. Knowing in which circumstances the endophyte favours or inhibits its host grass is important for understanding this symbiosis, as well as for applying it to plant breeding.

Materials and methods

Plant material

Two half-sib lines of *F. rubra* (SAN, PEN), each one consisting of endophyte-infected (E+) and endophyte-free (E−) seeds, were used for the experiment. Each line was developed from a single *F. rubra* plant originally infected by *E. festucae* (Zabalgogeazcoa *et al.*, 2006a). The mother plants of each line were collected at two locations about 40 km apart in semiarid natural grasslands (dehesas) of the province of Salamanca, in western Spain. Each mother plant was divided into six ramets, half of which were treated with a systemic fungicide to kill the endophyte. After verifying the infection status, fungicide treated (E−) and non-treated (E+) ramets were transplanted 60 cm apart in a field plot in a research farm in Salamanca province. These ramets have been maintained, and every year they were harvested for seed. Seeds produced by different ramets belonging to the same maternal line and infection status were pooled and stored until their use in experiments. Endophyte infection frequency in each population was determined by analysing stained seeds ($n = 30$) by light microscopy (Bacon and White, 1994). Results of infection were 100% for E+ biotypes, and 0% for E− biotypes. According to the method used for their production, E+ and E− seeds of each line have the same maternal background and can be considered as half-sibs. Nonetheless, taking into account that this species is self-incompatible and wind pollinated, each pool of seeds should have a genetic variability. The PEN and SAN plants have distinct phenotypes (Zabalgogeazcoa *et al.*, 2006a), but also the fungi infecting the E+ seeds of each line are likely to be different because of the genetic diversity observed in *E. festucae* populations at the habitat where these plants were obtained (Arroyo *et al.*, 2002).

Seed germination in the presence of arsenic

Two different lines of *F. rubra* (SAN, PEN) were used. Each line had infected (E+) and endophyte-free (E−) seeds. Seeds were incubated under five different As concentrations: 0, 6, 12, 25 and 50 mg L^{-1}. Five mL of a solution of sodium arsenate in distilled water of each concentration was used to soak a germination paper disk (Filter Lab) placed inside a 9 cm Petri dish. Seeds were surface disinfected by immersion for 10 min in a solution of 50% domestic bleach (2·5% active chlorine) containing 0·01% Tween 80 and rinsed with sterile water. Then, thirty seeds of each plant line (SAN, PEN) and infection status (E+, E−) were placed in each Petri dish. The experiment consisted of a total of eighty Petri dishes, from the factorial combination of two lines (SAN, PEN), two infection types (E+, E−) and five As doses (0, 6, 12, 25, and 50 mg L^{-1}) with four replications. All the dishes were placed at random in a controlled environment chamber, at a constant temperature of 12°C, 60% relative humidity, and in dark. The temperature was chosen because we previously observed better germination rates at 12°C than at 25°C (Gundel *et al.*, 2011). Germinated seeds were counted daily until no more radicles emerged. At this point, 15 d after sowing, all the non-germinated seeds were transferred to new dishes containing germination paper soaked with 5 mL of distilled water and incubated as above. Seeds germinating in this new As-free condition after one week were considered as inhibited, and the remaining seeds were considered dead. Seed germination was estimated as the ratio of germinated seeds to all sown seeds. Seed mortality was estimated as the ratio of dead seeds to all seeds placed in each Petri dish. The proportion of inhibited seeds was estimated as the ratio of inhibited seeds to all live seeds (germinated + inhibited). Radicle length was measured at the end of the experiment, 15 d after sowing the dishes. All germinated seeds were scanned, and the length of their radicles was measured from the digital images using WinRhizo software (Régent Instruments Inc., Québec, Canada).

Data analyses

Differences among treatments in seed germination, seed mortality, seed inhibition, and radicle length were analysed using a three-way ANOVA. *Festuca rubra* line, endophyte infection, and As concentrations were considered as fixed factors. Prior to the statistical analyses, the normality of each data set was tested using a Kolmogorov–Smirnov test, in case a transformation was necessary. Differences between means after the ANOVA were made using the LSD procedure with a 0·05 significance level. Statistica 5.0 (StatSoft, Tulsa, OK, USA) software was used for the statistical calculations.

