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Plant damage, seed production and persistence of the fungal endophyte *Epichloë occultans* in *Lolium multiflorum* plants under an herbivore lepidopteran attack and ozone pollution

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Abstract. Plants are expected to face novel challenges as consequence of human-driven global change. Outbreaks of pests and higher incidence of contaminants are increasing. Plants can improve tolerance to stress factors through associations with symbiotic microorganisms. Certain grasses establish persistent and asymptomatic symbioses with *Epichloë* fungal endophytes, which are known to confer protection against Herbivores and improve plant tolerance to abiotic stress factors. Nonetheless, accumulating evidence suggests the symbiosis outcome is context-dependent. We evaluated the capacity of the endophyte fungus *E. occultans* in protecting the annual grass *Lolium multiflorum* against a spontaneous larva attack of the generalist herbivore *Agrotis ipsilon* under episodic exposure of plants to ozone. Symbiotic and non-symbiotic plants were individually grown outdoors and exposed to ozone at different stages resulting in four treatments: control (plant never exposed to ozone), plant exposed to ozone at vegetative stage, plant exposed to ozone at reproductive stage, and plant exposed to ozone at both stages. After the last exposure, there was an outbreak of *A. ipsilon* larvae. We evaluated herbivore damage, seed production per plant, and endophyte transmission to the seeds. Frequency of attacked plants was irrespective of both the endophyte and ozone exposure. However, the damage level per plant was only reduced by the endophyte. Seed production was slightly lower in endophyte-symbiotic plants and not affected by ozone. Interestingly, herbivore damaged and undamaged endophyte-symbiotic plants contributed equally to seed production. However, in plants exposed to ozone once at vegetative or reproductive stage, endophyte-free undamaged plants had higher seed production than endophyte-free damaged plants. Ozone treatments did not affect the transmission efficiency of endophytes to the seeds. Mean endophyte transmission efficiency per plant was 95%. Medium doses of ozone seem to have undetectable effects on grass-endophyte symbiosis, not affecting the defensive mutualism nor the persistence of the symbiont across generations.

[Keywords: symbiosis, defensive mutualism, grass-endophyte symbiosis, context-dependency, resistance, tolerance]

Resumen. Daño, producción de semillas y persistencia del hongo endófito *Epichloë occultans* en plantas de *Lolium multiflorum* bajo un ataque de herbívoros lepidópteros y contaminación por ozono. Las plantas enfrentan nuevos desafíos debido al cambio global impulsado por las actividades humanas. Mientras los brotes de plagas y la incidencia de contaminantes están en aumento, las plantas pueden mejorar su tolerancia al estrés asociándose con microorganismos. Ciertas gramíneas establecen simbiosis persistentes y asintomáticas con endófitos fúngicos *Epichloë*, y así obtienen protección contra herbívoros y tolerancia al estrés abiótico. No obstante, los resultados de la simbiosis dependen del contexto ecológico. Evaluamos la capacidad del hongo endófito *E. occultans* para proteger el pasto anual *Lolium multiflorum* contra un ataque de larvas del herbívoro generalista *Agrotis ipsilon* bajo exposición episódica de las plantas al ozono. Cultivamos plantas simbióticas y no-simbióticas en macetas y las expusimos a ozono en diferentes etapas, resultando en cuatro tratamientos: control (nunca expuesta al ozono), planta expuesta al ozono en etapa vegetativa, planta expuesta al ozono en etapa reproductiva y planta expuesta al ozono en ambas etapas. Luego, hubo un brote de larvas de *A. ipsilon*. Evaluamos el daño causado por las larvas, la producción de semillas por planta y la transmisión del endófito a las semillas. La frecuencia de plantas atacadas fue independiente de la presencia del endófito y del ozono. Sin embargo, el nivel de daño por planta sólo fue afectado y reducido por el endófito. La producción de semillas fue ligeramente menor en las plantas con endófito y no afectada por el ozono. Curiosamente, las plantas simbióticas dañadas y no-dañadas presentaron igual producción de semillas. No obstante, las plantas no-simbióticas no-dañadas produjeron más semillas que las dañadas cuando fueron expuestas una vez a ozono en etapa vegetativa o reproductiva. El ozono no afectó la eficiencia de transmisión de los endófitos a las semillas, la cual fue, en promedio, 95%. El ozono, en dosis medias, parece tener efectos indetectables sobre la simbiosis planta-endófito.

