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Impact of ozone on the viability and antioxidant content of grass seeds is affected by a vertically transmitted symbiotic fungus

P.E. Gundel [a,*], N. Sorzoli [a], A.C. Ueno [a], C.M. Ghersa [a], C.E. Seal [b], D.A. Bastías [a], M.A. Martínez-Ghersa [a]

[a] IFEVA-CONICET, Department of Ecology, College of Agronomy, University of Buenos Aires, Av. San Martín 4453, Buenos Aires C1417DSE, Argentina
[b] Department of Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, West Sussex, RH17 6TN, UK

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ABSTRACT

Ozone gas is a rising pollutant in the troposphere and is a consequence of human-driven global change. As a novel environmental stressor, interest in the impact of ozone on symbiotic systems is increasing. Focusing on the symbiosis between grasses and *Epichloe* species, we evaluated the effect of ozone exposure on the relative fitness of symbiotic and endophyte free plants and its transgenerational effects including seed performance and endophyte persistence. Endophyte symbiotic and endophyte free *Lolium multiflorum* plants were exposed to high ozone concentration at pre-anthesis. Seed production of symbiotic plants was 23% higher than that of endophyte free plants, being positively correlated with number of spikes and independent of ozone. Seed viability was negatively affected by the endophyte and improved by ozone. A dramatic negative effect of ozone on endophyte viability was manifested only after 25-day seed storage under accelerating ageing conditions. On average, seeds from plants exposed to ozone had higher levels of reduced glutathione (GSH), whilst seeds from symbiotic plants were associated with higher content of glutathione disulfide (GSSG). Consistent with the pattern of seed viability dynamics, the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) was higher (i.e. less negative) in E+ seeds and slightly lower (i.e. more negative) in seeds from plants exposed to high ozone. The relationship between endophyte symbiosis and ozone stress with the levels of tocochromanol antioxidants was unclear, and irrespective of seed or endophyte viability. The concentration of some tocochromanols were not affected, whereas others were positively (β-tocopherol) or negatively (α-tocotrienol and γ-tocopherol) affected by the endophyte, or positively affected (γ-tocopherol) by ozone alone. The fungal symbiont modified the effect of ozone exposure in the maternal environment and thus, grass seed viability and antioxidant content. Although the grass-endophyte symbiosis may promote plant yield under rising ozone levels associated with global change, it may be at the expense of seed viability and endophyte persistence.

1. Introduction

The rising level of air contaminants is one component driving global climate change (IPCC, 2007). There is a general interest from the scientific community to understand the mechanisms by which biological systems, from individuals to ecosystems, will respond to such environmental changes (Kiers et al., 2010; Saikkonen et al., 2012). Specifically, it has been advised that positive interactions will be lost whilst at the same time, there would be an increment of negative ones such as plagues and diseases (Kiers et al., 2010). Symbiotic interactions have attracted attention not only because of their ecological role but also due to their potential use in sustainable agriculture (Tikhonovich and Provorov, 2009; Kiers et al., 2010). The importance of the mutualism between cool-season grasses and *Epichloë* species resides in that they are a potential factor of phenotypic variation, which can be selected to improve quality, persistence and productivity of forage cultivars (Johnson et al., 2013; Gundel et al., 2013). However, there is scarce information on how this symbiosis can cope with the novel factors of climate change.

The grass–endophyte symbiosis is a facultative interaction for plants, but obligate for the fungi. Variation in relative fitness between endophyte-symbiotic and endophyte free plants, and in

* Corresponding author. Tel.: +54 11 4 524 8000; fax: +54 11 4 514 8730.
E-mail addresses: gundel@agro.uba.ar (P.E. Gundel), sorzoli@agro.uba.ar (N. Sorzoli), aueno@agro.uba.ar (A.C. Ueno), ghersa@agro.uba.ar (C.M. Ghersa), c.seal@kew.org (C.E. Seal), dabastia@agro.uba.ar (D.A. Bastías), martinez@agro.uba.ar (M.A. Martínez-Ghersa).