Results

Seed germination, mortality and inhibition

Seed germination and seed mortality were significantly affected by the As treatments and by a two-way interaction between plant line and endophyte infection status (Table 1). Only the As doses of 25 and 50 mg L^{-1} depressed germination (Figure 1). Seed

Table 1 Summary of ANOVA results of the effects of plant line (PEN and SAN), endophyte status (E+ and E−) and arsenic concentrations (0, 6, 12, 25, and 50 mg L^{-1}) on the percentage of seed germination, seed mortality, seed inhibition and radicle length of *Festuca rubra*. Bold type indicates statistically significant probabilities ($P < 0{\cdot}05$).

		Seed germination		Seed mortality		Seed inhibition		Radicle length	
Effect	**df**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Line (L)	1	9·66	**0·0029**	7·44	**0·0083**	3·45	0·0674	62·15	**0·0000**
Endophyte (E)	1	9·48	**0·0031**	23·60	**0·0000**	1·25	0·2674	18·30	**0·0000**
Arsenic (A)	4	10·77	**0·0000**	3·60	**0·0108**	12·41	**0·0000**	4·35	**0·0037**
L × E	1	6·23	**0·0153**	9·44	**0·0031**	0·21	0·6452	2·48	0·1203
L × A	4	1·28	0·2894	1·09	0·3699	1·18	0·3309	0·16	0·9581
E × A	4	1·22	0·3128	1·22	0·3132	0·33	0·8555	0·52	0·7197
L × E × A	4	1·96	0·1118	1·95	0·1130	0·80	0·5311	3·02	**0·0246**
Error	60								

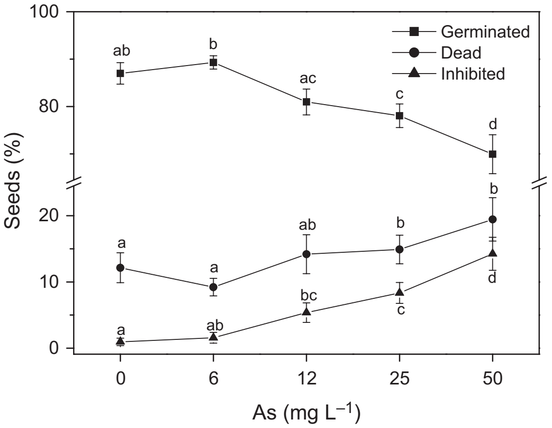

Figure 1 Inhibition of germination caused by increasing As concentrations. Seeds which germinated after being transferred to water at the end of the experiment were considered inhibited. Each data point is a mean ± s.e. of both *Festuca rubra* lines and infection types ($n = 16$). For each variable, means having the same letter in common are not significantly different ($P < 0{\cdot}05$).

mortality was only significantly affected by the 50 mg L^{-1} dose (Figure 1).

The statistically significant line × infection status interaction (Table 1) indicated that the endophyte effect depended on the plant–fungus combination, as differences in germination and mortality between E+ and E− seeds were significant only for the SAN seeds, not for the PEN ones (Figure 2). Mortality and germination rates were very similar for E− seeds of both lines, but the presence of the endophyte significantly depressed the germination and increased the mortality rate of SAN seeds.

Seed inhibition did not differ significantly between plant lines (mean percentage of inhibited seeds: PEN = 4·81 ± 0·93%; SAN = 7·39 ± 1·44%), or due to the infection status of seeds (E+ = 5·33 ± 1·33%; E− = 6·87 ± 1·11%). Seeds were significantly inhibited with As doses > 6 mg L^{-1} (Figure 1). The percentage of inhibited seeds increased with As concentration, and at 50 mg L^{-1}, the amount of inhibited seeds was about fourteen times greater than that of the control.

Radicle growth

The average length of the radicles, measured fifteen days after seed sowing, was significantly different between lines, endophyte infection status and As treatments. In addition, a significant three-way interaction (line × endophyte infection × As) affecting the radicle length was detected (Table 1, Figure 3). Radicles were longer in the PEN (9·65 ± 0·60 mm) than in the SAN line (5·10 ± 0·34 mm) and also were significantly longer in E+ (8·61 ± 0·65 mm) than in E− (6·14 ± 0·49 mm) seeds. Radicle length decreased with increasing As concentration, but the 6 mg L^{-1} dose did not differ significantly from the control. In the presence of As, the difference in length between E+ and E− radicles was more pronounced for the PEN than for the SAN line, but for both lines E+, seeds had longer radicles at most As levels (Figure 3). With the 50 mg L^{-1} dose, the difference between E+ and E− seeds was significant for the PEN, but not for the SAN line.

