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Ontogenetic and trans-generational dynamics of a vertically transmitted fungal symbiont in an annual host plant in ozone-polluted settings

Andrea C. Ueno[1] | Pedro E. Gundel[1] | Claudio M. Ghersa[1] | Patricia V. Demkura[1] | Stuart D. Card[2] | Wade J. Mace[2] | María Alejandra Martínez-Ghersa[1]

[1]IFEVA, Facultad de Agronomía, Departamento de Recursos Naturales y Ambiente, CONICET, Universidad de Buenos Aires, Av. San Martín 4453, Buenos Aires, C1417DSE, Argentina

[2]Forage Science, AgResearch Limited, Grasslands Research Centre, Palmerston North, Private Bag 11008, New Zealand

Correspondence
A. C. Ueno, IFEVA, Facultad de Agronomía, Departamento de Recursos Naturales y Ambiente, CONICET, Universidad de Buenos Aires, Av. San Martín 4453, Buenos Aires, C1417DSE, Argentina.
Email: aueno@agro.uba.ar

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Abstract

Tropospheric ozone is an abiotic stress of increasing importance in the context of global climate change. This greenhouse gas is a potent phytotoxic molecule with demonstrated negative effects on crop yield and natural ecosystems. Recently, oxidative stress has been proposed as a mechanism that could regulate the interaction between cool-season grasses and *Epichloë* endophytes. We hypothesized that exposure of *Lolium multiflorum* plants, hosting endophytes to an ozone-polluted environment at different ontogenetic phases, would impact the trans-generational dynamics of the vertically transmitted fungal symbiont. Here, we found that the ozone-induced stress on the mother plants did not affect the endophyte vertical transmission but it impaired the persistence of the fungus in the seed exposed to artificial ageing. Endophyte longevity in seed was reduced by exposure of the mother plant to ozone. Although ozone exposure did not influence either the endophyte mycelial concentration or their compound defences (loline alkaloids), a positive correlation was observed between host fitness and the concentration of endophyte-derived defence compounds. This suggests that fungal defences in grass seeds were not all produced *in situ* but remobilized from the vegetative tissues. Our study reveals ozone trans-generational effects on the persistence of a beneficial symbiont in a host grass.

KEYWORDS

alkaloid, defensive mutualism, fungal endophyte, grass, ozone, symbiosis

1 | INTRODUCTION

Human activities are altering the Earth's biosphere at an unprecedented rate with industrialization and intensification of production activities driving the accumulation of certain chemicals in water, soil and air to unsuitable levels of contamination (Hough & Derwent, 1990; IPCC, 2013; Tilman, Cassman, Matson, Naylor, & Polasky, 2002). One such molecule, ozone (O_3), when present in the troposphere (the lowest layer of the atmosphere near the Earth's surface) is a powerful greenhouse gas that contributes to global warming. Tropospheric ozone gas is produced by a different chemical process than stratospheric ozone. In the stratosphere, ozone is formed via a natural process involving sunlight and oxygen molecules; while in the troposphere, ozone is formed by photochemical reactions between naturally occurring gases and those from sources of pollution such as volatile organic compounds (VOCs), carbon monoxide (CO) and nitrogen oxides (NOx). These ozone

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precursors are largely generated from motor vehicle exhaust, industrial emissions and chemical solvents. In addition, tropospheric ozone is a major pollutant, being a component of smog, and a potent phytotoxic molecule with demonstrated negative effects on crop yield and natural ecosystems (Ainsworth, 2017; Fuhrer, 2009; Mills et al., 2018). The concentrations of tropospheric ozone have more than doubled since the industrial revolution, and there is a growing consensus that levels within the troposphere will continue to increase, therefore putting at risk global food security (Ainsworth, 2017; Ainsworth, Yendrek, Sitch, Collins, & Emberson, 2012; Mills et al., 2018).

Pollutants, such as ozone, not only directly affect plant species but also the symbiotic organisms they associate with (Chen, Liu, Wurihan, Card, & Ren, 2017; Compant, van der Heijden, & Sessitsch, 2010; Kiers, Palmer, Ives, Bruno, & Bronstein, 2010; Menéndez, Gundel, Lores, & Martínez-Ghersa, 2017; Sandermann Jr, 1996). This study focused on the effects of ozone on the dynamics of an asexual vertically transmitted fungal endophyte, *Epichloë occultans*, that colonizes the cool-season grass, *Lolium multiflorum*. This grass is an important forage species that thrives in different communities of the Pampa grasslands in Argentina (Perelman, Chaneton, Batista, Burkart, & León, 2007). Vertically transmitted symbionts are integrated into the life cycle of the host, implying mutual dependence and shared benefits; features that likely promote mutualistic interactions through the partner's fidelity (Bright & Bulgheresi, 2010; Ewald, 1987; Sachs, 2013; Sachs, Essenberg, & Turcotte, 2011). However, the symbiosis can be impaired and potentially lost if disrupted by environmental factors causing plant stress (Kiers et al., 2010; Sachs, 2013). The symbiosis between cool-season grasses (subfamily Pooideae) and *Epichloë* fungal endophytes (family Clavicipitaceae) has co-evolved over thousands of years (Leuchtmann, Bacon, Schardl, White Jr., & Tadych, 2014; Semmartin, Omacini, Gundel, Hernández-Agramonte, & Thrall, 2015). The symbiosis is not necessarily vital for the survival of all individuals within plants of a given grass population despite can possess several fitness-related benefits. Although most variable on wild species than domesticated ones (Saikkonen, Wäli, Helander, & Faeth, 2004), the most documented endophyte-mediated benefit is the resistance to avian, invertebrate and mammalian herbivory mediated by fungal-derived alkaloids (Bastías, Martínez-Ghersa, Ballaré, & Gundel, 2017; Pennell et al., 2016, 2017; Saikkonen, Gundel, & Helander, 2013; Schardl, Grossman, Nagabhyru, Faulkner, & Mallik, 2007).