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endophyte transmission efficiency determines its frequency and persistence in populations (Clay and Schardl, 2002; Gundel et al., 2008, 2011; Leuchtmann et al., 2014). The endophyte grows systemically in the apoplast of above ground tissues and it colonizes the developing seeds at flowering (i.e. vertical transmission) (Clay and Schardl, 2002; Majewska-Sawka and Nakashima, 2004; Christensen et al., 2008). The fungus remains quiescent in seeds and after germination, it is found in the seedlings (Clay and Schardl, 2002; Gundel et al., 2011; Card et al., 2014). The symbiosis is associated with higher plant resistance to herbivory and higher tolerance to factors of oxidative stress (Malinowski and Belesky, 2000; Clay and Schardl, 2002; Vila-Aiub et al., 2005), although it can often generate costs to the plant (Cheplick et al., 1989; Gundel et al., 2009a, 2010, 2012a). Relative to the vegetative stages, much less is known about the grass–endophyte interaction at the seed stage. The temporal dynamics of the frequency of viable endophyte in seed populations can result from the co-occurrence of two processes: (i) the viability of the fungus relative to host seeds, and (ii) the relative viability of endophyte–symbiotic and endophyte free seeds (Gundel et al., 2008, 2011). Compared to host seeds, the endophyte has shown a higher susceptibility to factors that control its viability (i.e. temperature and humidity), presenting as a consequence, lower longevity (Rolston et al., 1986; Welty et al., 1987; Gundel et al., 2009a, 2010). On the other hand, a negative effect of the endophyte on seed longevity has been observed under harsh storage conditions of high temperature with relative humidity (Gundel et al., 2009a, 2010, 2012a). It is unclear, however, whether seed or endophyte longevity could be more negatively affected under new scenarios of global climate change.

Ozone is a gaseous molecule (O_3) whose level is rising in the troposphere due to anthropic action and often, above the damage threshold for living organisms (i.e. 40 ppb, U.S.E.P.A., 2006). Ozone is a secondary contaminant produced via reactions catalysed by solar radiation and primary pollutants such as nitrogen oxides, sulphate oxides, carbon monoxide, hydrocarbons and volatile organic compounds. Therefore, it exhibits daily and seasonal dynamics with peaks at noon and during the summer (Booker et al., 2009; Schnell et al., 2009). Ozone enters the plant through the stomata and diffuses into the apoplast reacting with cellular components and producing reactive oxygen species (ROS) (Fiscus et al., 2005). Although ozone plays a role in signalling at the cellular level, it can generate different levels of oxidative stress depending on the intensity and frequency of exposure. Episodic exposure to ozone can elicit the activation of the antioxidant machinery that regulates oxidative stress, while chronic exposures to high ozone concentration may scale up from damage at cellular and tissue to the individual and crop level (Kanofsky and Sima, 1995; Sandermann et al., 1998; Kangasjärvi et al., 1994, 2005; Booker et al., 2009).

The antioxidant machinery which regulates oxidative stress involves ROS scavenging enzymes and molecules (Bailly, 2004; Kranner et al., 2006). In orthodox seeds (i.e. tolerant to desiccation; Roberts and Ellis, 1989), the antioxidants glutathione and tocochromanols have been associated with seed ageing and other vital quality parameters (germination rate, viability, among others) (Kranner et al., 2006; Seal et al., 2010). Glutathione is a water soluble antioxidant found in the cell cytoplasm in the reduced form (γ-glutamyl-cysteinyl-glycine or GSH) in unstressed conditions. With the increment of ROS, 2GSH can donate electrons and form glutathione disulphide (GSSG), a reversible reaction which is catalysed by the enzyme glutathione reductase (Kranner et al., 2006; Seal et al., 2010). A negative linear relationship has been found between the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) and the viability of orthodox seeds (Kranner et al., 2006). The relationship of tocopherols, which are lipid-soluble antioxidants associated to cell membranes, and seed viability has been more erratic with some studies finding a positive correlation between seed viability loss and a reduction in α-tocopherol content (Senaratna et al., 1988; Sattler et al., 2004; Seal et al., 2010).