[Palabras clave: simbiosis, mutualismo defensivo, simbiosis pasto-endófito, resistencia, tolerancia]

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INTRODUCTION

Worldwide alterations on plant production caused by global change factors is a hot topic in past and current literature, and some impressive changes are predicted to happen over the next century (Fuhrer 2003; Thomson et al. 2010; IPCC Climate Change 2014). Outbreaks of insect plagues and diseases are expected to increase because of global warming (de Sassi and Tylianakis 2012). Previous studies have shown that the increase of CO_2 in the atmosphere usually stimulates plant production (Long at al. 2004); however, it can also lead to an elevation of the global surface temperature and potentiate drought events (Dai 2011; IPCC Climate Change 2014). Less investigated, ozone (O_3) is another gas increasing in the troposphere which causes oxidative stress on biological systems (Holopainen et al. 2002; Valkama et al. 2007; Lindroth et al. 2010). Although ozone can elicit certain signaling pathways in plants and increase the resistance against herbivores and pathogens (Kangasjärvi et al. 1994; Sandermann et al. 1998; Menéndez et al. 2009), these effects may be conditional if defensive mechanisms are mediated by symbiotic microorganisms (Ueno et al. 2016).

The use of symbiotic microorganisms as biological control agents for pests and diseases has significantly grown worldwide as an alternative to reduce chemical products in agriculture (Azevedo et al. 2000). As part of this group, *Epichloë* fungal endophytes (family Clavicipitaceae) of grasses are considered a powerful biotechnological tool on the control of diseases, arthropod pests, mammals and birds (Kundau and Bacon 2008; Gundel et al. 2013a; Johnson et al. 2013; Finch et al. 2015; Lugtenberg et al. 2016). An advantage of this symbiosis is that *Epichloë* fungi occur in aboveground tissues and can be maternally inherited with high efficiency (Schardl 2010; Rudgers et al. 2009; Gundel et al. 2011); therefore, once plant cultivars have been inoculated, seed companies have to secure the endophyte persistence in the commercial product, that is 'the seed' (Johnson et al. 2013; Gundel et al. 2011, 2013a).

Several important species of cool-season grasses used for turf and forage (of genera *Festuca* and *Lolium*) usually form this association with fungal endophytes which by producing a variety of alkaloids, offer effective mechanisms of protection against herbivores to host plants (Clay and Schardl 2002; Rudgers et al. 2009; Saikkonen et al. 2013; Bastias et al. 2017a). Although endophytes can also enhance plant tolerance to abiotic stress in general (reviewed in Malinowski and Belesky 2000; Hamilton et al. 2012), it has been observed that previous exposure of plants to certain abiotic factors (e.g., drought, herbicide, ozone) can affect the endophyte-mediated anti-herbivore mechanism in host plants (Miranda et al. 2011; Gundel et al. 2012; Ueno et al. 2016). Therefore, the performance of fungal endophytes as an effective biological control tool requires that the symbiotic interaction between plant and fungus to be stable in time and under varying environmental conditions. Global change modelling forecasts an increase of the occurrence of extreme environmental events such as drought and flooding as well as an increase of tropospheric contaminants (Fuhrer 2003; Thomson et al. 2010; Dai 2011; IPCC Climate Change 2014) which could destabilize the symbiotic interactions and lead, in certain cases, to extinction (Brosi et al. 2010; Kiers et al. 2010).

Raising of the tropospheric concentration of ozone is considered a relevant issue caused by global change (Ashmore and Bell 1991; Booker et al. 2009). High concentrations of ozone in the air can negatively affect the human respiratory system, causing for instance, chronically respiratory diseases (U.S. EPA 2006). Originally, this problem was found to be concentrated in urban areas; however, it was soon realized that high concentration of ozone was expanding into rural areas, negatively affecting not only the air quality but crop production and natural systems as well (Mauzerall and Wang 2001; Fiscus et al. 2005). Usually studied in combination with CO_2 and temperature, ozone effects on plant interactions with herbivores and diseases have received certain attention (Holopainen 2002; Valkama et al. 2007; Lindroth 2010; Mikkelsen et al. 2015). As ozone concentration is highly variable in both space and time, plants and animals can be exposed for short episodic periods. Ozone can alter the interaction of plants with herbivores directly if both, plants and animals, are exposed to the contaminant or indirectly when herbivores land on plants previously exposed to the gas (Holopainen 2002). Ozone can affect plant structure and growth (Heagle et al. 1999; Martínez-Ghersa and Radosevich 2009), modifying chemical composition and activating (Sandermann et at. 1998) or deactivating (e.g., Ueno et al. 2016) defense mechanisms. Although ozone effects on plant-enemy interactions can depend on the affected defense mechanism against different feeding guilds of herbivores, it could risk the effectiveness of biological control agents increasing the dependency of crops on pesticides.

