Symbiosis (2012) 58:73–80
DOI 10.1007/s13199-012-0194-y

# Antioxidants in *Festuca rubra* L. seeds affected by the fungal symbiont *Epichloë festucae*

**Pedro E. Gundel · Cyd E. Hamilton · Charlotte E. Seal · Marjo Helander · María Alejandra Martínez-Ghersa · Claudio M. Ghersa · Beatriz R. Vázquez de Aldana · Iñigo Zabalgogeazcoa · Kari Saikkonen**

Received: 16 August 2012 / Accepted: 15 October 2012 / Published online: 23 October 2012


**Abstract** Vertically transmitted fungal endophytes can be beneficial for host grasses. While the alkaloid-mediated mechanism for herbivore resistance has been widely studied, underlying physiological mechanisms for increased tolerance to abiotic stress remain scarcely explored. In this study we used three maternal lines of perennial grass *Festuca rubra* to examine the role of antioxidants in endophyte-mediated effects on seed viability over long-term storage. Uncolonized plants (E−) were generated by removing the endophyte from ramets of naturally endophyte-colonized (E+) plants. The E+ and E− ramets were planted in a common garden in Salamanca, Spain. Seeds produced in 2009, 2010 and 2011 were harvested at maturity, dried and stored at 10 °C until 2011 when we tested seed and endophyte viability, and measured antioxidants. Seed viability and α-tocopherol antioxidant were negatively affected by the endophyte in two maternal lines. In these same lines, the endophyte viability was lowest at the longest storage time. In the maternal line that showed the highest negative effect of endophyte on seed viability, the pattern of glutathione was opposite to that observed for tocopherols since it was higher for E+ than for E− seeds. In all maternal lines, the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) and % glutathione disulphide (GSSG) increased with storage time but there was no clear pattern associated with endophyte symbiosis. Whether these parameters are good predictors of seed and endophyte longevity in storage and natural conditions should be further explored.

**Keywords** Symbiosis · Systemic endophytes · Red fescue · Cellular redox environment

**Electronic supplementary material** The online version of this article (doi:10.1007/s13199-012-0194-y) contains supplementary material, which is available to authorized users.

P. E. Gundel (✉) · C. E. Hamilton · K. Saikkonen
MTT Agrifood Research Finland, Plant Production Research,
31600 Jokioinen, Finland
e-mail: gundel@agro.uba.ar

P. E. Gundel · M. A. Martínez-Ghersa · C. M. Ghersa
IFEVA - CONICET – Faculty of Agronomy,
Buenos Aires University (UBA),
Av. San Martín 4453 (C1417DSE),
Buenos Aires, Argentina

C. E. Seal
Seed Conservation Department, Royal Botanic Gardens,
Kew, Wakehurst Place,
West Sussex RH17 6TN, UK

B. R. Vázquez de Aldana · I. Zabalgogeazcoa
Instituto de Recursos Naturales y Agrobiología, CSIC. Apartado,
Salamanca, Spain

M. Helander
Section of Ecology, Department of Biology, University of Turku,
20014 Turku, Finland

## 1 Introduction

Many plant species have extended their ecological niche through symbiosis with microorganisms, often acquiring new capabilities such as protection against enemies and tolerance to abiotic stresses (Clay and Schardl 2002; Rodriguez and Redman 2005; Rudgers et al. 2009; Harman 2011). Particularly interesting is the case in which grasses associate with asymptomatic fungal endophytes which are vertically transmitted through host seeds. The symbiosis between Pooideae grasses and epichloae fungal endophytes is widespread in a variety of environments ranging from arid/semiarid to cool-temperate humid grasslands and as it is facultative for plants but obligate for the endophytes (Clay and Schardl 2002;

Saikkonen et al. 2004; Schardl et al. 2007; Rudgers et al. 2009), simple population models predict a positive net effect on host grasses for the symbiosis to persist (Saikkonen et al. 2002; Gundel et al. 2008).

As seed-transmitted microorganisms, special attention has been paid to the effect of the plant-endophyte symbiosis at the host seed and seedling stages. Basically, two types of approaches can be found addressing this issue: experiments that evaluate seed germination and seedling growth, and storage experiments that evaluate seed and endophyte viability. Results have been variable demonstrating the complexity of the symbiosis between endophytes and their grass hosts, and their dependence on environmental conditions (Saikkonen et al. 2006, 2010a). For example, Wäli et al. (2009) found that the effects of the endophyte colonization on seed germination and seedling stage of *Festuca rubra* and *F. ovina* in sub-arctic Finland was interactively dependent on the species of grass, host genetic background and/or mother plant habitat. They suggested that these traits were adaptive to the habitat specific conditions leading to the detected variable outcomes in grass–endophyte interactions (Wäli et al. 2007). Gundel et al. (2006) demonstrated that although the colonization with *Neotyphodium occultans* inhibited seed germination in *Lolium multiflorum* under low water potential, it improved seed survival. Similarly the germination of *F. rubra* seeds colonized by *Epichloë festucae* was inhibited more than their uncolonized counterparts at high temperature combined with lower water potential but seeds had higher survival (Gundel et al. 2011).