Discussion

Germination is the first crucial step for the establishment of a homogeneous stand of plants in a given

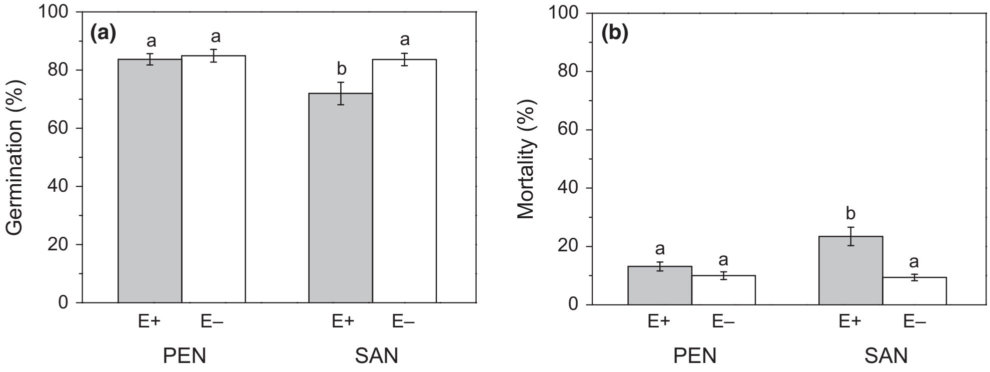

Figure 2 Interaction between infection status (E+ and E−) and *F. rubra* lines (PEN, SAN) on seed germination (a) and mortality (b). Values are means (± s.e.) of all the arsenic treatments ($n = 20$). In each graph, means sharing the same letter are not significantly different ($P < 0{\cdot}05$).

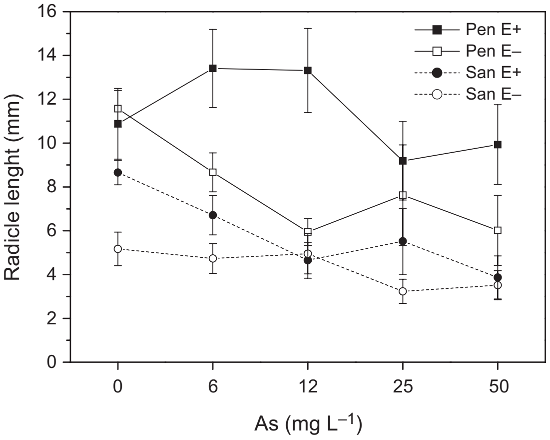

Figure 3 Radicle length in seedlings of two lines of *Festuca rubra* (PEN, SAN), infected or uninfected by *Epichloë festucae* (E+, E−), at five different concentrations of arsenic. Values are means ± s.e. (n = 4).

condition. In our work, we were interested in observing how the presence of As, an abiotic stress factor, affects the germination of two genotypes of *Festuca rubra* in the presence or absence of a symbiotic fungal endophyte in their seeds. The results of the experiments showed that the germination of *F. rubra* seeds decreased in the presence of As, but only when its concentration was > 12 mg L^{-1}. This decrease in germination due to As toxicity was expressed in two ways: as the death of some seeds and as the inhibition of germination in other seeds that could germinate when transferred to water after the arsenic treatment.

The grass–endophyte interaction is not an exception to the context-dependent outcomes often observed in symbiotic interactions (see Bronstein, 1994; Thompson, 2005). Endophyte symbiosis affected the germination of *F. rubra* in the presence of As, but only in PEN line. In this plant line, the presence of the endophyte had negative effects, decreasing germination and increasing mortality. A similar interaction between *Festuca rubra* genotype and endophytic symbiosis was observed in a previous study of germination under low water potential and supra-optimal temperature (Gundel *et al.*, 2011). In that system, as in the one here described, the presence of the endophyte was deleterious for some plant–endophyte combinations, but apparently neutral for others at the seed stage. A related epichloid endophyte (*Neotyphodium* sp.) was found to reduce the germination rate and total germination of *Lolium multiflorum* seeds, only under low water potentials but, at the same time, it promoted seed survival (Gundel *et al.*, 2006a,b). In contrast, epichloid endophytes have been reported to favour germination in *Lolium perenne* and *Festuca arundinacea* (Clay, 1987; Pinkerton *et al.*, 1990), and in conditions of water stress, the endophytic infection favoured germination in *Festuca sinensis* and *Elymus dahuricus* seeds (Zhang and Nan, 2010; Peng *et al.*, 2013). These observations suggest that epichloid endophytes can affect germination rates in their hosts, but particular characteristics of the endophyte, plant and environment are important determinants of the sign of this effect.