As we previously stated, *Epichloë* fungal endophytes have been proposed as effective biological control agents as they have been shown to protect host plants against a variety of harmful pests (Kuldau and Bacon 2008). Nonetheless, their success could be limited by natural environmental variability and the increasing incidence of contaminants in the context of climate change (IPCC Climate Change 2014). Here, we explored the capacity of the endophyte *Epichloë occultans* to protect host grass *Lolium multiflorum* against the generalist herbivore *Agrotis ipsilon* (common name: black cutworm) under ozone-enriched atmospheric conditions. A previous study showed that plants of the same grass species lost the endophyte-conferred protection against the generalist aphid *Rhopalosiphum* y *padi* (bird cherry-oat aphid) when exposed to ozone (Ueno et al. 2016). However, here we may expect a different response pattern since *A. ipsilon* larvae are chewers and they can respond differently to alkaloids and hormone defense mechanism of plants (Saikkonen et al. 2013; Bastias et al. 2017a). Given that fungal endophytes protect host grasses not only against herbivore but also against abiotic stress factors (Hamilton et al. 2012), our prediction is that endophyte will be an effective anti-herbivory mechanism under ozone contamination. We also assessed the stability of the symbiotic interaction by comparing the relative fitness of endophyte-symbiotic *vs.* endophyte-free plants under experimental enrichment with ozone and herbivory from a spontaneous lepidopteran outbreak, and explored the consequences for symbiosis persistence by checking the endophyte transmission to the next generation (see Gundel et al. 2008, 2011).

Materials and Methods

Study system

Epichloë occultans (Moon et al. 2000), previously known as *Neotyphodium occultans* (Leuchtmann et al. 2014), in association with Italian ryegrass (*Lolium multiflorum* Lam.) produces loline alkaloids, which protect the host plants against a wide range of insect pests (Gundel et al. 2012; Ueno et al. 2016; Bastias et al. 2017b). *Agrotis ipsilon* Hufnagel is a generalist caterpillar herbivore of cool season grasses. It can feed and develop in some endophyte grass associations (Williamson and Potter 1997). However, previous research has shown that development of *A. ipsilon* larvae in perennial ryegrass (*Lolium perenne* L.) and *Schedonorus arundinaceous* can be affected by fungal endophytes (Kunkel and Grewal 2003; Baldauf et al. 2011). In plants infected with *Epichloë festucae* var. lolii (formerly *N. lolii*), the development of the larvae was slower compared to development in endophyte free plants (Kunkel et al. 2003; Bixby and Potter 2010).

Plant material

Native to Mediterranean, *L. multiflorum* is cultivated as a high-quality forage grass in temperate regions of the world. Naturalized populations in grasslands of the Argentine Pampas exhibit high incidence of endophytes (Gundel et al. 2009). The population used in this work was brought as seeds from a successional field from Inland Pampa (36°00′ S - 61°5′ W). Seeds were hand harvested and once in the laboratory, the fungal incidence was evaluated by looking for the endophyte in 100 seeds under microscope using the seed squash technique (Card et al. 2011). After confirming that the endophyte incidence was very high (95%), one gram of seeds was treated with a systemic fungicide to kill the fungus (Vila-Aiub et al. 2005). Fungicide treated and untreated seeds were sown in independent 1 m^2 plots as to obtain fresh seeds from the F1 (for both seed lots, 1 g [~500 plants/plot] of seeds was homogeneously distributed in each plot). At flowering, plants could exchange pollen as to avoid genetic differentiation between both seeds lots (Gundel et al. 2012). At maturity, seeds were harvested, and the endophyte incidence checked as before.

Experiment

Seeds of *L. multiflorum* symbiotic (E+) and non-symbiotic (E-, without endophyte) were incubated under optimal conditions for germination (alternating temperature: 15/25 °C; photoperiod: light/dark [12/12 h]) (ISTA 1999). Young seedlings were individually transplanted into 1.5 L plastic pots filled with a mix of black organic soil (50%), peat (25%) and sand (25%). The initial number of plants per biotype was 150 plants (150 E+ and 150

E-) that were randomly placed in a common garden at the College of Agronomy, University of Buenos Aires (34°35′28.48′′ S - 58°28′47.56′′ W). The pots were watered periodically as to avoid water deficit.

