



Can Aphid Herbivory Induce Intergenerational Effects of Endophyte-conferred Resistance in Grasses?

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Abstract

Plants have evolved mechanisms to survive herbivory. One such mechanism is the induction of defences upon attack that can operate intergenerationally. Cool-season grasses (sub-family Pooideae) obtain defences via symbiosis with vertically transmitted fungal endophytes (genus *Epichloë*) and can also show inducible responses. However, it is unknown whether these herbivore-induced responses can have intergenerational effects. We hypothesized that herbivory by aphids on maternal plants induces the intergenerational accumulation of endophyte-derived defensive alkaloids and resistance intensification in the progeny. We subjected mother plants symbiotic or not with *Epichloë occulta*, a species known for its production of anti-insect alkaloids known as lolines, to the aphid *Rhopalosiphum padi*. Then, we evaluated the progeny of these plants in terms of loline alkaloid concentration, resistance level (through herbivore performance), and shoot biomass. Herbivory on mother plants did not increase the concentration of lolines in seeds but it tended to affect loline concentration in progeny plants. There was an overall herbivore-induced intergenerational effect increasing the endophyte-conferred defence and resistance. Symbiotic plants were more resistant to aphids and had higher shoot biomass than their non-symbiotic counterparts. Since maternal herbivory did not affect the loline concentrations in seeds, the greater resistance of the progeny could have resulted from an inherited mechanism of epigenetic regulation. It would be interesting to elucidate the origin of this regulation since it could come from the host or the fungal symbiont. Thus, endophyte-driven differential fitness between symbiotic and non-symbiotic plants might be higher as generations pass on in presence of herbivores.

Keywords *Epichloë* endophytes · herbivory resistance · induced resistance · fungal alkaloids · *Lolium multiflorum* · plant defense

Introduction

The continuous challenge imposed by the great diversity of herbivores on plant populations and species has driven the evolutionary fixation of strategies and phenotypic traits of defence (Agrawal and Heil 2012; Johnson et al. 2015). Whereas strategies may involve changes in plant demography and growth habits that diminish the probability of attack, defensive traits – either constitutive or inducible, protect plants from being consumed and interestingly, can eventually be transmitted intergenerationally (Agrawal and Heil 2012; Johnson et al. 2015; Karban and Baldwin 1997; Kessler and Kalske 2018). Plant defences against herbivory can be also boosted, or provided, by mutualistic symbiotic microorganisms (Pieterse et al. 2014; Pineda et al. 2010; Schardl et al. 2007, 2013). Despite some of these beneficial microorganisms are maternally inherited (Shade et al. 2017;

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Jonkers et al. 2022), their role in transmitting intergenerational effects of defence in plants attacked by herbivores is almost unexplored (Gundel et al. 2017, 2020).

Plants respond to herbivory by activating specific hormonal signalling pathways – namely the jasmonic acid (JA) or salicylic acid (SA) mediated defence pathways, that can stimulate the production of secondary metabolites (e.g., alkaloids, cyanogenic glycosides, terpenoids) with a specialised role in resistance (Erb and Reymond 2019; Mithöfer and Boland 2012; Robert-Seilaniantz et al. 2011). The type of resistance mechanism and its effectiveness depends on the plant and herbivore species as well as the plant ontogenetic stage, plant organ, and environmental condition (Agrawal and Weber 2015; Barton and Koricheva 2010). The hormones also mediate the plant's capacity to increase the level of defence upon herbivore attack (i.e., induced resistance) and interestingly, remain in a primed state that allows the plant to display a faster and stronger response in case the same enemies are return (priming) (Conrath et al. 2015; Frost et al. 2008; Karban and Baldwin 1997; Martinez-Medina et al. 2016; Züst and Agrawal 2016). Plants can also have the capacity to convey these herbivore-activated defence responses to their progeny and therefore the progeny of plants that have experienced herbivory are more resistant than progeny from plants that had not (Agrawal 2001; Agrawal et al. 1999; Holeski 2007; Martinez-Medina et al. 2016; Kafle and Wurst 2019). Still, there are many open-ended questions regarding the prevalence and adaptive significance of herbivore-induced intergenerational effects in plants and the possible mechanisms responsible for this phenomenon. Some of the underlying mechanisms of the intergenerational effects can be associated with the direct maternal contributions to the seed (organelle and plasmid associated DNA, reserves, secondary metabolites, seed coat, etc.) while others can be linked to inherited epigenetic regulations at the embryo nuclear genome (Herman and Sultan 2011; Holeski et al. 2012; Roach and Wulff 1987; Tricker 2015). Priming by epigenetic mechanisms which may involve DNA methylation, histone modification, and non-coding RNAs, would allow plants to memorise and stay ready for a subsequent attack (Bruce et al. 2007; Conrath et al. 2015; Martinez-Medina et al. 2016). Those mechanisms are likely to play a potential role in connecting the plant's maternal history with progeny phenotype and fitness.