The persistence and abundance of asexual *Epichloë* endophytes, those that are strictly vertically transmitted, in natural populations are mainly determined by the effect of the symbiont on host fitness and the efficiency with which the symbiont is transmitted to the next plant generations (Afkhami & Rudgers, 2008; Gundel et al., 2008; Ravel, Michalakis, & Charmet, 1997). Comparing the performance of endophyte-colonized plants with endophyte-free plants has been the most common experimental approach to understand the mechanisms influencing the endophyte incidence in plant populations. Very few studies have focused on underlying factors that affect the transmission process and persistence of the endophyte (Gundel et al., 2008; Gundel, Rudgers, & Ghersa, 2011; Ravel et al., 1997; Shymanovich & Faeth, 2019). Inefficiencies in the vertical transmission process mean that a host plant can produce a variable proportion of both endophyte-colonized and endophyte-free offspring (Afkhami & Rudgers, 2008; Gibert & Hazard, 2013; Gundel, Rudgers, et al., 2011). In addition, endophyte-colonized seeds can germinate and produce endophyte-free seedlings due to a loss of fungal viability (Gundel, Martínez-Ghersa, Batista, & Ghersa, 2010; Gundel, Martínez-Ghersa, Garibaldi, & Ghersa, 2009; Welty, Azevedo, & Cooper, 1987). The vertical transmission process can be disrupted at certain critical ontogenetic phases (e.g., at plant reproduction) by many environmental factors, either abiotic or biotic (Gundel et al., 2008; Gundel, Rudgers, et al., 2011). For example, defoliation of endophyte-colonized *L. multiflorum* plants at the phase of culm elongation (close to flowering) produced a high proportion of endophyte-free seeds (García Parisi, Casas, Gundel, & Omacini, 2012). The incidence of oxidative stress factors, which occurs very early in the plant cycle (i.e., seedling), did not affect the vertical transmission to seeds (Gundel et al., 2012), suggesting that the plant and the endophyte were able to recover from the stress.

Elevated temperature and humidity are detrimental to endophyte within stored seed and the grass seed itself. While the endophyte presence has been found to affect seed viability and govern changes in relative abundance of sugars (Gundel et al., 2010; Zhang, Mace, Matthew, & Card, 2019), endophyte viability generally declines at a faster rate than seed viability (Gundel et al., 2009, 2010; Welty et al., 1987). In addition, there is a strong genetic component controlling the survival of endophytes in seed, explaining some of the observed differences in endophyte status within grass populations (Gundel et al., 2010; Hill & Roach, 2009; Tian et al., 2013). Although the majority of studies investigating endophyte-derived alkaloids are concerned with the vegetative plant, this function has also been linked to the protection of reproductive structures and the seed (Faeth, 2009; Finch, Pennell, Kerby, & Cave, 2016; Pennell et al., 2017; Uchitel, Omacini, & Chaneton, 2011), which can extend to protection of the developing seedling (Gundel, Rudgers, & Whitney, 2017; Gundel, Seal, et al., 2018; Justus, Witte, & Hartmann, 1997; Ruppert, Matthew, McKenzie, & Popay, 2017; A. V. Stewart, 1985). Recent work has focused on the effects of the environment explored by mother plants on endophyte transmission and alkaloid production (Gundel et al., 2017; Gundel, Seal, et al., 2018). Despite its implication for seed ecology, no information exists on the factors that affect alkaloid concentrations in seed (Gundel et al., 2017; Gundel, Seal, et al., 2018). In the plant, however, the concentrations of endophyte mycelia and alkaloids follow the seasonal plant growth, although different alkaloids can peak at slightly different stages (Bluett, Thom, Clark, Macdonald, & Minneé, 2005; Fuchs, Krischke, Mueller, & Krauss, 2017). The concentration of alkaloids can be positively correlated to the concentration of endophyte mycelia present (Justus et al., 1997; Rasmussen et al., 2007), with the concentration of certain alkaloids responding positively to herbivory (Fuchs, Krischke, Mueller, & Krauss, 2017; Fuchs, Krischke, Mueller, Krauss, & Biere, 2017; Sullivan et al., 2007). However, it is not clear whether there is a decoupling between fungal hyphae and alkaloid concentration, or it is simply an induction in the synthesis of the product

(Bastías et al., 2018; Ryan, Rasmussen, Xue, Parsons, & Newman, 2014). Even though the concentration of alkaloids in seed can vary between plant and endophyte species, plant populations and fungal strain (Bylin et al., 2014; Justus et al., 1997; TePaske, Powell, & Clement, 1993; Vazquez de Aldana, Zabalgogeazcoa, Garcia Ciudad, & Garcia Criado, 2003), it is unknown whether this is related to the concentration of endophyte hyphae within the seed and/or if this depends on the environmental conditions that the mother plant was exposed to before seed maturation.