When plants had 4-5 tillers, they were placed into open top chambers equipped with an ozone generator (spark discharge-type ozone generator) (Hogsett et al. 1985) capable of deliver and maintain different concentrations. Two chambers were enriched with ozone (~70 ppb) and two were kept at natural air condition. In plants trials, this ozone concentration is considered a medium-level-dose (Hansen et al. 2019). Plants were exposed to ozone four hours per day (from 11 am to 15 pm) during a month (August 6 - September 6) at vegetative stage and another exposure from October 6 to November 6 at reproductive stage. The assignment of each plant to a given treatment was at random. Thus, the set of plants in each ozone treatment was independent. For example, plants assigned to receive ozone at reproductive stage, were placed in control chambers during plant exposure to ozone at vegetative stage but placed in ozone-enriched chambers during plant exposure to ozone at reproductive stage. Before, between, and after these periods in which ozone were not applied, all the plants were kept outside the chambers, in the common garden. The plants assigned to a given treatment were periodically rotated among chambers as to avoid any potential bias associated to a chamber effect. The number of plants in each ozone treatment for both biotypes was between 30 and 40 except for the control (never exposed to ozone) in which there were only 15 plants. Besides the control, the resulting treatments were ozone exposure at vegetative stage (Vegetat), ozone at reproductive stage (Reprod), and ozone at both reproductive and vegetative stages (Veg&Rep), respectively. After the last ozone exposure, the plants were randomly placed back into the common garden until maturity was reached.

After the last ozone treatment (at plant reproductive stage), a spontaneous outbreak of larvae of the Lepidoptera noctuid moth *Agrotis ipsilon* affected the plants over the whole experiment. The damage caused by the larvae was the typical cut on leaves and tillers. We recorded the number of damaged plants and the severity of the damage as the proportion of tillers affected over the total number of tillers per plant. By the end of the growing cycle, all the plants were individually harvested to estimate the total seed production. To evaluate the stability of the symbiotic association to the next generation, we estimated in E+ plants the efficiency with which the endophyte was vertically transmitted to the seeds. Seeds from endophyte-free plants were also evaluated just to confirm the endophyte symbiotic status (E-). We checked the endophyte presence in 10 seeds from 10 randomly selected plants (independently of the herbivore damage) from each ozone treatment (including the control) under light microscope (seed squash technique) (Card et al. 2011).

Statistics

To analyze the frequency of plants damaged by the herbivore, we fitted a generalized linear model in R (R Core Team 2016) with a binomial distribution and logit link function. The variable 'number of damaged plants over the total number of plants' was used as the dependent variables and endophyte and ozone were treated as fixed factors. To analyze the damage severity in attacked plants, we fitted a second generalized linear model with a binomial distribution and logit link function. The variable 'number of damaged tillers from the total number of tillers per plant' was used as the dependent variable with endophyte and ozone were treated as fixed factors, and number of tillers per plant (a measure of plant size) was used as a covariate. Significance was tested with Type II Likelihood Ratio Test (car package; Fox and Weisberg 2011). We corroborated overdispersion assumption with the function glm.binomial.disp from dispmod library. To analyze the seed production per plant we fitted a linear model with a normal distribution, endophyte and ozone as fixed factors, and number of damaged tillers per plant as a covariate. We corroborated homoscedasticity and normality assumptions with Levene test and QQplot. To analyze relative seed production between undamaged and damaged plants we calculated confidence intervals for each treatment quotient with the online GraphPad calculation page (http://www.graphpad.com/quickcalcs/) based on Fieller method (Fieller 1940). The ozone effect on the endophyte transmission efficiency (proportion of endophyte-symbiotic seeds out 10 evaluated seeds per plant) was tested with a generalized linear model with a binomial distribution and logit link function.

Results

The frequency of plants damaged by the herbivore was not affected by either the endophyte (X_1=1.206; P=0.272) or the ozone (X_3=1.176; P=0.759) treatments (Figure 1), although it depended positively on the number of tillers per plant (X_1=36.738; P<0.001) (data not shown). However, the severity of the damage –the proportion of affected tillers per plant– was mainly reduced by the endophyte presence (X_1=11.821; P<0.001) and associated to total number of tillers per plant (X_1=4.451; P<0.034); while it was not affected by ozone treatments (X_3=2.445; P=0.485) (Figure 1). On average, E+ plants presented a lower level of damage than E- plants (E+ ~16.13 *vs.* E- ~21.80).