Recently, it has been proposed that ROS may play a role in regulating the symbiotic interaction between plants and fungi (Rodriguez and Redman, 2005; White and Torres, 2010; Hamilton et al., 2012; Hamilton and Bauerle, 2012). Molecular studies suggested that the redox balance in plants would regulate the stability of the mutualistic symbiosis between grasses and fungal endophytes (Tanaka et al., 2006; Eaton et al., 2011). A screen to identify symbiotic genes isolated a fungal mutant that altered the interaction from mutualistic to antagonistic (Tanaka et al., 2006). This mutant has a single-copy plasmid insertion in the coding region of a NADPH oxidase gene, noxA. The fungal biomass in these associations is increased dramatically while the plants infected with the noxA mutant become severely stunted, show precocious senescence, and eventually die. ROS accumulation was detected in the endophyte extracellular matrix in wild-type but not in noxA mutant associations. This led to the hypothesis of a dual role for ROS in the grass–endophyte interaction. ROS could increase the permeability of the plant membranes favouring nutrient leaching to the apoplast and could also be involved in controlling the endophyte growth within the host (White and Torres, 2010; Eaton et al., 2011). Both roles would in turn lead to the activation of the plant's anti-stress defence system by increasing the antioxidant content (White and Torres, 2010; Hamilton et al., 2012; Hamilton and Bauerle, 2012). The antioxidant hypothesis proposes that there would be a higher production of antioxidant compounds, some of them of fungal origin (e.g. mannitol, proline; Richardson et al., 1992; Rasmussen et al., 2008), that would explain the higher stress tolerance usually found in symbiotic plants compared to endophyte free ones (Rodriguez and Redman, 2005; Malinowski and Belesky, 2000; White and Torres, 2010; Hamilton and Bauerle, 2012). However, information relating the endophyte-symbiosis to plant stress tolerance and antioxidant content is certainly scarce (Hamilton et al., 2012) and there is much less in relation to the endophyte effects on seed viability (Gundel et al., 2012b).

Here, we studied the effect of ozone on the persistence of the symbiosis between a grass and an endophyte of the *Epichloë* species. Specifically, we evaluated the effect of ozone exposure on the relative fitness of symbiotic and endophyte free plants and endophyte transmission efficiency. Using this framework, we explored the consequences of ozone exposure at pre-anthesis on the plant vegetative and reproductive stages. We hypothesized that (1) exposure of plants to ozone at pre-anthesis would negatively affect seed production, with lower impact on endophyte symbiotic plants than on endophyte free plants, but it would not affect the endophyte transmission to seeds because the fungus is found in the ovaries before seed formation (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004; Sugawara et al., 2004); (2) oxidative stress caused by ozone at pre-anthesis would stimulate the production of antioxidants promoting seed longevity only in non-symbiotic plants since the fungus increases stress tolerance mediated by antioxidants; and (3) the stress triggered by ozone in the apoplast of plants might adversely affect the endophyte and thus, its own longevity in the seeds.

2. Materials and methods

2.1. Study system and biological material

We worked with the symbiosis between the annual grass *Lolium multiflorum* L. and its natural endophyte fungus, *Epichloë occultans* (≡*Neotyphodium occultans* C.D. Moon, B. Scott and M.J. Chr. Mycologia 92:1113. 2000; Leuchtmann et al., 2014). Naturalized populations in Argentine Pampa grasslands present high frequencies of endophyte symbiotic individuals (Gundel et al.,

2009b). Seeds of *L. multiflorum* presenting 95% of endophyte symbiotic individuals (based-on evaluating 100 previously stained and squashed seeds under microscope (×10, ×40); see Bacon and White, 1994) were collected in 2010 from a grassland in Inland Pampa, Argentina. Half were treated with a systemic fungicide (Baytan, Bayer, Buenos Aires, Argentina; F 150 g a.i. kg^{-1}) in order to kill the fungus. The fungicide was applied to the seeds as a slurry for 1 h prior to planting. Two seed lots, fungicide-treated and untreated, of about 2.5 g ($N \approx 1250$ seeds) were sown in 1 m^2 contiguous plots at the experimental field of Agronomy College, University of Buenos Aires, to produce new seeds. Pollen-mediated gene flow between the two stands of plants was allowed in order to diminish genetic differentiation between fungicide-treated and untreated seed lots. Having checked the effectiveness of the fungicide, two comparable endophyte symbiotic (E+) and endophyte free (E−) seed lots were achieved for the experiment. These seed lots presented a 95% and 5% of endophyte symbiotic seeds, respectively, based on 100 seeds evaluated as indicated before.