Results from storage experiments have consistently shown that fungal endophytes lose viability faster than seed hosts (Rolston et al. 1986; Welty et al. 1987; Wheatley et al. 2007; Gundel et al. 2009, 2010, 2012; Hill and Roach 2009). In addition, the association of endophyte-colonization with reduced seed longevity in some host genotypes of *L. multiflorum* has been proposed to be driven by changes in seed water content (Gundel et al. 2009, 2010, 2012). Post-maturation conditions (predominantly temperature and water content) have a profound effect on the decline in seed germination rate and maximum percentage of germination, and this may vary depending on the species, population, prevailing growth conditions and the presence of symbionts (Walters et al. 2005; Gundel et al. 2009, 2010, 2012; Seal et al. 2010). Even in dry seeds, several products of oxidative stress (ROS: reactive oxygen species) can positively accumulate with ageing time, causing damage to macromolecules like nucleic acids, proteins and lipids, affecting membrane integrity and functioning (Bailly 2004; Kranner et al. 2010). Nonetheless, ROS production is controlled at least to a certain point, by enzymatic scavengers and antioxidants such as glutathione (γ-glutamyl-cysteinyl-glycine; GSH), tocopherols and carotenoids (Bailly 2004; Kranner et al. 2010; Seal et al. 2010). Although dependent on the species, population and growth/storage conditions, losses in seed viability can be correlated with decreases in GSH and α-tocopherol concentrations, and changes in the intracellular redox state. In particular, the concentration-dependent redox couple glutathione disulphide (GSSG)/GSH, in which GSH donates electrons to detoxify free radicals forming GSSG before re-reduction back to GSH by the enzyme glutathione reductase, can be accurately defined by the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) which increases to more oxidising values as seed viability is lost (Kranner et al. 2006; Seal et al. 2010). As a membrane-bound antioxidant, α-tocopherol is linked to another typical symptom found in aged seeds, an increment of solute leakage (Tammela et al. 2005; Seal et al. 2010). Ultimately, death of imbibed seeds can occur as a result of ageing and increased susceptibility to latent pathogenic fungi.

Recently, it has been proposed an improved antioxidant machinery may be the reason for the higher stress tolerance exhibited by symbiotic organisms compared to non-symbiotic counterparts; a hypothesis that has been proposed for positive symbiotic interactions in general (Rodriguez & Redman 2005) and for the grass-endophyte system in particular (White & Torres 2010; Hamilton et al. 2012). In this article, we explored the pattern in the antioxidants, α-tocopherol, α-tocotrienol, and glutathione in relation to the endophytic symbiosis in seeds of the perennial grass *Festuca rubra* (red fescue). The dynamics of these antioxidant parameters have been found to be reliable descriptors of seed quality (see Seal et al. 2010). Given the symbiotic outcome is highly controlled by the interaction between the partners' genotype and environmental conditions (Ahlholm et al. 2002; Gundel et al. 2010, 2011; Saikkonen et al. 2010b; Wäli et al. 2007, 2009), our experimental design consisted of seeds from three maternal lines of the host grass with colonized (E+) and uncolonized (E−) counterparts produced under common garden conditions during three consecutive years. Since evaluations of seed viability, endophyte viability and antioxidants were performed in 2011, the seeds produced in 2009, 2010 and 2011 had 24, 12 and 1 months of storage time, respectively. We are aware that our storage time can be affected by the variation among years in the productive environmental conditions; however, our study provides evidence about the proposed underlying antioxidant mechanisms of the effect of fungal endophyte on host seed eco-physiology.

## 2 Material and methods

Three maternal lineages of *Festuca rubra* (RAB, SAN, and PEN), each consisting of endophyte colonized (E+) and endophyte uncolonized (E−) plants, were used for the experiment. Three mother plants of *F. rubra* naturally colonized with the fungus *Epichloë festucae* were collected from different locations (40 km apart) in semiarid grasslands

(dehesas) of the province of Salamanca, Spain. Each plant was divided into six ramets, and half of them were treated with a systemic fungicide to remove the fungus. After verifying their colonization status, untreated (E+) and fungicide treated (E−) ramets were transplanted to soil in the research farm in Salamanca. Seeds of the experimental plants were harvested in 2009, 2010 and 2011. The threshed seeds were pooled per maternal line and colonization status. Colonization status of seed lots was confirmed by checking for the endophyte presence in seed under light microscope (Saha et al. 1988; see also Gundel et al. 2011). The seeds were air-dried and stored at 10 °C. At the time of the seed and endophyte viability experiments and antioxidant analysis, the seeds had been stored at 10 °C for 24, 12 and 1 month.

### 2.1 Viability experiment

Seed viability was determined by incubating seeds under optimal germination conditions (ISTA 1999). Four replicates of forty seeds per population were sown on filter paper in Petri dishes (9 mm diameter) moistened with 5 ml of distilled water. The Petri dishes were placed in a growth chamber set to cycle alternating temperatures of 12/15 °C in synchrony with night/day light cycles (180±10 $\mu mol m^{-2} s^{-1}$). The Petri dishes were sealed with a plastic film to prevent drying, and their positions in the chamber were randomized. Three different categories of seeds were recorded every 3 days: germinated seeds (hence viable), dead seeds (soft and moldy aspect), and non-germinated seeds (but apparently healthy). Germinated and dead seeds were removed from the dish once they were identified. The counting continued until there was no further germination.