Using invertebrate herbivores (either sap-sucking or chewing insects) or actions that simulated herbivory (clipping leaves with scissors or punching holes with a paper punch) or using elicitors of antiherbivore defence pathways (e.g., spraying plants with methyl jasmonate) as maternal treatments, studies have shown evidence of intergenerational induction of defences at physical, chemical and gene expression level in the progeny. For instance, increased density of trichomes was reported in leaves of common yellow

monkeyflower (*Erythranthe guttata*, formerly *Mimulus guttatus*) and radish (*Raphanus sativus*) due to exposure of mother plants to simulated or actual herbivory, respectively (Holeski 2007; Sobral et al. 2021). Transcriptome-based studies have revealed that progeny plants display different patterns of expression in genes related to defence signalling pathways and synthesis of secondary compounds (Colicchio et al. 2015; Verhoeven et al. 2018; Sobral et al. 2021). Noteworthy, even though progeny plants expressed a high resistance level due to the maternal history of herbivory, high levels of defence hormones or accumulation of secondary metabolites might not be evident at the seed stage. In a clear link with an herbivore-induced upregulation of defence against herbivores, seeds produced by *Arabidopsis thaliana* plants attacked by the red spider mite (*Tetranychus urticae*) showed high levels of jasmonic acid-isoleucine (Singh et al. 2017). An early work investigating infestations of rapeseed (*Brassica napus*) by the cabbage aphid (*Brevicoryne brassicae*), informed that herbivory increased the concentration of glucosinolates, secondary metabolite defence compounds, in the seeds produced from these plants (Lammerink et al. 1984). However, Rasmann et al. (2012) showed that neither the concentration of defence hormones (e.g., JA, SA) nor glucosinolates were different between seeds produced by *Arabidopsis* plants challenged or not by caterpillars. All information together suggests that increased resistance observed in progeny plants is due to their maternal history regarding herbivores and therefore linked with the transgenerational priming of defences (Holeski et al. 2012; Rasmann et al. 2012; Singh et al. 2017; Colicchio et al. 2015; Verhoeven et al. 2018; Sobral et al. 2021).

Mutualistic microbial symbionts that are inherited from mother plants (i.e. vertically transmitted via seed) are especially interesting to study because the fitness of both partners is closely linked, even beyond the ontogenetic cycle of the host (Gundel et al. 2011; Panaccione et al. 2014; Jonkers et al. 2022). The mutualistic symbiosis between cool-season grasses (Poaceae, subfamily Pooideae) and vertically transmitted fungal endophytes of the genus *Epichloë* (Family Clavicipitaceae), is proposed to be shaped by herbivory-driven selection forces, which is consistent with the observed high frequency of symbiotic plants in populations under high herbivore pressure (Clay et al. 2005; Selosse and Schardl 2007; Schardl et al. 2013). The main underlying mechanism is the endophyte-conferred resistance via the production of bioactive alkaloids (Bastías et al. 2017; Panaccione et al. 2014; Saikkonen et al. 2013; Schardl et al. 2013). While the production of certain fungal-derived alkaloids is confined to some *Epichloë* species or strains (Schardl et al. 2013; Caradus et al. 2022), their concentrations can depend on host and endophyte genotype (e.g., Helander et al. 2016; Hewitt et al. 2020) and the amount of *Epichloë* mycelial biomass in plant tissue (e.g.,