We investigated the consequences of exposing endophyte-colonized plants to a high level of ozone by assessing different aspects of performance: Endophyte vertical transmission efficiency, endophyte persistence in seeds and seed alkaloid concentration. We hypothesized that ozone-caused oxidative stress at the plant reproductive phase would be more detrimental for endophyte than that at the plant vegetative phase since, at this moment, fungus is colonizing the ovaries and growing into developing seeds. In addition, we explored the relationship between parameters of fungal endophyte performance in the plants' offspring with individual mother plant fitness. Since the plant partners' ecological fitness is aligned, we predicted that endophyte transmission, mycelial concentration and alkaloid concentration in seed are positively correlated to the number of plant seed produced within a given year (i.e., host fitness).

2 | MATERIALS AND METHODS

2.1 | Plant material

We worked with a wild *L. multiflorum* population initially collected in 2012 from a successional grassland (Argentina; 36° 00′ S, 61° 5′ W). *Lolium multiflorum* (Italian ryegrass) is an annual cool-season grass species naturalized in Pampa grasslands, where populations usually show high levels of endophyte incidence (Gundel et al., 2009; Vila-Aiub, Martínez-Ghersa, & Ghersa, 2003). In a recent work, we confirmed that our experimental population harbours the common endophyte of annual ryegrasses, *Epichloë occultans* (Bastías et al., 2017; Moon, Scott, Schardl, & Christensen, 2000). In order to maintain the population and multiply the seeds for experimentation, the population is yearly cultivated during the normal growing season for the species (autumn–winter–summer), in a 1 m^2 plot with a sowing density of 100 seeds/plot. As *L. multiflorum* is obligately outcrossing species, plants can exchange pollen not only with individuals from the population but also with wild plants from the surroundings. At plant ripening, seeds are harvested and stored in cold (4°C) and dry conditions. In addition, the endophytic status of the population is checked by assessing 100 seeds using seed squash technique (Latch & Vaughn, 1995).

2.2 | Experimental procedures

Endophyte-colonized *L. multiflorum* plants (E+) were grown individually within 3 L pots filled with a mixture of potting soil (50%), perlite (25%) and peat moss (25%) during the normal growing season (autumn–winter–spring) 2015. Prior to sowing seeds for the experiments, the endophytic status of the seeds was confirmed by assessing the presence or absence of endophyte on the endosperm side of the seed (Gundel, Ueno, Panteix, & Iannone, 2018). The method allows determining the endophyte presence without compromising the seed and endophyte viability. They were kept under natural conditions and watered as required. Plants were exposed to ozone during the vegetative (tillering) or reproductive (spikes emerging from tillers) phases.

For treatment of the vegetative phase, 64 individual plants (3 months since sowing/≈5 tillers each) were randomly distributed among eight ozone chambers (eight plants/chamber). These 8 m^3 open-top chambers were cylinders (2 m ø) with transparent PVC plastic film and equipped with air conditioning to control the temperature (Hogsett, Tingey, & Holman, 1985). Four chambers were randomly assigned to the low-ozone treatment (≤10 ppb) while the others, remaining, were assigned to the high-ozone treatment (≈130 ppb). Based on the natural occurrence of ozone peaks of about 100 ppb (The Royal Society, 2008), the use of ozone concentrations between 100 and 130 ppb is common in plant experimental eco-physiology (see Abeli et al. (2017), Khaling et al. (2015), Ueno et al. (2016)). The contrasting levels of ozone were generated by a spark discharge-type ozone generator (OZ5000, Dobzono, AR), with the concentration of ozone within each chamber recorded by monitoring the 254 nm absorbance on an API-Teledyne model 450 ozone monitor (Teledyne Advanced Pollution Instrumentation Inc., USA). Ozone-enriched air was distributed among the chambers through a pipe system with delivery to each chamber regulated by a valve and mixed with a constant flow of activated carbon-filtered ambient air. The ozone generator was turned on at noon to simulate the natural peaks of the gas in the troposphere. After 2 weeks of ozone treatment, the plants were randomly placed back to the common garden. Approximately 2 months later, the same procedure was repeated with another set of 64 plants that were at the reproductive phase (spikes emerging from tillers). After finishing the ozone exposure, all the plants were returned to the common garden and maintained with watering until they reached maturity. The experiment was a full factorial design with three factors and two levels: Endophyte treatment (E+ and E−), ozone treatment (O_3+ and O_3−) and plant ontogenetic phase at ozone exposure (vegetative and reproductive). At the end of the growing cycle, all mature spikes per plant were harvested, threshed and seed production estimated. The number of seeds per plant was estimated by weighing three batches of 100 seeds each. Since there was no significant variation in seed weight due to the treatments (mean 1,000-seed weight ≈1.94 ± 0.10 g), seed production per plant was mainly explained by variation in seed number (data not shown).