2.2. Experimental design

In 2011, E+ and E− *L. multiflorum* plants were grown during the natural growing period (from April to December, Southern Hemisphere) individually in 2 L pots filled with equal parts of black organic soil, sand and peat moss. The plants were kept outdoors and watered periodically to avoid stress. At pre-anthesis phenological stage, 128 plants (64 E+ and 64 E−) were equally distributed in eight open top chambers (8 E+ and 8 E− independent plants were randomly assigned to each chamber). An open-top chamber consisted of a mushroom-shaped aluminium structure (2.5 m height, 8 m^3 volume) with crystal PVC walls placed at the Agronomy College, University of Buenos Aires (for more details, see Landesmann et al., 2013). Each chamber received ambient air pumped through activated charcoal. In half of the chambers, ambient air was mixed with ozone generated by a spark discharge-type generator (Hogsett et al., 1985). The systems thus provided two ozone concentrations: charcoal-filtered ambient air (less than 10 ppb) (hereafter: low-ozone) and high ozone level air (≈120 ppb, hereafter: high-ozone). This study included 4 treatments: endophyte symbiosis (E+ or E−) nested within the ozone treatment (low-ozone or high-ozone) with four chambers each (blocks). High ozone level air simulated highly contaminated areas where several days of ambient smog exposure involving 1-h ozone concentration peaks in the range of 120–190 ppb might occur throughout the growing season (Booker et al., 2009). The level of ozone was monitored (Teledyne-API, model 450).

Ozone treatment was applied at pre-anthesis to avoid significant reduction in seed production (Martinez-Ghersa et al., 2008). Ovary colonization by the endophyte is prior to flowering, but it continues growing after flower fertilization and seed development (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004; Sugawara et al., 2004). Before ozone application, 5–10 tillers per plant were labelled with a wire ring to ensure that the seeds collected for the seed experiment (see below) were produced by spikes that were close to flowering when exposed to ozone. Plants were exposed to ozone for two hours during five consecutive days at noon (time of high solar radiation) to simulate the natural ozone peak in the troposphere. Plants were then moved back outdoors until they reached maturity. Seeds from ring-labelled and non-labelled spikes were harvested and threshed to estimate production of seeds per plant. In addition, endophyte transmission from plant-to-seed was estimated at the plant level (10 seeds $plant^{-1}$) by microscopic observation of seeds (see Bacon and White, 1994). Seeds from ring labelled spikes were grouped per symbiotic status and open-top chamber, and stored dry at 10 °C until used in the following experiment.

Seed and endophyte longevity were estimated through an accelerated ageing experiment (Roberts and Ellis, 1989). Seeds were previously characterized in terms of 1000-seed weight and seed water content (SWC). The 1000-seed weight was estimated by weighing 5 batches of 30 seeds each, for every symbiotic status and open-top chamber. For each combination of symbiotic status and open-top chamber, the SWC was estimated by weighing 30 seeds, drying them for an hour at 130 °C and reweighing (ISTA Vigour Test Committee, 1995). SWC is expressed as a percentage (%) on a fresh weight basis as: g H_2O 100 g^{-1} fresh weight. The accelerated ageing experiment consisted of storing seeds under high relative humidity (75%) and temperature (40 °C). Relative humidity was controlled with NaCl saturated salt solution contained in open glass jars within hermetic plastic boxes (described in Gundel et al., 2009a). About 1000 seeds (≈2 g) of every symbiotic status were stored in these plastic boxes, suspended above the solutions within each glass jar. Eight plastic boxes (one per open-top chamber) were placed in a growth chamber set at 40 °C. Seed viability was periodically evaluated by incubating seeds under optimal conditions for germination of this species [15/25 °C alternating temperature (12 h/12 h)] (ISTA Vigour Test Committee, 1995). Thirty seeds per symbiotic status, from each plastic box (open-top chamber), were sown in a Petri dish (9 cm diameter, 5 ml distilled water). Seedlings produced from the endophyte symbiotic seeds were transplanted into soil for further endophyte assessment. Endophyte viability was determined by evaluating the status of the seedling to indicate successful endophyte transmission from seed-to-seedling. Endophyte viability was evaluated by microscopic observation of the base of the pseudostem of 3–4 week old seedlings stained with Rose Bengal (Belanger, 1996; Bacon and White, 1994).

2.3. Seed antioxidant determination

Glutathione and tocopherol antioxidants were measured in seeds from each symbiotic status and open-top chamber combination. For determination of antioxidants, freeze-dried seeds 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. Tocopherols and tocotrienols were determined following a modified procedure of Bagci et al. (2004). To three replicates of 100 mg of ground material, 500 μl heptane (HPLC-grade, Fisher, UK) was added and vortexed for 30 s, before centrifugation at 13,000 g and 4 °C for 40 min. The supernatant was collected and the pellet was re-suspended in 500 μl heptane, and vortexed and centrifuged as before. The supernatants from the two centrifugation steps were combined and centrifuged prior to normal phase high performance liquid chromatography (HPLC) analysis (Jasco, Great Dunmow, Essex, UK). α-, β-, γ- and δ-Tocopherol and α-, β-, γ- and δ-tocotrienol were separated on a Supelcosil LC-Diol column (Supelco Analytical, Sigma–Aldrich, Bellefonte, USA) of 250 mm length, 3.0 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: 330 nm) and quantified using standards (Sigma–Aldrich, Poole, Dorset, UK) prepared in heptane.