Freitas et al. 2020; Ueno et al. 2020). Although it can be positively correlated with mycelial biomass (Rasmussen et al. 2007; Ueno et al. 2020), the concentration of alkaloids varies among plant organs, phenological stages, and environmental conditions (Fuchs et al. 2017a; Gundel et al. 2018a; Justus et al. 1997). Still, there are well-characterized *Epichloë*-plant associations in which the endophyte effectively protects the host grass against certain herbivore species (Bastías et al. 2017; Panaccione et al. 2014; Saikkonen et al. 2010). For example, it is well established that *Epichloë* endophytes that can synthesize loline alkaloids (potent insecticidal and feeding-deterrents with no known toxic effects on mammals), protect their host grasses against aphids of the species *Rhopalosiphum padi* (Bastías et al. 2017; Scharldt et al. 2007; Simons et al. 2008; Ueno et al. 2016; Wilkinson et al. 2000). The greater the concentration of lolines, the more resistance is achieved to this aphid (the lower insect performance) (Wilkinson et al. 2000). It has been observed that plant exposure to simulated herbivory (e.g., clipping with scissors) can increase the concentration of loline alkaloids and consequently, the level of resistance in endophyte symbiotic plants (Bultman et al. 2004; Gonthier et al. 2008). Since *R. padi* has shown high sensitivity to endophyte presence, it has been used extensively to determine the level of plant resistance [e.g., (Bastías et al. 2018; Bubica Bustos et al. 2020; Bultman et al. 2004; Gundel et al. 2012; Sullivan et al. 2007; Gonthier et al. 2008; Ueno et al. 2016)]. Although in experiments where no symbiotic microorganisms were involved, this generalist aphid could induce defences during the ontogenetic cycle of certain plant species (Karban and Baldwin 1997; Züst and Agrawal 2016). However, it is unknown whether these aphids can modulate the synthesis of lolines and the *Epichloë* conferred defence in host grasses. Although endophyte-derived alkaloids are commonly detected at high concentrations in grass seeds (Gundel et al. 2018a; Ueno et al. 2020), it is also unknown whether herbivore-induced resistance observed in plants during the ontogenetic scale is transmitted to the plant's progeny.

In this article, we asked the following questions: (i) Does aphid herbivory on maternal plants increase the mycelial density and concentration of alkaloids from *Epichloë* endophytes in grass seeds? and (ii) Does aphid herbivory on maternal plants increase the endophyte-conferred defence and resistance in the progeny plants? We addressed these questions by conducting a two-generation experiment in which *Lolium multiflorum* mother plants symbiotic and non-symbiotic with *Epichloë occultans*, a loline-producing endophyte, were challenged with a sap-sucking insect, the aphid *R. padi*. Then, we evaluated the amount of endophyte-conferred defence in the plant's progeny (specifically on the seeds and on the plants) and the level of resistance through herbivore performance (only on the progeny plants). With a focus on the direct effects of insect herbivory on plant defence and performance mediated by fungal endophytes,

the main question is whether there is a relationship between mother plants and their progeny concerning insect herbivory. Although aphids can induce resistance in non-symbiotic plants (Züst and Agrawal 2016), we expected this aphid-induced intergenerational effect would increase the differential fitness between endophyte-symbiotic (E+) and non-symbiotic (E-) progeny plants. Our work shows that the intergenerational effect of aphid herbivores on the level of resistance in plants is mediated by vertically transmitted *Epichloë* fungal endophytes.

Materials and Methods

Plant Material We worked with the mutualistic symbiosis between the host grass *Lolium multiflorum* Lam. and its fungal endophyte *Epichloë occultans* (Moon et al. 2000). *Lolium multiflorum* (Poaceae) is an annual species commonly known as Italian ryegrass. The grass originates from the Mediterranean region, although the species is now globally distributed. Extant populations in different grassland communities of the Pampa region, Argentina, present high frequencies of plants symbiotic with *E. occultans* (Bastías et al. 2017; Gundel et al. 2009). Although *L. multiflorum* is cultivated as a forage crop and used as a turf grass, the grass species is also recognised as an important weed within cereal crops such as wheat (*Triticum aestivum* L.) (Beckie and Jasieniuk 2021).