2.3 | Endophyte transmission from plant to seeds

To evaluate the consequences of ozone exposure on endophyte transmission from colonized grass mother plants to seed, a group of 10 seed per spike from all spikes per plant were assessed for the

presence of *E. occultans* using microscopic observation as described by Latch and Vaughn (1995). The endophyte transmission efficiency was calculated as follows:

$$\%\ endophyte\ transmission\ efficiency = \frac{number\ of\ E\ +\ seed}{total\ number\ of\ evaluated\ seed} \times 100$$

2.4 | Dynamics of fungal endophyte viability in grass seed

To evaluate the consequences of ozone exposure on the endophyte persistence in seed, we assessed endophyte viability after the seeds were subjected to an accelerated ageing regime. It consists of storing seeds under deteriorate conditions (high temperature and humidity) and allow to compare different seed lots and study underlying mechanisms of longevity (Domergue, Abadie, Limami, Way, & Tcherkez, 2019). Here, the experimental conditions for seed artificial ageing were 40°C and relative humidity close to 75%, which have been shown to be detrimental to endophyte survival (Card, Rolston, Lloyd-West, & Hume, 2014; Gundel et al., 2009, 2010). Seed produced by all the plants was pooled by each treatment combination (plant ontogenetic phase at ozone exposure and ozone level) (low O_3 and high O_3), resulting in 16 batches. Seeds were stored in eight plastic boxes 13 × 13 × 14 cm (length, width and height) and within the base of each box set four open glass jars (100 cm^3 each), each containing a saturated solution of sodium chloride. This solution generated a nominal relative humidity of 75% (Meriaux, Wagner, Ducournau, Ladonne, & Fougereux, 2007). A plastic net (0.5 cm mesh) separated the seed from the NaCl solution. Thirty seed were placed in 1.5 ml Eppendorf tubes with pre-drilled holes (1 mm ø) in the lids to allow air exchange. A total of 24 tubes (4 treatments by 6 sampling times) were placed in each box. Each plastic box was hermetically closed and only opened at specific sampling times, 0 (no accelerated ageing), 7, 15, 22, 36 and 44 days, to assess endophyte viability. For every sampling time, the seeds were scattered in Petri dishes (90 mm ø, AGB-Indulab, AR) on filter paper with 5 ml of distilled water and incubated under optimal conditions for germination (15:25°C, 12:12 light: dark). Five-days old seedlings were subsequently transplanted to 30-cell seedling-trays (cell size: 2 cm ø and 7 cm depth) filled with a potting mix. The endophyte presence was evaluated each time in 25 seedlings obtained from viable seeds. However, the viability of the seeds was decreasing as time passed under artificial ageing. Therefore, by the end of the experiment, we had to increase the number of seeds as to be able to have enough number of seedlings to evaluate the endophyte viability. When plants were at the 2–3 tiller stage, they were assessed for endophyte within the first 3–4 mm of the leaf sheaths (Christensen, Bennett, & Schmid, 2002; Gundel, Ueno, et al., 2018). The presence of mycelia at this plant stage is an indication that the endophyte was alive in the seed. The proportion of seedlings, containing endophyte, was calculated as follows:

$$\%\ endophyte\ viability = \frac{number\ of\ E\ +\ seedlings}{total\ number\ of\ evaluated\ seedlings} \times 100$$

2.5 | Concentration of endophyte mycelia in seed

The concentration of *E. occultans* mycelia within seed tissue was estimated based on qPCR of genomic DNA levels isolated from endophyte-colonized seed using 7,500 Real-Time PCR System (Applied Biosystems, Foster City, USA) following the manufacturer's standard method for absolute quantification using FastStart Universal SYBR Green Master Mix (Roche Applied Science, DE) and primers at a final concentration of 500 nM (see the sensitivity's response of qPCR method in Figure S1 in Data S1). DNA extraction from 10 seeds of each treatment combination was performed using a CTAB method modified from Murray and Thompson (1980) and C. N. Stewart (1997) (see protocol in Data S1). Primers suitable for qPCR were designed for a fragment of *E. occultans* and *L. multiflorum* specific gene, encoding *translation elongation factor 1-alpha* (tef1; GenBank Acc. # AF457541; Moon, Craven, Leuchtmann, Clement, & Schardl, 2004) and *histone h3 exon 1-2* (H3; GenBank Acc. # AB205017; Takahashi, Oishi, Ikeda, Takamizo, & Komatsu, 2006), respectively. Primer sequences are listed in Table S1 in Data S1. The concentration of endophyte mycelia was expressed as the level of amplification of the fungal-specific gene per amplification level of the gene of the host plant (linearly related to plant biomass).