Endophyte viability was estimated on 1-month-old seedlings using the technique by Saha et al. (1988) with minor changes. All the germinated seeds per dish were transplanted to one pot filled with soil and peat (50/50) and kept in a greenhouse until they were evaluated. Of each seedling, the first 5 mm of the base was severed and incubated for≈14 h in NaOH (5 %) to soften the tissues. After that, they were carefully spread on a glass slide and dyed with two drops of aniline blue, squashed, set aside for 10 min and covered with a coverglass and the endophyte examined under light microscope (20×, and 40×).

### 2.2 Antioxidant measurements

For determination of antioxidants, freeze-dried seeds from each combination of maternal line, endophyte colonization status, and storage time were thawed over silica gel and ground to a fine powder using a micro-dismembrator (Retsch MM200, Germany) where the grinding capsule was frozen in liquid nitrogen. α-Tocopherol and α-tocotrienol were determined following a modified procedure of Bagci et al. (2004). Three replicates of 5 mg of ground material were first extracted in 1 ml heptane (HPLC-grade, Fisher, UK) and centrifuged at 13,000 g and 4 °C for 20 min, and then, re-suspended in 1 ml heptane and centrifuged as before. The supernatants from the two centrifugation steps were combined and centrifuged prior to normal-phased high performance liquid chromatography (HPLC) analysis (Jasco, Great Dunmow, Essex, UK). α-Tocopherol and α-tocotrienol were separated on a Supelcosil LC-Diol column (Supelco Analytical, Sigma-Aldrich, Bellefonte, USA) of 250 mm length, 4.6 mm internal diameter and 5 μm particle size with heptane: tert-butyl methyl ether (97.5 % : 2.5 % (v/v)) as a mobile phase at a flow rate of 1 $ml min^{-1}$. The compounds were detected with a fluorescence detector (excitation: 295 nm; emission: 325 nm) and quantified using standards (Sigma Aldrich, Poole, Dorset, UK) prepared in heptane.

For glutathione determination, three replicates of 30 mg ground seeds were extracted in 1 ml of 0.1 M HCl with 0.5 % (v/v) Triton X-100 and 30 mg polyvinylpolypyrrolidone and centrifuged as before for 40 min. The pellet was re-suspended in 1 ml 0.1 M HCl with 0.5 % (v/v) Triton X-100 and centrifuged for a further 40 min. The supernatants from the two centrifugation steps were combined and centrifuged before determining GSH and GSSG following the procedure described in Kranner and Grill (1996). Thiols of low-molecular-weight were separated by reversed-phase HPLC on an HiQsil RP18 column (150×2.1 mm i.d., 3 μm particle size; KyaTech), and detected fluorimetrically (excitation: 380 nm; emission: 480 nm) with a gradient elution of 0.25 % (v/v) acetic acid in distilled water at pH 3.9/methanol. Glutathione was separated from other low-molecular-weight thiols cysteine, cysteinyl-glycine and γ-glutamyl-cysteinyl. Standards of these low-molecular-weight thiols (Sigma Aldrich, Poole, Dorset, UK) at different concentrations were prepared to construct calibration curves. Calculation of $E_{GSSG/2GSH}$ followed the formulas given in Schafer and Buettner (2001) and Kranner et al. (2006) using the Nernst equation:

$$E_{GSSG/2GSH} = E^{0'} - \frac{RT}{nF} \ln \frac{[GSH]^2}{[GSSG]}$$

where R is the gas constant (8.314 $JK^{-1} mol^{-1}$); T is temperature in K; n is number of transferred electrons; F is the Faraday constant ($9.6485 \times 10^4$ $Cmol^{-1}$); $E^{0'}$ is the standard half-cell reduction potential at pH 7 [$E^{0'}_{GSSG/2GSH} = -240$ mV]; [GSH] and [GSSG] are molar concentrations of GSH and GSSG, estimated using seed WCs. The density of water, approximated as 1 $g ml^{-1}$, and the amount of water per gram of seed were used in the calculations of molar concentrations of GSH and GSSG.

### 2.3 Statistical analysis

The variables maternal line (RAB, SAN and PEN), endophyte colonization status (E+ and E−) and storage time (24, 12 and 1 month) were considered as fixed explanatory variables. All analyses for proportion of germinated seeds, proportion of endophyte-colonized seedlings as a surrogate of seed and endophyte viability, and antioxidants were analyzed using the GLIMMIX procedure (SAS/STAT 9.2, The GLIMMIX Procedure 2008), with type III fixed effects, and degrees of freedom corrected via a Satterthwaite correction. The GLIMMIX procedure accommodates squared contrasts present among different treatment means (including variance components) when models include both random and fixed effects and when the data set is balanced (Satterthwaite 1946), as in these analyses. Additionally, the GLIMMIX procedure fits statistical models to data sets which produce non Gaussian distributions and because it includes random effects specific to both subject and population averages, variable variances are accommodated. Note, the antioxidant measured were: α-tocopherol, α-tocotrienol, total glutathione (GSH+GSSG), percentage of GSSG and $E_{GSSG/2GSH}$. Proportion of seed and endophyte viability were arc-sine transformed for getting normality and homogeneity of variances. Tukey's tests ($P<0.05$) were performed to highlight the differences between E+ and E− within maternal lines.