Seed production of individual plants was affected by the endophyte (F_1=5.522; P=0.019) and not by ozone (F_3=2.538; P=0.058) (Figure 2). Seed production was 8.4% lower in symbiotic plants than in endophyte-free plants which represents, on average, 100 seeds less per plant. Individual seed production was negatively related with the number of damaged tillers per plant (F_1=8.347; P=0.004).

The analysis of the relative contribution of damaged and undamaged plants to

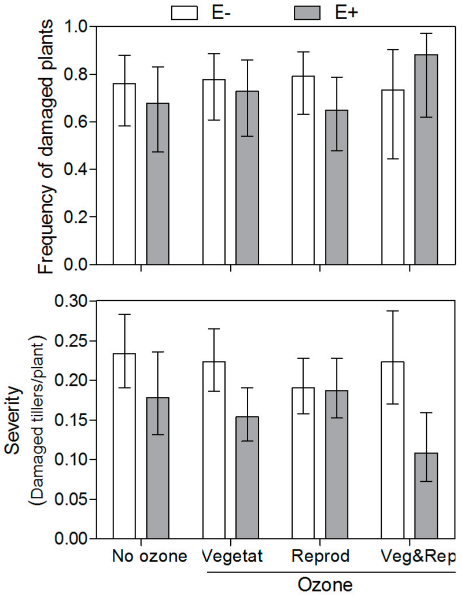

Figure 1. Frequency of damaged plants (upper panel) and severity of the attack (lower panel) by larvae of the herbivore *Agrotis ipsilon* to *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E-) with the fungus *Epichloë occultans* exposed to ozone at different stages: vegetative (Vegetat), reproductive (Reprod) or both (Veg&Rep), and no exposed (No ozone). Values are mean±95% CI.

Figura 1. Frecuencia de plantas dañadas (panel superior) y severidad de daño (panel inferior) causado por el ataque de larvas del herbívoro *Agrotis ipsilon* en las plantas de *Lolium multiflorum* simbióticas (E +) y no simbióticas (E-) con el hongo endófito *Epichloë occultans* y expuestas a ozono en diferentes etapas: vegetativa (Vegetat), reproductiva (Reprod) o ambas (Veg&Rep), y no expuestas (sin ozono). Los valores son promedios±95% IC.

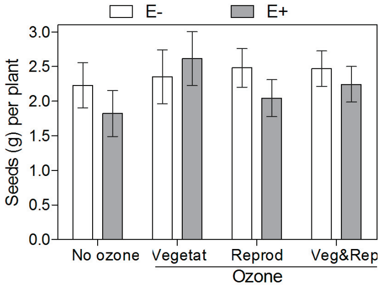

Figure 2. Seed production per plant (g) of *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E-) with the fungus *Epichloë occultans* exposed to ozone at different stages: vegetative (Vegetat), reproductive (Reprod) and both (Veg&Rep), and no exposed (No ozone). Values are mean±95% CI.

Figura 2. Producción de semillas por planta (g) de *Lolium multiflorum* simbióticas (E+) y no simbióticas (E-) con el hongo *Epichloë occultans*, expuestas a ozono en diferentes etapas: vegetativa (Vegetat), reproductiva (Reprod) o ambas (Veg&Rep), y no expuesta (sin ozono). Los valores son promedios±95% IC.

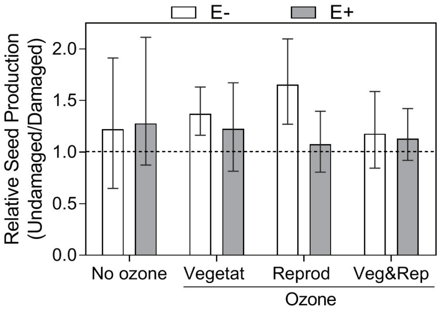

Figure 3. Seed production of undamaged relative to damaged plants (relative seed production) by the herbivore *Agrotis ipsilon* larvae to *Lolium multiflorum* symbiotic (E+) and non-symbiotic (E-) with the fungus *Epichloë occultans* exposed to ozone at different stages: vegetative (Vegetat), reproductive (Reprod) or both (Veg&Rep), and no exposed (No ozone). Values are mean±95% CI. The dashed line at Y=1 means that both type of plants (herbivore-damaged and undamaged) had the same production of seeds.