2.6 | Alkaloid concentration in seed

E. occultans is known to produce loline alkaloids in its host *L. multiflorum*, i.e., *N*-acetylnorloline (NANL), *N*-acetylloline (NAL) and *N*-formylloline (NFL). These alkaloids were measured using gas chromatography (GC) (Bastías et al., 2018; Moore, Pratley, Mace, & Weston, 2015). For the analysis of lolines, fresh, lyophilized and ground seed material (50 mg) was extracted for 1 hr with 50 μl of 40% methanol, 5% ammonia and 1 ml of the 1,2-dichloroethane (containing 54.8 ng ml^{-1} 4-phenylmorpholine as an internal standard). Then, the samples were centrifuged (8,000 g, 5 min) and the supernatant transferred to glass GC vials via a 10 μm filter for analysis. The analysis was conducted on a GC-flame ionization detector (GC2010Plus, Shimadzu Corporation, Japan) equipped with a ZB-5 capillary column (30 m × 0.32 mm × 0.25 μm film; Phenomenex, Torrance, CA, USA). The detection limit using this technique was 25 μg g^{-1} DW (dry weight).

2.7 | Statistical analysis

The effect of plant exposure to ozone (low and high) in two ontogenetic phases (vegetative and reproductive) on vertical endophyte

transmission efficiency and endophyte viability in seeds was analysed with mixed-generalized linear-effect models (lme4 package; Bates, Mächler, Bolker, & Walker, 2015), where the response variables assumed a binomial distribution with a logit link function (Crawley, 2007). In the case of vertical endophyte transmission efficiency, the model included a seed production of plant as a covariate. For the viability of endophyte in seed under conditions of accelerated ageing, a generalized linear mixed-effects model that specified the binomial family (link-"logit") (lme4 package; Bates et al. (2015)) was used. The ozone treatment, the ontogenetic phases (vegetative and reproductive), the time under accelerating ageing condition (0, 7, 15, 22, 36 and 44 days) and their interactions were considered categorical and fixed variables, while the plants nested within chambers were considered as random effects. No over-dispersion was observed.

The concentration of fungal mycelia and endophyte-derived alkaloids (total lolines, and the derivatives NAL, NFL and NANL) were analysed with a mixed linear-effects model (nlme package; Pinheiro, Bates, DebRoy, Sarkar,, & R Core Team, 2018) where the maternal history of ozone exposure and ontogenetic phases were considered as fixed effects and the plant nested within chambers as random effects. For total lolines, a varldent variance structure was used to meet ANOVA assumptions (Zuur, Ieno, Walker, Saveliev, & Smith, 2009).

Finally, the relationships between the concentration of fungal mycelia and total lolines in the seeds, and these two variables with production of seeds at individual plant level, were estimated by linear mixed-effects model using the nlme package and normal distribution (Pinheiro et al., 2018), considering the same random structure. Conditional R^2 (i.e., proportion of variance explained by both the fixed and random factors) was reported for each relationship (Nakagawa & Schielzeth, 2013). For all analyses, we reduced the model estimated by maximum likelihood, removing non-significant terms in a stepwise fashion (single term deletion strategy), and selected the optimal models following Zuur et al. (2009). All analyses were run with the statistical program R (R Core Team, 2018).

3 | RESULTS

3.1 | Endophyte transmission to seed

Exposing endophyte-colonized *L. multiflorum* plants to ozone at the plant's vegetative and reproductive phases did not affect the transmission of *E. occultans* to seed (χ^2 = 0.01, p = .934). The vertical transmission efficiency of endophyte across all treatments was very high with an average of 97.5% (Table 1). Moreover, this response variable was not related to variation in seed production per plant (χ^2 = 3.25, p = .071).

3.2 | Concentration of endophyte mycelia in seed

The concentration of fungal mycelia in seed was not influenced by host plant exposure to ozone ($F_{1,6}$ = 0.18, p = .681). The average of relative abundance of fungal DNA in seed expressed as "ng DNA fungus/ng DNA plant" was 3, with a minimum value of 2.6 and a maximum value of 3.4 (Table 1).

3.3 | Alkaloid concentration in seed

Neither the concentration of total loline alkaloids ($F_{1,6}$ = 0.14, p = 0.718), nor that of each individual loline (NFL: $F_{1,6}$ = 0.13, p = .726; NANL: $F_{1,6}$ = 0.27, p = .616; NAL: $F_{1,6} = 0.83 \times 10^{-3}$, p = .977) in the seeds were affected by the exposure of plants to high concentrations of ozone. The most abundant loline type was NFL with ≈87% of the total, followed by NANL (≈11%) and finally NAL (≈2%) (Table 1).