For glutathione determination, 50 mg of ground seeds were extracted in 1 ml of 0.1 M HCl with 0.5% (v/v) Triton X-100 and 50 mg polyvinylpolypyrrolidone and centrifuged as above 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-

Table 1
Number of spikes, seed production, 1000-seed weight and endophyte transmission to seeds in endophyte symbiotic and endophyte free *Lolium multiflorum* plants exposed to high and low ozone concentration at pre-anthesis. Numbers represent mean values ($n = 4$) and standard error is in parentheses.

Ozone level	High-ozone		Low-ozone	
Endophyte symbiotic status	E+	E−	E+	E−
Spikes (n plant^{-1})	29.75 (2.40)	22.53 (1.10)	27.87 (0.89)	22.62 (1.04)
Seed production (g plant^{-1})	3.74 (0.26)	2.90 (0.19)	3.46 (0.33)	2.95 (0.44)
1000-seed weight (g)	2.15 (0.04)	2.16 (0.11)	2.14 (0.07)	2.15 (0.06)
Proportion of symbiotic seeds	0.99 (<0.01)	–	0.99 (<0.01)	–

molecular-weight were separated by reverse-phase HPLC on a HiQsil RP18 column (250 × 2.1 mm i.d., 5 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 J K^{-1} mol^{-1}); T is temperature in K; n is number of transferred electrons; F is the Faraday constant (9.6485 × 104 C mol^{-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 the SWC. 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.

2.4. Statistics

Statistical analyses were run in R (R Development Core Team, 2011) using packages for mixed models (e.g. lme4 and nlme: Bates et al., 2012; Pinheiro et al., 2012). Seed production per plant as affected by symbiosis and ozone treatment were modelled with a generalised linear mixed model with E+ and E− plants nested within open-top chamber and chamber as random factor. ANOVA assumption for number of spikes per plant, a predictor variable in the model, was achieved through a varPower variance structure. Effects of symbiosis and ozone on seed viability were modelled with a generalised linear mixed model with endophyte nested in chamber, and open-top chamber as a random factor. The effect of ozone on endophyte viability was analysed in a similar way. Since viability is a proportion based on a binary response variable (live/dead), we used a logit link function (Crawley, 2007 Zuur et al., 2009). With the same hierarchy structure, the content of different antioxidants was analysed with linear mixed models.

3. Results

3.1. Plant performance and endophyte transmission to seeds

The number of spikes and seed production per plant was higher in endophyte symbiotic plants than in endophyte free plants and was irrespective of the ozone treatment in both cases (Table 1). Seed production was ≈23% higher in endophyte symbiotic plants compared to endophyte free plants ($F_{1,114} = 5.603$, $P_{endophyte} = 0.019$) which was also positively related to number of spikes per plant ($F_{1,114} = 65.882$, $P_{spikes} < 0.001$). Endophyte symbiotic plants presented on average, six more spikes compared to endophyte free plants. The average weight of produced seeds was not affected by either the endophyte nor ozone treatment. The transmission efficiency of the endophyte from plant to seed was very high (0.99) and was not affected by the ozone treatment (Table 1).

3.2. Seed and endophyte fungus longevity

Placing the seeds and fungus under stress from ageing was critical to see phenotypic differences; at 0 days there was no apparent difference in the viability of both the seed and the fungus following ozone exposure. Seed viability of *L. multiflorum* (Fig. 1) was negatively affected by the endophyte ($F_{1,89} = 20.924$, $P_{endophyte \times time} < 0.001$), with the maximum difference between E− and E+ seed viability occurring after 75 days of ageing. There was a tendency for ozone to increase seed viability (Fig. 1), although the effect was not statistically significant ($F_{1,94} = 2.514$, $P_{ozone} = 0.116$). SWC under accelerated ageing conditions (relative humidity: 75%, and temperature: 40 °C) did not differ between either biotypes [E+ = 13.05% (±0.65), E− = 13.00% (±0.71)] or treatment of ozone [high-ozone = 12.91% (±0.59), and low-ozone = 13.13% (±0.75)] (Average of $n = 4$ along experimental time (5 times) ± SE; Appendix A, Fig. 1S).