Since 2012, endophyte-symbiotic (E+) and non-symbiotic (E-) grass plants are cultivated for the annual production of seeds for experimental purposes in the experimental field of the Faculty of Agronomy, University of Buenos Aires (34°35'S, 58°35'W). Every year, 50 E+ and 50 E- seeds are selected and sown in soil previously cleaned from weeds, with positions within the line (5 rows, 8 m each) assigned at random (distance between plants: 35 cm). After harvest, endophyte presence is assessed by microscopic observation of previously softened and stained seeds (Card et al. 2011; Gundel et al. 2018b). Briefly, seeds were soaked in solution of NaOH (5%) for 8 h, squashed on a glass slide, stained with Bengal rose, gently pressed with a coverslip, and observed under a light microscope (x100). Seeds were confirmed as E+ once hyphae characteristic of *Epichloë* were observed (Gundel et al. 2018b). Since *L. multiflorum* is obligately cross-pollinated while *E. occultans* is seed transmitted only, with this design we promote pollen exchange between E+ and E- plants at flowering to avoid genetic differentiation between biotypes (Gundel et al. 2012). At maturity, seeds are hand harvested at individual plant level. Before pooling seeds from E+ or E- plants, seeds are again assessed for endophyte presence using the same technique as described previously. The seeds used in this paper were produced in 2016.

Insect Material Approximately 50 individuals of *R. padi* (Hemiptera: Aphididae) were collected in spring 2017 from various weedy grasses growing in the surroundings of the Faculty of Agronomy, University of Buenos Aires, Argentina. Commonly known as bird cherry-oat aphid, this insect is a phytophagous pest able to infest a wide range of cereal and pasture grasses (Van Emden and Harrington 2007). The aphids were maintained for five days in Petri dishes (≈ 10 aphids each) containing fresh leaf pieces of oat (*Avena sativa* L.), a period that is enough to identify and remove any individuals infected with primary parasitoids. After that, ten healthy aphids (5 nymphs and 5 apterous adults) were then placed and reared on young oat plants (≈ 6 tillers) (Dixon and Glen 1971) within a growth chamber set at constant environmental conditions (Temperature: $23\text{ }^{\circ}\text{C}$ (± 1); Photoperiod: L16:D8 h; Radiation: $150\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$). Six oat plants were grown in 1 L pots filled with a commercial potting-mix and without water deficit. Every time oat plants showed symptoms of deterioration, they were replaced by new ones. This general procedure (that is the aphid collection and the rearing) was repeated every year to maintain a suitable aphid population for the experiments presented here.

General Experimental Design We carried out a two-year experiment (2017 and 2018) in which we first challenged *L. multiflorum* mother plants with and without endophytes to herbivory by aphids, and second, we evaluated the consequences of this history on the level of resistance to the same herbivore in the progeny (Fig. 1). The experiment was

carried out under natural environmental conditions in the experimental field at the Agronomy Faculty, University of Buenos Aires, Argentina ($34^{\circ}35'\text{S}$, $58^{\circ}35'\text{W}$). In an area of 9 m^2 ($3 \times 3\text{ m}$), we set a table at 20 cm height where the experimental plants were kept and managed. The underneath vegetation was cut down in order to avoid attracting pests and diseases.

Herbivory Treatments on Maternal Plants During the first year, 7 E+ and 7 E- *L. multiflorum* plants were grown individually in 3 L pots filled with a commercial potting mix (Sustrato Profesional Exterior F546, Hi-Soil®, Argentina). The sowing of seeds that originated those plants was in mid-autumn. In early winter, when the plants had approximately 8–10 tillers (tillering stage), they were cloned in two. For the cloning, each original plant was removed from the soil and carefully split in two, making sure each new plant had a well-developed root system. All cloned plants were planted in a new pot and received the same care (protection against plagues and watering on demand) until advanced spring when they were challenged by aphids. The two clones from each original mother plant were randomly assigned to one of the following herbivory treatments: challenged by aphids or not (control). At the moment of herbivory treatment application, plants were at the stem elongation stage.