## 3 Results

Seed viability depended on the three-way interaction among endophyte colonization status, maternal line and storage time ($F_{4,36}=18.12$, $P<0.001$). However, the expected tendency for gradual decreasing of seed viability along with the prolonged storage time was not observed for any of the seed groups (See table 2, Supplementary material). Nonetheless, the endophytic colonization was associated with a negative effect on seed germination in the maternal lines RAB (E+: 0.66 vs E−: 0.86) and SAN (E+: 0.87 vs E−: 0.96) but not in PEN (≈ 0.95) (Fig. 1a).

Endophyte viability interactively depended on maternal line and storage time ($F_{4,16}=10.10$, $P=0.003$). The negative relationship between the proportion of viable endophyte and time of storage was only apparent for RAB and SAN but not for PEN maternal line (Fig. 2).

Contents of α-tocopherols and α-tocotrienol were affected by the three-way interaction among endophyte colonization status, maternal line and time of storage ($F_{4,24}=17.90$, $P<0.001$). However, there was not any clear pattern of association between tocopherol content with storage time (data not shown). Only for the RAB maternal line did endophyte colonized seeds show consistently lower levels of both α-tocopherols (E+: 46.99 nmol $gDW^{-1}$ vs E−: 101.72 nmol $gDW^{-1}$) and α-tocotrienol (E+: 60.46 nmol $gDW^{-1}$ vs E−: 127.35 nmol $gDW^{-1}$) antioxidants than endophyte uncolonized seeds across all years (Fig. 1b and c). No clear effect of endophyte colonization status was observed in the tocopherol contents of the other two maternal lines (Fig. 1b and c).

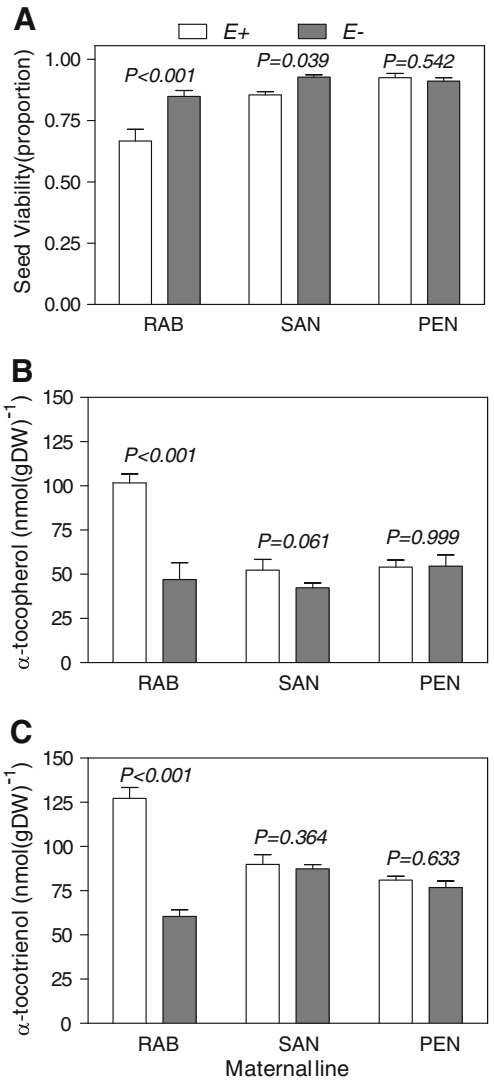


**Fig. 1** Proportion of viable seeds (panel **a**), and concentrations of α-tocopherol (panel **b**) and α-tocotrienol (panel **c**) of endophyte-colonized (E+: *white bars*) and uncolonized (E−: *dark bars*) seeds for three maternal lines (RAB, SAN and PEN) of the grass *Festuca rubra*. The seeds were produced in a common garden (Salamanca, Spain) during 3 years (2009, 2010 and 2011) and stored dry at 10 °C until the experiment in 2011, representing three storage times (24, 12 and 1 months). Values are means±SEM ($n=12$ for seed viability and $n=9$ for tocopherols). *P*-values of Tukey test are shown to highlight the differences between E+ and E− within each maternal line

Total glutathione (GSH+GSSG) in seeds was found to be dependent on the three-way interaction among endophyte colonization status, maternal line and storage time ($F_{4,24}=18.98$, $P<0.001$). However, there was a clear pattern of having low