The effect of ozone treatment on the viability of endophyte was evaluated only in seedlings generated by seed lots produced by endophyte-symbiotic plants (E+) exposed to both conditions of low and high ozone concentrations. As accelerated ageing reduced the amount of seeds and hence seedlings on which to evaluate the endophyte presence (i.e. endophyte viability), we only have data for 0 days (non-aged seeds) and 25 days ageing. A negative effect of ozone on endophyte viability was manifested only when seeds were stored under accelerated ageing conditions ($F_{1,12} = 3.365$, $P_{ozone \times time} = 0.016$)(Fig. 2). The proportion of the endophyte fell about 40% in seeds stored for 25 days at accelerated ageing conditions, produced by plants exposed to high-ozone concentration in comparison to seeds produced by plants kept under low-ozone atmosphere. On the other hand, the proportion of endophyte was unaffected by ozone in seedlings generated by seeds that were not stored under deteriorating conditions (Fig. 2).

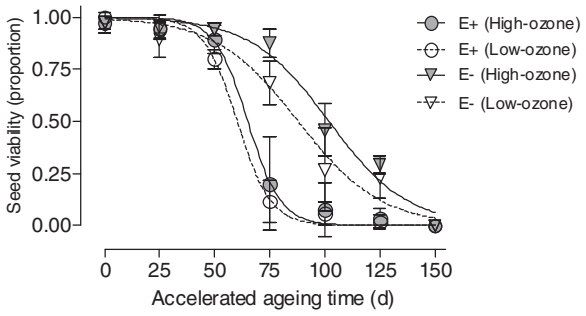

Fig. 1. Viability dynamics of endophyte-symbiotic (E+) and endophyte free (E−) seeds produced by *Lolium multiflorum* plants exposed to low (low-ozone) and high (high-ozone) ozone concentrations at pre-anthesis, under accelerated ageing conditions. Symbols are mean values ($n = 4$) ± SE.

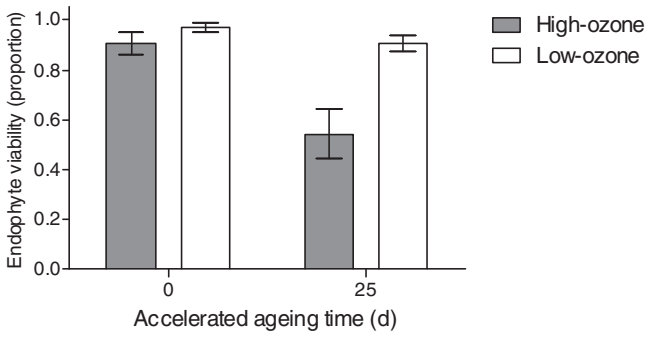

Fig. 2. Proportion of seedlings with fungal endophyte (number of seedlings with endophyte/total evaluated seedlings) generated by seeds produced by *Lolium multiflorum* plants under low (low-ozone) and high (high-ozone) ozone concentrations, and exposed to accelerated ageing conditions for 0 and 25 days. Symbols are mean values ($n = 4$) ± SE.

3.3. Seed antioxidants

In seeds at day 0, the antioxidant content of glutathione and tocochromanols was examined. Ozone promoted the content of reduced glutathione in seeds (GSH) ($F_1 = 5.335$, $P_{ozone} = 0.039$), whereas the endophyte was associated with a higher content of glutathione disulfide (GSSG) ($F_1 = 14.779$, $P_{endophyte} = 0.002$) (Fig. 3). The glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) was higher (i.e. less negative value) in the seeds of E+ plants ($F_1 = 5.218$, $P_{endophyte} = 0.041$) and marginally lower (i.e. more negative value) for seeds produced by plants exposed to high ozone treatment ($F_1 = 4.291$, $P_{ozone} = 0.060$) (Fig. 3).