3.4 | Dynamics of fungal endophyte viability in grass seed

The viability of the fungal endophyte in host seed declined with increasing periods of accelerated ageing and followed a negative

TABLE 1 Effects of ozone exposure (low: ≈0 ppb, and high: ≈130 ppb) on the performance of the endophyte *Epichloë occultans* within *Lolium multiflorum* plants

Response variable		Ozone treatment	
		Low	High
Endophyte transmission frequency (%)		97 (±0.01)	98 (±0.004)
Relative abundance of fungal DNA		3.35 (±0.83)	2.99 (±0.43)
Loline alkaloid concentration (μg g^{-1} DW)	NFL	657.60 (±58.45)	684.90 (±55.40)
	NANL	82.57 (±7.09)	89.94 (±9.03)
	NAL	9.53 (±1.58)	8.77 (±0.99)
	Total	749.70 (±63.63)	783.70 (±63.31)

Note: Performance was measured in terms of the vertical endophyte transmission efficiency to seeds (n = 64), the concentration of endophyte mycelia in seed (n = 20) and the concentration of endophyte-derived loline alkaloids in seed [total = *N*-formylloline (NFL) + *N*-acetylnorloline (NANL) + *N*-acetylloline (NAL)] (n = 20). Values are means ± *SE*.

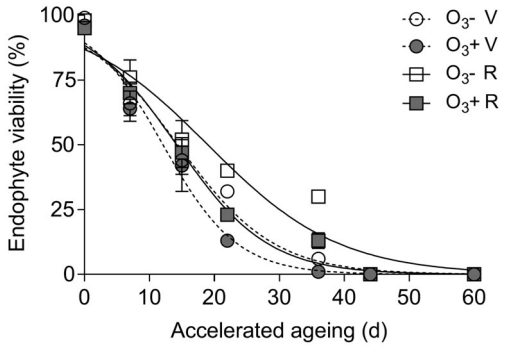


FIGURE 1 Viability dynamics of the endophyte *Epichloë occultans* in seed stored under accelerated ageing conditions (40°C and 75% RH) harvested from *Lolium multiflorum* plants exposed to low (≈0 ppb) or high (≈130 ppb) concentrations of ozone (white and grey symbols, respectively) at vegetative (V) and reproductive phases (R) (circle and square symbols, respectively). The viability of the endophyte was determined by the observation of hyphae in young seedlings from the seeds and is expressed as a proportion of "number of seedlings with endophyte/number of evaluated seedlings". Symbols are means ± *SE* (*n* = 4, 25 seedlings each), and the lines correspond to the fitted models for each population

sigmoidal curve (Figure 1). The dynamics of endophyte viability depended on whether the mother plants had or had not been exposed to ozone. Specifically, endophyte viability declined at a faster rate in seed produced by plants exposed to ozone at either of the two assessed life phases, vegetative or reproductive ($\chi 2 = 13.15$, $p = .022$) than seeds from control plants (i.e., those not exposed to ozone). On average, the time at which endophyte viability reached 50% survival was 13 and 17 days in seeds produced by plants exposed to high- and low-ozone concentrations, respectively (Figure 1).

3.5 | Relationships between parameters of performance of the fungal endophyte in the offspring and mother plants

None of the relationships explored in this section were dependent on plant exposure to ozone (see Data S1). The concentration of total lolines was positively related to the concentration of fungal mycelia in seed ($F_{1,30} = 9.30$, $p = .005$) (Figure 2a). Similarly, the concentration of alkaloids (total lolines) in seed was positively related to plant seed production ($F_{1,26} = 4.76$, $p = .035$) (Figure 2b). However, the concentration of endophyte mycelia in seed was not related to plant seed production ($F_{1,29} = 0.01$, $p = .928$) (Figure 2c).