Twenty apterous adult aphids were placed on each grass clone assigned to the herbivore treatment. To contain the aphids, each plant was enclosed within a protective plastic

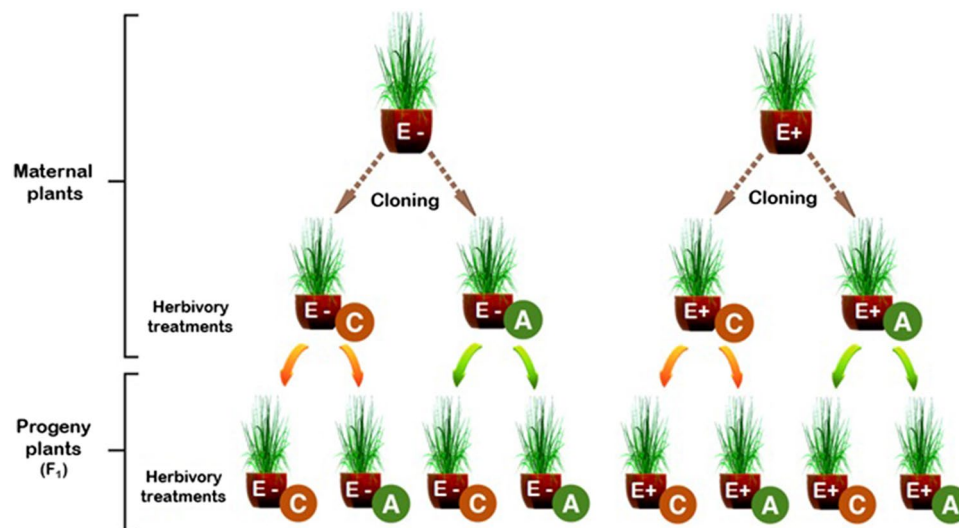


Fig. 1 Schematic depicting the full experimental manipulation of plants, endophyte symbiotic status, and herbivory treatment. The upper part of the figure represents the maternal *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E-) with the fungal endophyte *Epichloë occulta*, which were split into two clones. One

clone was challenged by the aphid *Rhopalosiphum padi* (green A circle) while the other was kept free of herbivores (orange C circle). The lower part shows the progeny plants that were challenged or not by the same aphid. Arrow colours indicate the history related to the plant herbivory treatment (green: herbivory, and orange: control)

cylinder made of 2 cm² mesh, 1 m long, surrounded by white fabric (0.05 mm mesh). The clones assigned to the control treatment were also enclosed with cylinders, but no aphids were added. *Rhopalosiphum padi* is usually placed in the base of leaf blades where it pierces and sucks phloem sap. The whole aphid population of a plant is formed by small populations that develop in the many but healthy leaves of the individual. Although we did not characterize the size of aphid populations, we know they grew well on each plant assigned to herbivory treatment. The herbivory treatment lasted until plants were between heading state and anthesis. After that, the aphids were removed from the herbivore-challenged plants, and the protective cylinders were removed from all plants, challenged and unchallenged by aphids. To make sure that every plant was completely free of aphids, a small brush was softly passed through the whole plant trying not to damage it. Plants from the control treatment were subjected to the same procedure. After aphid removal, the plants were let to complete the flowering and seed filling stages. Plants were then watered and protected from any threat until the end of the growing cycle by early summer.

Mature seeds were harvested from individual plants. All spikes from a single plant were threshed and the seeds were stored under dry, cold (5 °C) conditions. The performance of mother plants was characterized by measuring shoot biomass, seed production, seed mass (1000-seed weight) and reproductive effort [i.e., the proportion of plant biomass that is allocated to reproduction; Reekie and Bazzaz (1987)]. To estimate the mean seed weight, three groups of 30 seeds per plant were weighed and then, with the average of these three, it was extrapolated to estimate the weight of 1000. Additionally, ten seeds per plant were assessed for determining the endophyte presence using the same technique as before (Card et al. 2011; Gundel et al. 2018b). Besides the presence/absence evaluation, the concentration of *Epichloë* fungal mycelia and endophyte-derived alkaloids were quantified for a batch of around 30 seeds per mother plant (for details see below *Quantification of Endophyte Mycelium and Fungal Alkaloids*).