Of the antioxidants analysed from the tocochromanol family, only α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, γ-tocotrienol and δ-tocopherol were detected, whereas β-tocotrienol and δ-tocotrienol were not detected. α-Tocopherol, γ-tocotrienol and δ-tocopherol were not affected either by endophyte ($F_1 = 3.000$, $P_{endophyte} = 0.108$; $F_1 = 0.218$, $P_{endophyte} = 0.649$; $F_1 = 3.943$, $P_{endophyte} = 0.070$, respectively) or ozone ($F_1 = 0.684$, $P_{ozone} = 0.414$; $F_1 = 0.267$, $P_{ozone} = 0.614$; $F_1 = 0.569$, $P_{ozone} = 0.465$, respectively) (Fig. 4). There was a strong positive association between the endophyte and the concentration of β-tocopherol ($F_1 = 77.379$, $P_{endophyte} < 0.001$). On the contrary, the endophyte was associated to a negative, although smaller in magnitude, effect on antioxidants α-tocotrienol and γ-tocopherol ($F_1 = 0.039$, $P_{endophyte} = 0.039$, and $F_1 = 4.829$, $P_{endophyte} = 0.048$, respectively). Plants exposed to high ozone showed an increase in the seed concentration of the antioxidant γ-tocopherol ($F_1 = 7.540$, $P_{ozone} = 0.017$; Fig. 4).

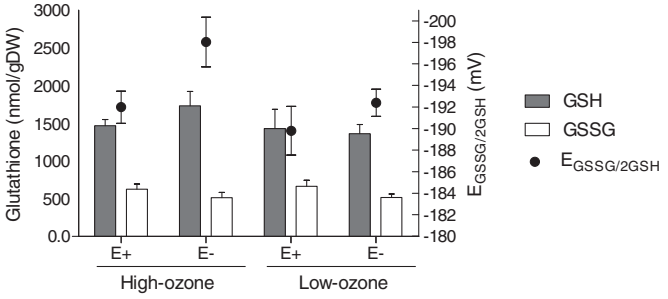

Fig. 3. Concentration of glutathione (GSH, gray bars) and glutathione disulphide (GSSG, white bars) and the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$, black circles) measured in seeds produced by *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E−) with fungal endophyte, exposed to low (low-ozone) or high (high-ozone) ozone concentration at pre-anthesis. The measurement corresponds to time zero of the accelerated ageing experiment. Values are means ($n = 4$) ± SE. Black symbols are displaced in order to avoid overlapping.

4. Discussion

The seed stage is critical for the persistence of annual species in general and for vertically transmitted symbionts in particular (Gundel et al., 2008, 2011). The frequency of symbiotic seedlings produced by a population of seeds depends on the differential viability of symbiotic and non-symbiotic seeds and endophyte viability relative to that of the host seeds (Gundel et al., 2009a, 2010, 2012a). With ozone levels altering with climate change, phenotypic variation in the grass–endophyte symbiotic relationship and the cost to both parties are important to understand, particularly if improvements are to be made to the quality and productivity of forage species.

The pre-anthesis exposure of plants to a high concentration of ozone did not affect the average seed weight nor the endophyte transmission from mother plants to seeds. This is likely to occur since vertically transmitted endophytes colonize the developing ovaries well before anthesis (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004; Sugawara et al., 2004). Endophyte symbiotic plants produced more spikes and seeds/spike than endophyte-free plants, independently of the ozone treatment, giving a yield advantage for plants of the mutualism. However, the seed quality was lower in those produced from the endophyte symbiosis, in terms of both viability and longevity. Therefore, more seeds may be produced from the symbiotic plants but fewer will germinate or persist in the soil seed bank. As high-ozone marginally improved seed quality, the presence of the endophyte had the largest (negative) influence on the seed.

This relationship between seed quality and ozone or endophyte was also mirrored in the redox state of glutathione. Glutathione is a major intracellular redox buffer and Kranner et al. (2006) demonstrated in a range of plant tissues, including seeds, and fungi across 13 orders, that a shift in $E_{GSSG/2GSH}$ towards more oxidizing (i.e. positive) values occurred as viability is lost. In this study, the high-ozone treatment stimulated the production of the antioxidant glutathione in its reduced form (GSH) and consequently, the $E_{GSSG/2GSH}$ was more negative, in seeds produced under this treatment. This may explain why seeds produced from the high-ozone treatment had higher seed quality than those under the low-ozone treatment. In contrast, the presence of endophyte was more detrimental on the cellular redox state (high concentration of GSSG and less negative $E_{GSSG/2GSH}$). As in other studies (see Seal et al., 2010; Gundel et al., 2012b), there was no straightforward relationship between abundance of tocopherols and viability of both seeds and endophytes. Whereas ozone had negligible effect on tocopherols (except for a very small one on γ-tocopherol), the fungal endophyte had huge positive effect on the concentration of β-tocopherol, but negative and of smaller magnitude on the α-tocotrienol and γ-tocopherol antioxidant. Causes and consequences of these changes are still uncertain and needs more research.