Figura 3. Producción relativa de semillas (producción de semillas de plantas no dañadas relativo a la de plantas dañadas por larvas del herbívoro *Agrotis ipsilon*) en plantas de *Lolium multiflorum* simbióticas (E+) y no simbióticas (E-) con el hongo *Epichloë occultans*, expuestas a ozono en diferentes etapas: vegetativa (Vegetat), reproductiva (Reprod) o ambas (Veg&Rep), y sin exposición (sin ozono). Los valores son promedios±95% IC. La línea discontinua en Y=1 significa que ambos tipos de plantas (dañadas y no dañadas por las larvas) tuvieron la misma producción de semillas.

average seed production per plant indicated that endophyte symbiotic plants tended to produce equally well under herbivore attack and at all ozone exposures. The same response pattern was observed in endophyte-free plants except when they were exposed to ozone only one time, at either the vegetative or the reproductive stage (Figure 3). In these two conditions, the seed production of endophyte-free plants was pulled-up by a higher contribution by undamaged individuals relative to the damaged counterparts (Figure 3). The frequency of endophyte presence in seeds produced by symbiotic plants was not affected by any of the ozone treatments (X_3=6.606; *P*=0.086) (Table 1).

Table 1. Vertical transmission efficiency of the endophyte fungus *Epichloë occultans* (proportion of endophyte-symbiotic seeds over total evaluated seeds) in *Lolium multiflorum* plants exposed to ozone at different stages: vegetative (Vegetat), reproductive (Reprod) or both (Veg&Rep), and no exposed (No ozone). Values are mean±95% CI.

Tabla 1. Eficiencia de transmisión vertical del hongo endófito *Epichloë occultans* (proporción de semillas con endófito del total de semillas evaluadas) en plantas de *Lolium multiflorum* expuestas a ozono en diferentes etapas: vegetativa (Vegetat), reproductiva (Reprod) o ambas (Veg&Rep), y no expuesta (sin ozono). Los valores son promedios±95% IC.

Symbiotic status	Ozone treatment		Vertical transmission efficiency (inf-sup, 95% CI)
E+	No ozone		0.94 (0.87-0.97)
	Ozone	Vegetat	0.99 (0.93-0.99)
		Reprod	0.95 (0.88-0.97)
		Veg&Rep	0.92 (0.84-0.95)

DISCUSSION

The frequency of attacked plants by the *A. ipsilon* larvae was independent of both the endophyte symbiosis and the ozone. However, the endophyte reduced the severity of damage in attacked plants, irrespectively of the previous exposure to ozone. Interestingly, this endophyte-mediated protection was not associated to a higher seed production of plants. In fact, seed production per plant was slightly lower in endophyte-symbiotic plants than in endophyte-free counterparts. When looking at the relative seed production of damaged and undamaged plants to the mean seed production, was interesting to notice that in two of the three ozone scenarios (i.e., Vegetat and Reprod), the high seed production of E- plants was due to the contribution of undamaged (not damaged) plants. In other words, herbivory by *A. ipsilon* larvae caused a significant reduction in seed production of endophyte free plants. This would also mean that on average, the endophyte tended to buffer the effect of herbivores' damage on host plant seed production. None of the ozone treatments impacted on the vertical transmission of the endophyte from mother plants to seeds.