Consequences of Maternal Herbivory on Progeny Performance In 2018, the consequences of the maternal history of herbivory were assessed at the progeny plant level for endophyte-conferred defences (concentration of fungal alkaloids) and resistance to herbivory (through the performance of the aphid *R. padi*). In late winter, two seeds per maternal herbivory treatment (control and aphid) and symbiotic status (E+ = 14 and E- = 14) were sown in 2 L pots filled with the same substrate as mother plants. The pots were randomly placed in a square grid (distance between adjacent plants: 20 cm) on a Table (20 cm height from the soil surface) that was established in the experimental field. In late spring (two and a half months after sowing), they were randomly

assigned to the following treatments: challenged by aphids or not (control) (Fig. 1). The plants were at tillering stage with about 5–6 tillers each. In the treatment herbivory, 10 apterous adult aphids were placed on each plant. The plants were enclosed within a protective cylinder as described earlier for mother plants but with modifications to fit the size of smaller pots. On day 21, when population growth dynamics stabilized, all aphids were collected from each plant using a small soft brush (the same procedure was performed on the control plants) and placed in Petri dishes (aphids from 1 plant = 1 dish). Aphid performance was characterized by population size, population structure (proportion of nymphs, apterous and winged), and individual weight per instar. We divided the Petri dishes into six equally sized segments and distributed the aphids homogeneously. In one-sixth, we counted the number of aphids and extrapolated this to the whole dish to estimate population size. In the same segment, we counted the number of individual aphids per instar to characterize the population structure. Individual aphid performance was estimated by weighing ten aphids per instar on a precision scale (± 0.001 g) and dividing this number by 10. Herbivore biomass was calculated by integrating variables of performance at the individual (individual weight per instar) and population (number of aphids in each instar) levels, following the numerator in the formula:

$$\text{Herbivore biomass} = \frac{\sum(\#_i \times W_i)}{\text{Shoot plant biomass}}$$

where $\#_i$ is the number of individuals for a given (i) instar, and W_i is the individual mean weight for the same given (i) instar. In addition, we divided the herbivore biomass by shoot plant biomass (denominator) in order to correct the herbivore performance variable by a unit of the resource offered.

We also sampled each plant for alkaloid quantification. From each plant, we took sections of tillers including 2–3 cm of leaf sheath and the whole leaf blade from 2 to 3 leaves; all was wrapped in aluminium foil and placed in liquid nitrogen (see below *Quantification of endophyte mycelium and fungal alkaloids*). Finally, we estimated the dry weight biomass of plant shoots. Each plant shoot within a paper bag was set to dry in an oven (60–70 °C) for 48 h and then weighed on a precision scale (± 0.001 g). Thus, the measurements of shoot biomass characterize the size of plants at the end of the herbivory treatment.

Quantification of Endophyte Mycelium and Fungal Alkaloids As we explained before, we evaluated the consequences of aphid herbivory on the endophyte vertical transmission efficiency by inspecting 10 seeds per mother plant (see '*Herbivory Treatments on Maternal Plants*'). Since we

did not observe failures in the vertical transmission process (all seeds produced by E+ mother plants were endophyte symbiotic), we proceeded to evaluate the biomass of fungal mycelia and alkaloids in those seeds. Those variables were only assessed in the seeds produced by the E+ mother plants. Each seed sample was ground in liquid nitrogen using a mortar and pestle, and transferred to a 2.5 ml plastic tube (250 mg of seeds minimum). Only loline alkaloids were quantified in the foliar tissue of progeny plants. Foliar tissues were freeze-dried, ground, and placed in plastic tubes (approx. 250 mg of vegetative tissue).