The magnitude of change in $E_{GSSG/2GSH}$ in response to ozone treatment was lower in seeds from E+ plants, and thus mitigated by the fungus. However, this did not have any obvious positive effect for the seed. Furthermore, the exposure of plants to ozone was costly to the endophyte. The longevity of the endophyte was impaired in seeds produced by ozone-treated plants affecting its transmission from seeds to seedlings. The frequency of established seedlings symbiotic with endophyte was affected under ozone, probably through two not-mutually exclusive processes: (i) a positive effect of ozone on seed viability, attenuated by the endophyte itself, and (ii) a negative effect of ozone on the viability of the endophyte. The first process would be in agreement with the

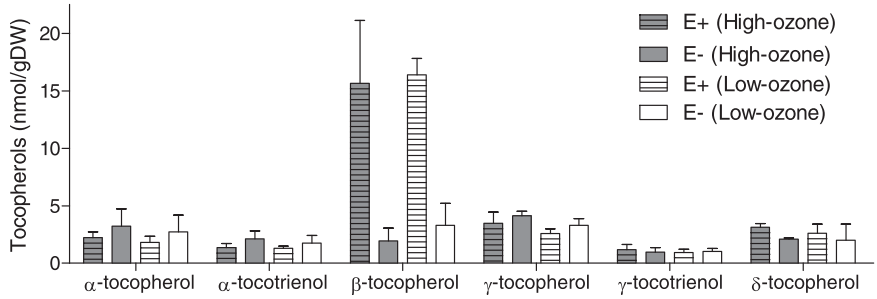

Fig. 4. Concentration of different tocochromanol compounds (α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, γ-tocotrienol and δ-tocopherol) measured in seeds produced by endophyte symbiotic (E+) and non-symbiotic (E−) *Lolium multiflorum* plants, exposed to low (low-ozone) or high (high-ozone) ozone concentration at pre-anthesis. The measurement corresponds to time zero of the accelerated ageing experiment. Values are means ($n = 4$) ± SE.

proposed protective role of endophytes on hosts (i.e., the antioxidant hypothesis; White and Torres, 2010; Hamilton et al., 2012; Hamilton and Bauerle, 2012), and the results suggest that the effects may be related to the antioxidant mechanism mediated by glutathione. Since the apoplast is the first place where the ozone produces oxidative stress and the apoplast is, in turn, where the fungus grows, ozone induced oxidative stress may affect the homeostasis of apoplastic medium and thus, the interaction stability. Furthermore, as plants exposed to ozone have been found to accumulate salicylic acid (SA) (Sharma et al., 1996) and this defence pathway is active against pathogenic biotrophic micro-organisms (Pieterse et al., 2009), the endophyte might be challenged by the ozone-triggered defence mechanism in plant. More experiments are needed in order to determine whether the mechanism by which ozone affects the fungal symbiont is mediated by phytohormones or by the oxidative stress.

In summary, the exploration of the antioxidant hypothesis mediating the grass–endophyte interaction at seed stage has yielded variable results depending on species, organism (host or fungus) and antioxidant. However, the association of fungal endophyte and antioxidant may be related to the specific location of the antioxidant and its function. Glutathione is a cytoplasmic and water soluble antioxidant proposed as a reliable marker of stress in orthodox seeds (Kranner et al., 2006; Seal et al., 2010), and our results show that glutathione can be affected by the endophyte, although a clear relationship with the viability of the fungus is not yet proven (Gundel et al., 2012b). Alternatively, the association of the endophyte to a higher level of some tocochromanol antioxidants (lipid-soluble and membrane-bound molecules) could explain the maintenance of membranes functionality in germinating seeds and the high survival of seedlings (Vila-Aiub et al., 2003; Gundel et al., 2012b). Additional experiments are needed in order to reveal the association of the endophyte presence with the role of the different antioxidants as well as the origin (i.e. plant or endophyte) of each antioxidant compound. For cool-season grasses such as *L. multiflorum*, the grass-endophyte symbiosis may promote seed yield in response to rising ozone levels associated with climate change but at the expense of seed viability. However, the ultimate cost is to the endophyte.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2015.01.001.

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