The variation in relation to species and host genotype for the plant tolerance to As is in accordance with other previous antecedents. The As sensitivity observed in *F. rubra* seeds was similar to that of wheat, whose germination in a system similar to ours was slightly reduced at a maximum dose of 20 mg L^{-1}, but not at lower concentrations (Li *et al.*, 2007). In contrast, some rice cultivars can be completely inhibited at concentrations of 8 mg L^{-1} (Abedin and Meharg, 2002). *Festuca rubra* seeds seem to have a relatively high tolerance to As at the germination stage because their germination rate was reduced only by 17% at the high As dose of 50 mg L^{-1}. A grass mixture containing seeds of this species showed a high germination rate and survival in As-contaminated soils where other plant species were inhibited (Zacarias *et al.*, 2012). Although lacking statistical significance, the lowest As dose (6 mg L^{-1}) tended to favour germination and decrease mortality. This stimulatory effect of a toxicant at low concentrations is known as hormesis, and it has been observed in other grasses like wheat (Li *et al.*, 2007; see also Kranner and Colville, 2011).

Root growth is a process very sensitive to the presence of As (Smith *et al.*, 2010). In rice seedlings, an As concentration of 8 mg L^{-1} caused a reduction of about 90% in the root length of all cultivars tested. This occurred even in cultivars whose germination rate was only slightly affected at this concentration (Abedin and Meharg, 2002). In wheat seedlings incubated with 20 mg L^{-1}, the 57% decrease observed in root length doubled the one that observed in shoot length (Li *et al.*, 2007). The root length data of our experiment indicated that the tolerance of As is relatively high in *F. rubra*, since seeds that germinated in 50 mg L^{-1} As had radicle lengths ranging between 10% and 36% smaller than those of the controls.

Although radicles of *F. rubra* were longer in PEN than in SAN seedlings, symbiotic seeds of both lines had radicles longer or equal to those of their non-symbiotic counterparts. Contrary to what was observed for germination, where the endophyte effect was negative, and only observed in one line, root growth was favoured by the presence of the endophyte in both *Festuca* lines at most As doses tested. Although only two lines were tested in our experiment, the results suggest that root growth can be improved by symbiosis with an endophyte. This improvement of radicle growth has also been observed when seeds of *Festuca sinensis* infected by a *Neotyphodium* endophyte were germinated in conditions of water stress (Peng *et al.*, 2013). *Epichloë* endophytes colonize aerial plant organs, but not roots (Schardl *et al.*, 2004). However, they have been found to affect plant biomass allocation, particularly a reduction in root biomass relative to aboveground biomass (Omacini *et al.*, 2012; Vázquez de Aldana *et al.*, 2013). In addition, *Epichloë* and related *Neotyphodium* endophytes are known to induce chemical changes in roots of their host plants, which produce greater amounts of secondary metabolites (i.e. phenolic compounds) than non-symbiotic plants (Malinowski *et al.*, 1998; Vázquez de Aldana *et al.*, 2011; Omacini *et al.*, 2012; Vázquez de Aldana *et al.*, 2013). The alterations that such compounds cause in the rhizosphere might be linked to the observed improvement of As tolerance in roots of E+ seedlings.

In plant roots, the high affinity phosphorus uptake system is induced by P deficiency. Both arsenate and phosphate are absorbed by this system. Therefore, the suppression of this uptake system results in a reduced As uptake and an increased tolerance to As in some plant species, although it might have consequences for plant nutrition unless other systems of P acquisition come into play (Marschner, 1995; Smith *et al.*, 2010). Mycorrhizal fungi can inhibit the high affinity phosphate/arsenate ports in roots of the grass *H. lanatus*, modifying As uptake and increasing the tolerance of host plants (González Chavez *et al.*, 2002). Whether similar modifications resulting in reduced As uptake occur in roots of symbiotic *F. rubra* seedlings is unknown, but such mechanisms could explain the observed increase in radicle length.

In conclusion, in the presence of As, endophyte infection was deleterious for the germination of one line of *F. rubra*, but apparently neutral for another line. In contrast, the endophyte infection increased root growth in both lines of *F. rubra*. Although only two different lines of *F. rubra* were tested in this work, the results indicate that endophytic symbioses could be used for the improvement of some characteristics of *F. rubra* cultivars used for reclamation of As-contaminated soils.

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