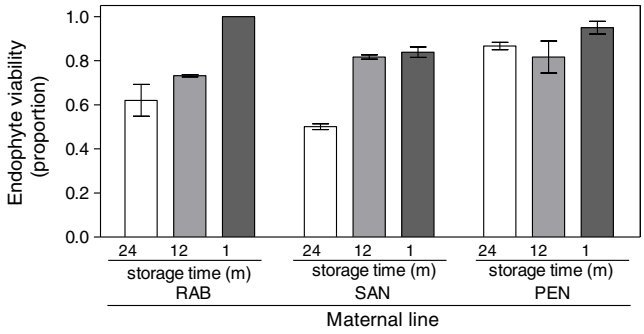


**Fig. 2** Proportion of seedlings colonized by the fungal endophyte *Epichloë festucae* (endophyte viability) for the three maternal lines (RAB, SAN and PEN) of the grass *Festuca rubra*. The seeds were produced in a common garden (Salamanca, Spain) during 3 years (2009, 2010 and 2011) and stored dry at 10 °C until the experiment in 2011, representing three storage times (24, 12 and 1 months). Values are means±SEM ($n$=3)

(1633.39 nmol $gDW^{-1}$) and high (2911.46 nmol $gDW^{-1}$) concentrations of total glutathione in seeds stored for 24 and 1 month, respectively (Fig. 3). This pattern matched with the variation in %GSSG (Table 1). In general, the $E_{GSSG/2GSH}$ followed an opposite pattern to total glutathione (Fig. 3), and was also found to be dependent on the three-way interaction among endophyte colonization, maternal line and time of storage ($F_{4,36}$=20.86, $P$<0.001). Except for SAN E− and PEN E+ which showed a low value in 2010, $E_{GSSG/2GSH}$ was the highest in seeds stored for 24 months (−169.53 mV), and the lowest in seeds stored for 1 months (−190.62 mV) (Fig. 3).

## 4 Discussion

The relative rate of viability loss between the seed and endophyte (i.e. comparing viability of seeds and the proportion of seedlings colonized by endophyte) has been well studied, mainly in *Schedonorus phoenix* (tall fescue), *Lolium perenne* (perennial ryegrass) and *L. multiflorum* (Italian ryegrass) (Rolston et al. 1986; Welty et al. 1987; Wheatley et al. 2007; Gundel et al. 2009; Hill and Roach 2009). However, the effects of endophyte on the rate of seed viability loss (i.e. comparing viability of colonized vs uncolonized seeds) has been scarcely studied and even less in wild species, like *F. rubra* (Gundel et al. 2009, 2010, 2012). The interactive effect of maternal line, endophyte colonization status, and storage time is in agreement with the general pattern of higher variability in the response of wild populations to the endophyte symbiosis when compared to cultivars (Saikkonen et al. 2004, 2006). Endophyte colonization was negatively associated with seed viability in the maternal lines RAB and SAN where interestingly, endophyte viability was found to be slightly lower as seed age or storage period increased. For a variety of environmental conditions [combining temperatures (5 to 40 °C) and relative humidity (5–75 %)] and host species, it has been observed that endophytes lose viability before seeds under storage (Rolston et al. 1986; Welty et al. 1987; Wheatley et al. 2007; Gundel et al. 2009; Hill and Roach 2009). The negative effect of the endophyte on seed longevity under storage has been previously observed in the grass-endophyte system, *L. multiflorum* and *N. occultans*, and as was found here, this effect depended on the host population (Gundel et al. 2009, 2010, 2012). In previous research we suggested that the negative interaction could be explained by the fungal mycelium altering the water content in the embryo environment thereby increasing seed ageing rate. Our present results suggest that the negative effect of endophyte may be also related to a reduction in a particular antioxidant (e.g. α-tocopherol). This pattern observed under storage may greatly contrast with what is observed in freshly dispersed, imbibed seeds and/or ecological conditions. Highly controlled experiments have shown the endophytes can increase germination requirements of the host while simultaneously improving seed survival (Gundel et al. 2006; Gundel et al. 2011). Consequently, it is important to consider the endophyte

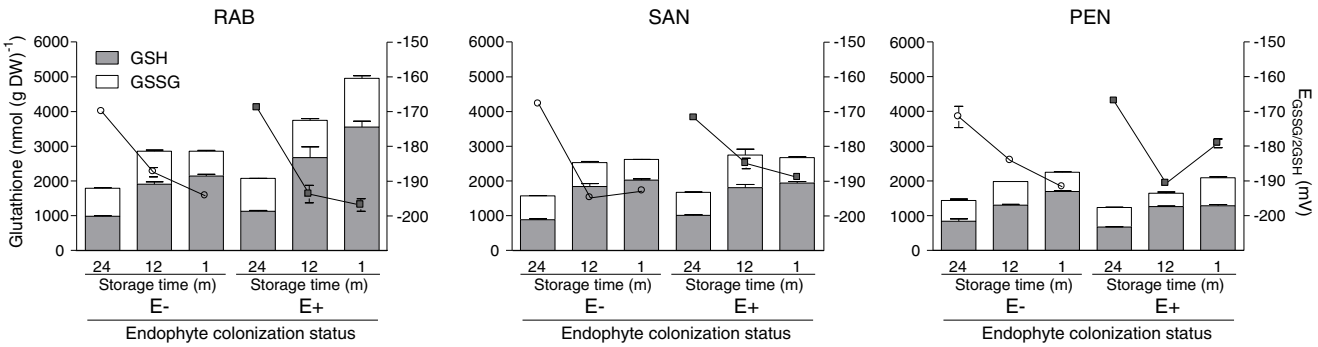


**Fig. 3** Concentrations of glutathione (GSH; *dark bar part*), glutathione disulphide (GSSG; *white bar part*) and the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$; black square and open circles symbols for endophyte-colonized (E+) and uncolonized (E−) by the fungal endophyte *Epichloë festucae*, respectively) in seeds of the three maternal lines (RAB, SAN and PEN) of the grass *Festuca rubra*. The seeds were produced in a common garden (Salamanca, Spain) during 3 years (2009, 2010 and 2011) and stored dry at 10 °C until the experiment in 2011, representing three storage times (24, 12 and 1 months). Values are means±SEM ($n$=3)