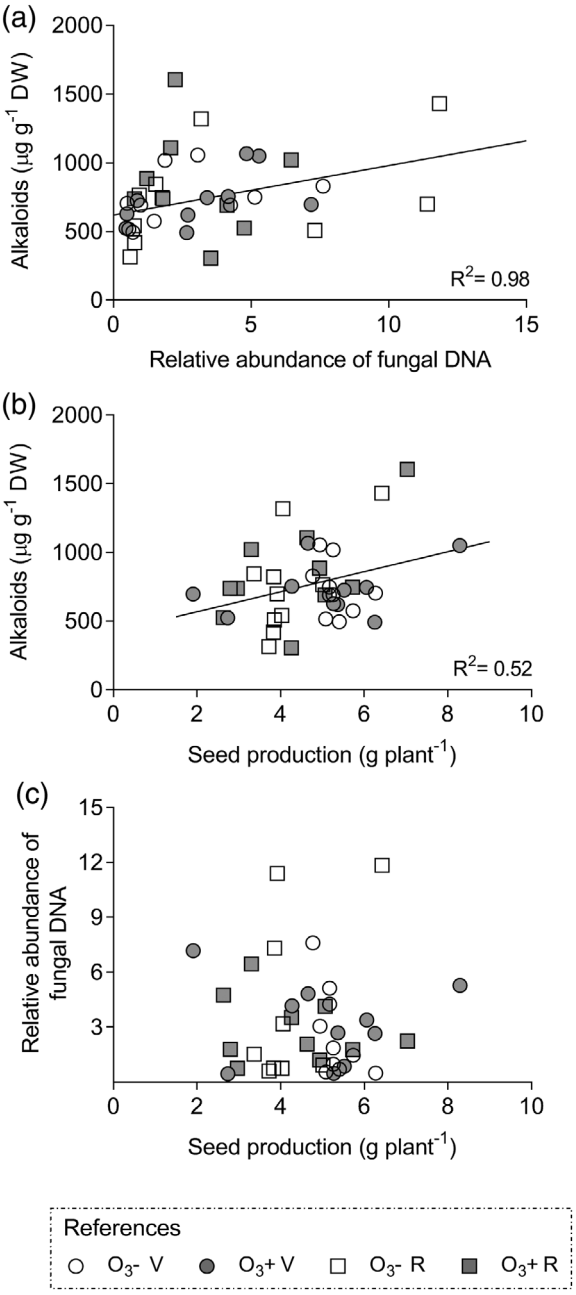


FIGURE 2 Relationships between performance parameters of the fungal endophyte *Epichloë occultans* and *Lolium multiflorum* host plant fitness exposed to low (≈0 ppb) and high (≈130 ppb) levels of ozone (grey and white symbols, respectively) at vegetative and reproductive phases (circle and square symbols): (a) concentration of total lolines in seed and endophyte mycelial concentration in seed, (b) concentration of total lolines in seed and host plant seed production, and (c) endophyte mycelial concentration in seed and host seed production. Each point corresponds to an individual plant (*n* = 10 per treatment). When statistically significant, the lines of fitted linear regressions are shown

4 | DISCUSSION

Based on previous studies showing that grass-endophyte mutualistic interactions are finely tuned by the oxidative balance at the tissue level (Eaton, Cox, & Scott, 2011; White & Torres, 2010), we predicted that ozone-induced oxidative stress would disrupt the highly-synchronized association between both fungal and plant partners. This disruption would have consequences for the endophyte transmission to – and persistence in – the seeds, and for any advantageous

trait conferred by these fungi to their grass hosts, i.e., protection from invertebrate herbivory via the production of insect deterrent and insecticidal endophyte-derived alkaloids (Schardl et al., 2007, 2013). Here, we found that exposure of endophyte-colonized *L. multiflorum* plants to high levels of ozone did not affect either the vertical transmission frequency of endophyte from plant to seeds or the amount of endophyte mycelia found within these seeds. The viability of seed-associated endophyte, produced from plants exposed to ozone, was, however, impaired particularly after increasing periods of accelerated ageing. This technique is designed to mimic the natural physiological ageing process of seed and is used to assess seed vigour and storage characteristics of particular fungal strains (Domergue et al., 2019). Therefore, the ozone effect was not apparent on growth variables, but it did affect fungal persistence. In addition, we found a positive relationship between the concentration of lolines and endophyte mycelia within seed, and a positive correlation between alkaloid concentration and plant fitness (seed production).

In local populations, the number of symbiotic individuals (i.e., incidence) is a measure of the endophytes ability to improve plant fitness and to transmit to successive generations. If the endophyte does not provide a benefit to the host plant (increased resistance to pest pressure, abiotic stress), then due to the energy sink, the endophyte represents, colonized individuals will be out-competed by non-colonized equivalents. To maintain colonized individuals in the population, the fungal endophyte also has to transmit through all the vital plant development phase transitions, as their existence and dispersal are completely dependent on the plant host (Gundel et al., 2008; Gundel, Garibaldi, Martínez-Ghersa, & Ghersa, 2011; Ravel et al., 1997). We found here that the endophyte transmission frequency from plant to seed was not disrupted by the mother plant's exposure to ozone. It is possible that the plant and the endophyte had fully recovered from the oxidative stress imposed by the exposure to ozone at the plant vegetative phases, resulting in nearly 100% transmission efficiency. In previous studies, we also found that the stress caused by sub-lethal doses of a herbicide (diclofop-methyl) at the seedling stage in *L. multiflorum* did not affect the transmission of the endophyte to the seed (Gundel, Garibaldi, et al., 2011; Gundel et al., 2012). Interestingly, even ozone applied during the reproductive phase of the plant did not have any effect on transmission efficiency, although this phase has been indicated as being critical in the transmission process (Gundel, Garibaldi, et al., 2011; Gundel, Rudgers, et al., 2011). A recent transcriptomic study with *Schedonorus arundinaceus* Schreb. (formerly *Festuca arundinacea*; common name: tall fescue) showed that the gene expression profile of the endophyte, *E. coenophiala*, during the reproductive phase is mostly related to proteins and enzymes (e.g., heat-shock proteins, catalase, superoxide dismutase) for the control of oxidative stress (Nagabhyru, Dinkins, & Schardl, 2019). This can likely explain the high resilience of the grass-endophyte symbiotic system –in terms of transmission efficiency and growth within the seed– at this critical phase when facing stressful situations.