The fungal alkaloids found in plant tissues usually dissuade herbivores from attacking hosts and/or negatively impact their performance (i.e., resistance; *sensu* Karban and Baldwin [2007]) (Saikkonen et al. 2013; Li et al. 2014; Bastias et al. 2017a). Besides the alkaloids, *Epichloë* fungal endophytes have been found to control the herbivore-mediated induction of volatile organic compounds which have been suggested to work as an indirect defense mechanism (Li et al. 2014; Fuchs and Krauss 2018). In our experiment, any endophyte-mediated effect in plant volatiles was not effective in repelling the larvae of the herbivore *A. ipsilon*. Both endophyte-symbiotic and endophyte-free *L. multiflorum* plants were equally visited by *A. ipsilon* larvae. Nonetheless, once plants had been visited, the endophyte reduced the severity of the attack, indicating E+ plants were more resistant that E- plants to *A. ipsilon* larvae. In another experiment but with the same grass-endophyte symbiosis, we had also found *E. occultans* endophytes to reduce the attack severity of *Spodoptera frugiperda* larvae (Bastias et al. 2018). All this together supports the wide spectrum effect of the fungal alkaloids lolines against lepidopteran larvae. Although evidence shows that outcomes of the grass-endophyte symbiosis is context-dependent (e.g., Davitt et al. [2011]), and despite the observed great variability in mean difference in damage of attacked E+ and E- plants in relation to ozone treatment, we did not detect a statistical interaction between the endophyte and the ozone. Ozone was previously found to disrupt the defensive mutualism on the same symbiotic system (*L. multiflorum* - *E. occultans*) but against *Rhopalosiphum padi* aphids (Ueno et al. 2016). Therefore, it is interesting that while ozone can induce resistance to herbivores in some plant species (Sandermann et at. 1998; Menéndez et al. 2009), it can have the opposite effect on resistance mechanisms based on a symbiotic microorganism.

Apart from driving changes in the chemical phenotype of host plants, the symbiotic association with fungal endophytes mediates also growth, architecture and carbon allocation of host plants (Cheplick and Cho 2003; Vila-Aiub et al. 2005; Faeth 2009; Gundel et al. 2013b). It has been also proposed that the endophytic symbiosis could mediate the resistance/tolerance trade-off, so endophyte symbiotic plants would exhibit a penalty in regrowth after defoliation (Partida-Martínez and Heil 2011; Saikkonen et al. 2013). Here, we observed that main seed production per plant was marginally diminished by the endophyte independently on the ozone condition. However, the higher seed production in E- plants was mainly explained by undamaged individuals, while in E+ plants damaged, and undamaged individuals contributed in a similar way. Therefore, the presence of fungal endophytes in *L. multiflorum* plants buffered the damage caused by the herbivorous larvae. In any case, this endophyte-mediated effect would have been related to the lower herbivore damaged on plants or a higher ability to compensate for the larvae-caused damage on plants. If endophyte-symbiotic plants are likely to exhibit penalty in regrowth after defoliation —as it is suggested—, the contribution of damaged E+ plants to the mean seed production per plant was likely due to the lower negative effect of the herbivorous larvae.

Some forage breeding programs are set on the use of fungal endophytes that not producing toxic alkaloids are still able to deliver benefits on host grasses (the so-called "safe endophytes") (Gundel et al. 2013a; Johnson et al. 2013). This strategy consists of inoculating elected fungal strains in plants of elite cultivars (Johnson et al. 2013). The main challenges for this strategy are: 1) it can be constrained by partners' incompatibility —so inoculated fungi are not able to establish stable associations—, being rejected by host plants or not vertically transmitted, and 2) fungal endophytes can be lost from commercial seeds during storage in warehouses (Latch and Christensen 1985; Gagic et al. 2018; Zhang et al. 2019). The use of natural associations, like the one we present here, are promising strategies to be commercially developed. In some forage chains, annual ryegrass is promoted as winter forage crop, although neither farmers nor technicians are aware of the presence of fungal endophytes. Several articles show the benefits of *E. occultans* on *L. multiflorum* plants (e.g., Vila-Aiub et al. 2005; Gundel et al. 2012; Bastias et al. 2017b). However, those benefits can be comprised by the occurrence of certain stress factors such as herbicides (Gundel et al. 2012), or ozone (Ueno et al. 2016). For example, despite ozone did not affect the endophyte vertical transmission to the seed —as it happened here—, the longevity of the fungus in still viable seeds can be severely impaired by exposing mother plants to ozone (Gundel et al. 2015; Ueno et al. 2020). Therefore, ozone effects on the grass-endophyte symbiosis seem to vary with plants stage at exposure (vegetative/reproductive), the processes evaluated (transmission to *vs.* persistence in the seeds), and depend also on the type of exposure (chronic or acute) and doses (low, medium and high). It would be important to study the effect of other factors extensively used in agriculture —e.g., glyphosate herbicide— to unveil how they affect the symbiosis outcome particularly in relation to the interaction between plants and herbivores. Although the symbiosis with endophyte can increase tolerance to herbicides (Vila Aiub and Ghersa 2001; Handayani et al. 2017), the indirect effect on the endophyte persistence could explain part of the incidence variability found in agroecosystems (e.g., see Vila Aiub et al. 2003; Yamashita et al. 2010).

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