**Table 1** Percentage of glutathione disulphide (%GSSG) in seeds of the three maternal lines (RAB, SAN and PEN) of the grass *Festuca rubra*, endophyte-colonized (E+) and uncolonized (E+) by the fungal endophyte *Epichloë festucae*. The seeds were produced in a common garden (Salamanca, Spain) during three years (2009, 2010 and 2011) and stored dry at 10 °C until the experiment in 2011, representing three storage times (24, 12 and 1 months). Values are means±SEM ($n$=3)

| Maternal line | Endophyte colonization status | Storage time (m) | %GSSG |
|---|---|---|---|
| RAB | E− | 24 | 44.79 (0.41) |
| | | 12 | 33.20 (1.44) |
| | | 1 | 25.08 (0.38) |
| | E+ | 24 | 45.64 (0.46) |
| | | 12 | 28.97 (1.78) |
| | | 1 | 28.42 (1.93) |
| SEN | E− | 24 | 43.54 (0.64) |
| | | 12 | 27.25 (0.84) |
| | | 1 | 22.61 (0.19) |
| | E+ | 24 | 39.56 (0.86) |
| | | 12 | 33.69 (3.00) |
| | | 1 | 27.30 (0.23) |
| PEN | E− | 24 | 41.48 (3.68) |
| | | 12 | 33.98 (0.33) |
| | | 1 | 24.44 (0.28) |
| | E+ | 24 | 45.34 (0.55) |
| | | 12 | 23.71 (0.98) |
| | | 1 | 38.66 (1.48) |

effect on the grass-endophyte system within an environmental context where they will be used. For example, the eventual negative effect of the endophyte in decreasing seed longevity may be a problem for breeders and seed companies which can be even more critical if non-toxic endophyte strains in the cultivar is considered as a commercial added value (Hill and Roach 2009; Gundel et al. 2012). In nature these host grass species usually form transient seed banks, and the positive endophyte effect on seed dormancy, alkaloid-mediated anti-herbivory, and antioxidant-mediated resistance to stress may have an important impact on seed fitness under diverse ecological scenarios (Ghersa and Martínez-Ghersa 2000; Wäli et al. 2007, 2009; Zhang and Nan 2010; Gundel et al. 2012; Hamilton and Bauerle 2012; Hamilton et al. 2012).

Due to the variable geographic incidence of the symbiosis in wild grass populations (Saikkonen et al. 2000; Wäli et al. 2007), the identification of the underlying mechanisms maintaining the symbiosis remains puzzling (Gundel et al. 2008; Rudgers et al. 2009; Saikkonen et al. 2010a). In fact, the well-documented anti-herbivore role of fungal alkaloids has not proven to be ecologically straightforward (Bush et al. 1997; Faeth 2002; Schardl et al. 2007; Saikkonen et al. 2010a). Recently, a new line of research is focusing on the potential role of antioxidants as a basic mechanism by which the endophyte symbiont could protect the host plants against stress (White and Torres 2010; Zhang and Nan 2010; Singh et al. 2011; Hamilton and Bauerle 2012; Hamilton et al. 2012). Along with ROS scavenging enzymes, antioxidants are critical to the maintenance of ROS produced as a result of biotic and abiotic stresses, by ensuring ROS levels do not become deleterious to cellular function (Kranner et al. 2010). Antioxidants such as the lipid-soluble and preferentially membrane-bound tocochromanols and glutathione, the major intracellular water-soluble antioxidant, have previously been identified as key components of protection against oxidative stress (Noctor and Foyer 1998; Kranner et al. 2010; Seal et al. 2010). In the maternal lines RAB and SAN where endophyte colonization was associated negatively with seed viability, a significant decrease in α-tocopherol concentration and α-tocotrienol (only in RAB line) was observed. α-Tocopherol has previously been shown to positively correlate with seed viability in many species (Seal et al. 2010 and references therein), protecting against non-enzymatic lipid peroxidation, thereby maintaining membrane integrity and cellular function in seeds during both storage and germination (Sattler et al. 2004). In contrast, the PEN maternal line did not show symptoms of decay since seed viability, endophyte viability and α-tocopherol and α-tocotrienol concentrations remained high and unaffected by the endophyte colonization. In summary, although highly dependent on the interaction between the colonization status and host genotype, there was for certain maternal lines a relationship between parameters of seed quality, the endophyte itself and the content of the tocochromanols.