The endophyte has to persist viable during the quiescent state of the seed to colonize the new seedling (i.e., seed-to-seedling transmission). The factors affecting endophyte viability in the seed have been the focus of intense research. Fungal endophytes generally lose viability at a faster rate compared to the host seed although plant and endophyte genotype, length of storage and certain environmental conditions (such as high temperature and relative humidity) can also impact endophyte survival (Christensen et al., 2002; Gundel et al., 2009, 2010; Gundel, Ueno, et al., 2018; Latch & Vaughn, 1995; Rolston, Hare, Moore, & Christensen, 1986). Although the relationship between endophyte viability and endophyte-derived alkaloids in seed is still unknown (Gundel, Rudgers, & Ghersa, 2011), certain evidence suggests that the endophyte-conferred protection to herbivores in seedlings remains even though the fungus was no longer alive (Hewitt, Mace, McKenzie, Matthew, & Popay, 2020; A. V. Stewart, 1985). Our work provides evidence that the variation in the longevity of the endophyte is not necessarily related to the mycelial concentration achieved by the fungus in the seed. However, our results suggest that the maternal environment can impact on the longevity of the endophyte in the seeds with high ozone levels reducing the longevity, irrespective of the ontogenetic phase of the plant life cycle at which ozone was applied. Evidently, even though no change in mycelial concentration in the seeds was detected, it is possible that an oxidative imbalance, resulting from ozone exposure, could have affected the viability of the endophyte. Previous research also failed to detect changes in antioxidant content in seeds produced by endophyte-colonized plants exposed to ozone (Gundel et al., 2015). Understanding why fungal endophytes lose viability more rapidly in seeds produced by ozone-stressed plants merits more research.

High concentrations of endophyte-derived alkaloids have been determined in seed (Gundel et al., 2018; Justus et al., 1997; TePaske et al., 1993; Vazquez de Aldana et al., 2003). These alkaloids are proposed to play an ecological role, protecting grass seed and seedlings against granivores and herbivores (Gundel, Seal, et al., 2018; Hewitt et al., 2020; A. V. Stewart, 1985; Uchitel et al., 2011). The concentration of endophyte-derived alkaloids may be directly related to the level of protection conferred by a particular endophyte genotype (Bastías et al., 2018; Wilkinson et al., 2000). However, factors that regulate the endophyte-derived alkaloid production have been mostly studied in green tissues of plants and scarcely in seed (Gundel et al., 2017; Gundel, Seal, et al., 2018). A positive correlation has been observed between the concentration of endophyte-derived alkaloids and endophyte mycelial concentration in vegetative tissues of tall fescue (*S. arundinaceus*) and perennial ryegrass (*Lolium perenne* L.) (Easton, Latch, Tapper, & Ball, 2002; Keogh, Tapper, & Fletcher, 2010; Rasmussen et al., 2007); a relationship that can be shifted if plants grow under high concentrations of CO_2 (Ryan et al., 2014). In our work, neither the concentration of alkaloids nor that of hyphae in seed was modified by the maternal history of exposure to ozone. Strikingly, when we correlated parameters associated with the performance of both partners, it was loline concentration not mycelial concentration that correlated positively with host plant fitness (the higher the seed production per plant, the higher concentration of loline alkaloids in seeds). Although changes in alkaloid concentration can also be explained by changes in the synthesis rate (Bastías et al., 2018; Ryan

et al., 2014), our results suggest that the accumulated lolines in seed are not all produced in situ but are mobilized from the vegetative tissue where other parts of the endophyte are metabolically active during seed filling. Loline alkaloids have shown to be highly mobile and able to be translocated long distances within the plants (Justus et al., 1997). Finally, our analysis is supported by a recent transcriptomic approach which found that genes for alkaloids biosynthesis (including those for lolines) are mostly expressed in vegetative tissues, such as the pseudo-stems, rather than in reproductive tissues, such as inflorescence primordia and ovaries (Nagabhyru et al., 2019).

The results from this study are relevant for understanding the ecological dynamics of the plant-endophyte symbiosis in natural and managed ecosystems. Vertical transmission efficiency, which also involves endophyte persistence in seed, is an important process controlling the incidence of *Epichloë* fungal endophytes in grass populations (Afkhami & Rudgers, 2008; Cavazos et al., 2018; Gundel, Garibaldi, et al., 2011; Gundel, Rudgers, et al., 2011). In addition, this study showed that there was an alignment of the partners' fitness; the higher the seed production, the higher multiplication rate of the fungus and higher concentration of alkaloids in seed. As it has been shown at the plant vegetative phases (e.g., Wilkinson et al., 2000), we may presume there is a direct link between alkaloid concentration in seed and the endophyte-conferred protection against granivores. It has been suggested that the higher seed production of endophyte-symbiotic plants compared to endophyte-free plants may be explained to the accumulation of the fungal defensive compounds in reproductive structures (Faeth, 2009; Gundel, Seal, et al., 2018). Here, the relationship between functional traits (in this case, the potential benefit of alkaloids) of the endophyte and the ecological fitness of host plants shows the synergism among the partners and contributes to explain why the symbiosis is so common in certain cool-season grass populations all over the world.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ORCID

Andrea C. Ueno https://orcid.org/0000-0002-7853-5270

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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