Alternatively, while there was no clear association between endophyte colonization and total glutathione (GSH + GSSG) concentration, results of percentage of GSSG, or $E_{GSSG/2GSH}$ did match the expected temporal variation according to time of storage (Seal et al. 2010). Even though there was no evidence of seed viability decay and storage time, the changes in the redox state of seeds showed ageing symptoms at the biochemical level. This suggests that endophyte colonization does not influence the glutathione redox state when seed viability was maintained at high levels. But seed viability loss and eventually seed death will occur once cells at a critical location or in a critical number die, and central to the cellular signaling network are changes in the cellular redox state (Kranner et al. 2010). Therefore, further investigation is needed to determine the changes in the glutathione redox state from endophyte colonization of seeds at lower viabilities.

The relative role and impact of antioxidants and fungal alkaloids as currencies of the mutualistic symbiosis between grasses and systemic fungal endophytes (Schardl et al. 2007; White and Torres 2010; Hamilton and Bauerle 2012; Hamilton et al. 2012) remain unanswered. While different alkaloids are specific or active from the perspective of specific

herbivores (Bush et al. 1997; Schardl et al. 2007), antioxidants appear to be part of a more generalist strategy in the face of oxidative stress. Besides, it is well known alkaloids are synthesized by the endophyte (Schardl et al. 2007) but it is unclear whether the antioxidants are a by-product of the symbiotic interaction or if they are synthesized by the plant or by the fungus in the symbiotum. As secondary compounds, antioxidants and alkaloids are not free of energetic costs and the likely trade-off may explain the observed variability in the endophyte effects on host grass herbivore resistance among populations as a result of local selection forces operating on host or endophyte populations separately or on both organisms involved as a symbiotic unit. Future assessments and experiments should address whether there is a relationship between antioxidant and fungal alkaloids levels in plants at different life stages including seeds, as well as the ecological role of each type of compound.

**Acknowledgments** This collaborative project has been funded by the Academy of Finland grant 137909 (MTT: 21030085), Turku University Foundation, and PICT 2010-1573 from Argentina. The Royal Botanic Gardens, Kew, receive grant-in-aid from DEFRA. We are grateful to Tiina Piltti for her technical assistance during the viability experiment.

## References

Ahlholm JU, Helander M, Lehtimäki S, Wäli P, Saikkonen K (2002) Vertically transmitted endophytes: effects of environmental conditions. Oikos 99:173–183

Bagci E, Bruehl L, Ozcelik H, Aitzetmuller K, Vural M, Sahim A (2004) A study of the fatty acid and tocochromanol patterns of some Fabaceae (Leguminosae) plants from Turkey I. Grasas y Aceites 55:378–384

Bailly C (2004) Active oxygen species and antioxidants in seed biology. Seed Sci Res 14:93–107

Bush LP, Wilkinson HH, Schardl C (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol 114:1–7

Clay K, Schardl C (2002) Evolutionary origin and ecological consequences of endophyte symbiosis with grasses. Am Nat 160:S99–S127

Faeth SH (2002) Are endophytic fungi defensive plant mutualists? Oikos 98:25–36

Ghersa CM, Martínez-Ghersa MA (2000) Ecological correlates of weed seed size and persistence in the soil under different tilling systems: implications for weed management. Field Crops Res 67:141–148

Gundel PE, Maseda PH, Vila-Aiub MM, Ghersa CM, Benech-Arnold RL (2006) *Neotyphodium* fungi effects on *Lolium multiflorum* seed germination in relation to water availability. Ann Bot 97:571–577

Gundel PE, Batista WB, Texeira M, Martínez-Ghersa MA, Omacini M, Ghersa CM (2008) *Neotyphodium* endophyte infection frequency in annual grass populations: relative importance of mutualism and transmission efficiency. Proc Roy Soc Lond B 275:897–905

Gundel PE, Martínez-Ghersa MA, Garibaldi LA, Ghersa CM (2009) Viability of *Neotyphodium* endophytic fungus and endophyte-infected and non-infected *Lolium multiflorum* seeds. Botany (ex Canadian Journal of Botany) 87(1):88–96

Gundel PE, Martínez-Ghersa MA, Batista WB, Ghersa CM (2010) Dynamics of *Neotyphodium* endophyte infection in ageing seed pools: incidence of differential viability loss of endophyte, infected seed, and non-infected seed. Ann Appl Biol 156:199–209

Gundel PE, Zabalgogeazcoa I, Vazquez de Aldana BR (2011) Interaction between plant genotype and the symbiosis with *Epichloë* fungal endophytes in seeds of red fescue (*Festuca rubra*). Crop & Pasture Science 62:1010–1016

Gundel PE, Martínez-Ghersa MA, Ghersa CM (2012) Threshold modelling *Lolium multiflorum* seed germination: effect of *Neotyphodium* endophyte infection and storage environment. Seed Sci Tech 40:51–62

Hamilton CE, Bauerle TL (2012) A new currency for mutualism? Fungal endophytes alter antioxidant activity in hosts responding to drought. Fungal Divers 54:39–49

Hamilton CE, Gundel PE, Helander M, Saikkonen K (2012) Endophytic mediation of reactive oxygen species and antioxidant activity in plants: a review. Fungal Divers 54:1–10

Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol 189:647–649

Hill NS, Roach PK (2009) Endophyte survival during seed storage: endophyte–host interactions and heritability. Crop Sci 49:1425–1430

ISTA (1999) International rules for seed testing. Seed Sci Tech 27 (Suppl):1–333

Kranner I, Grill D (1996) Determination of glutathione and glutathione disulfide in lichens: a comparison of frequently used methods. Phytochem Anal 7:24–28

Kranner I, Birtic S, Anderson KM, Pritchard HW (2006) Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? Free Radic Biol Med 40:2155–2165

Kranner I, Minibayeva FV, Beckett RP, Seal CE (2010) What is stress? Concepts, definitions and applications in seed science. New Phytol 188:655–673

Noctor G, Foyer CH (1998) Ascorbate and glutathione: Keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279

Rodriguez R, Redman R (2005) Balancing the generation and elimination of reactive oxygen species. Proc Natl Acad Sci 102 (9):3175–3176

Rolston MP, Hare MD, Moore KK, Christensen MJ (1986) Viability of *Lolium* endophyte fungus in seed stored at different seed moisture contents and temperature. N Z J Exp Agric 14:297–300

Rudgers JA, Afkhami ME, Rua MA, Davitt AJ, Hammer S, Huguet VM (2009) A fungus among us: broad patterns of endophyte distribution in the grasses. Ecology 90:1531–1539

Saha DC, Jackson MA, Johonson-Cicalese JM (1988) A rapid staining method for detection of endophytic fungi in turf and forage grasses. Phytopathology 78:237–239

Saikkonen K, Ahlholm J, Helander M, Lehtimäki S, Niemeläinen O (2000) Endophytic fungi in wild and cultivated grasses in Finland. Ecography 23:346–352

Saikkonen K, Ion D, Gyllenberg M (2002) The persistence of vertically transmitted fungi in grass metapopulations. Proc Roy Soc Lond B 269:1397–1403

Saikkonen K, Wäli P, Helander M, Faeth SH (2004) Evolution of endophyte-plant symbioses. Trends Plant Sci 9:275–280

Saikkonen K, Lehtonen P, Helander M, Koricheva J, Faeth SH (2006) Model systems in ecology: dissecting the endophyte-grass literature. Trends Plant Sci 11:428–433

Saikkonen K, Saari S, Helander M (2010a) Defensive mutualism between plants and endophytic fungi? Fungal Divers 41:101–113

Saikkonen K, Wäli PR, Helander M (2010b) Genetic compatibility determines endophyte-grass combinations. PLoS One 5(6):e11395

SAS Institute Inc (2008) SAS/STAT® 9.2 User's Guide. SAS Institute Inc, Cary

Satterthwaite FE (1946) An approximate distribution of estimates of variance components. Biom Bull 2(6):110–114

Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, DellaPenna D (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. Plant Cell 16:1419–1432

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulphide/glutathione couple. Free Radical Biology and Medicine 30:1191–1212.

Schardl CL, Grossman RB, Nagabhyru P, Faulkner JR, Mallik UP (2007) Loline alkaloids: currencies of mutualism. Phytochemistry 68:980–996

Seal CE, Zammit R, Scott P, Flowers TJ, Kranner I (2010) Glutathione half-cell reduction potential and a-tocopherol as viability markers during the prolonged storage of *Suaeda maritima* seeds. Seed Sci Res 20:47–53

Singh LP, Gill SS, Tuteja N (2011) Unraveling the role of fungal symbionts in plant abiotic stress tolerance. Plant Signaling Behavior 6(2):175–191

Tammela P, Salo-V n nen P, Laakso I, Hopia A, Vuorela H, Nygren M (2005) Tocopherols, tocotrienols and fatty acids as indicators of natural ageing in Pinus sylvestris seeds. Scand J For Res 20:378–384

Wäli PR, Ahlholm J, Helander M, Saikkonen K (2007) Occurrence and genetic structure of the systemic grass endophyte Epichloë festucae in fine fescue populations. Microb Ecol 53:20–29

Wäli PR, Helander M, Saloniemi I, Ahlholm J, Saikkonen K (2009) Variable effects of endophytic fungus on seedling establishment of fine fescues. Oecologia 159:49–57

Walters C, Hill LM, Wheeler LJ (2005) Dying while dry: kinetics and mechanisms of deterioration on desiccated organisms. Integr Comp Biol 45:751–758

Welty RE, Azevedo MD, Cooper TM (1987) Influence of moisture content, temperature, and length of storage on seed germination and survival of endophytic fungi in seeds of tall fescue and perennial ryegrass. Phytopathology 77:893–900

Wheatley WM, Kemp HW, Simpson WR, Hume DE, Nicol HI, Kemp DR, Launders TE (2007) Viability of endemic endophyte (*Neotyphodium lolii*) and perennial ryegrass (*Lolium perenne*) seed at retail and wholesale outlets in south-eastern Australia. Seed Sci Tech 35:360–370

White JF Jr, Torres MS (2010) Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol Plant 138:440–446

Zhang YP, Nan ZB (2010) Germination and seedling anti-oxidative enzymes of endophyte-infected populations of *Elymus dahuricus* under osmotic stress. Seed Sci Tech 38:522–527