The concentration of loline alkaloids [NAL (*N*-acetyllooline), NANL (*N*-acetylnorloline) and NFL (*N*-formyllooline)] was quantified using a modification of the method described in Moore et al. (2015). Seeds from the threshed mother plants were placed in individual aluminium drilling envelopes, freeze-dried, and frozen to -80 °C until required. The material was then ground using a mortar and pestle in liquid nitrogen until a homogeneous powder was obtained and this powder was stored in 1.5 ml plastic tubes at -20 °C. 50 mg per sample was extracted for 1 h with 50 µl of 40% methanol/5% ammonia and 1 ml of 1,2-dichloroethane (containing 54.8 ng.ml⁻¹ of 4-phenylmorpholine as an internal standard). After centrifugation (5 min at 8000 G) the supernatant was transferred to amber glass GC vials with a 10 µm filter. The analysis was performed with a gas chromatography flame ionization detector (GC2010Plus, Shimadzu Corporation, Japan) equipped with a ZB-5 capillary column (30 m long, 0.32 mm internal diameter and 0.25 µm thick, Phenomenex, Torrance, CA, USA). The detection limit was 25 µg.g⁻¹ dry weight.

The biomass of endophyte mycelia was assessed in 10 seeds per plant using an enzyme-linked immunosorbent assay (ELISA) developed by AgResearch NZ (Faville et al. 2015). ELISA plates were coated with *E. festucae* var *lolii* antigen, and 1% Bovine serum albumin was utilised as a blocking agent. Samples were quantified using standard curves prepared with *E. festucae* var *lolii* endophyte standard. The presence of the endophyte in the seed sample extracts was indicated by inhibition of specific *E. festucae* var *lolii* antibodies binding to the coating antigen which was determined using a commercial anti-rabbit-HRP conjugate and TMB substrate for HRP. Curve fits of mean absorbance versus the log of the analyte concentration were performed by four-parameter curve fit and results were reported as *E. festucae* var *lolii* immunoreactive equivalents (IRE) in µg/mg dry weight.

Statistical Analysis All analyses were carried out using R software (R Core Team 2020). For the assumption of the

homogeneity of variance and normality, data were checked graphically by inspecting the residuals plotted against fitted values, and against each predictor variable in all models. Mother plants were characterized in terms of shoot biomass, seed production, 1000-seed weight and reproductive effort. Also, endophyte mycelia and endophyte-derived alkaloids were estimated in the produced seeds. All response variables were analysed with a mixed linear effects model using lmer function [lme4 package; Bates et al. (2015)]. The herbivory treatments (control and aphid), endophyte symbiotic status (E+ and E-) and the herbivory treatments × endophyte symbiotic status interaction were considered as fixed factors, whereas the original plant was considered a random effect to address the cloning effect (genetically identical) in the experimental set-up.

To evaluate shoot biomass, variables of individual aphid performance (mean weight per instar) herbivore biomass, and concentration of endophyte-derived alkaloids (NAL, NANL, NFL, and total lolines) associated with progeny plants, mixed-effects models were performed using lmer/glmer from the lme4 package (Bates et al. 2015). In all models, maternal herbivory treatment (Control or Aphids), endophyte symbiotic status (E+ or E-), progeny herbivory treatment (Control or Aphids), and their interactions were the predictor variables, whereas the original plant was considered a random effect to address the cloning effect (genetically identical plant) within the experimental set-up. The same fixed and random factors described above were used. The concentration of endophyte-derived alkaloids was log-transformed (base 10) to meet the normality of the error distribution. For aphid population size and structure [# per instar (nymphs, apterous and winged adults)], we fitted generalized linear mixed-effects models using the function glmer.nb from R package lme4 (Bates et al. 2015). Tests of significance for main effects were obtained using ANOVA function of the car package (Fox and Weisberg 2011). Finally, Tukey's *post hoc* comparisons were performed with the emmeans package (Lenth 2019).

Results

Consequences of Aphid Herbivory and Symbiosis with Fungal Endophytes on Mother Plants The shoot biomass of grass mother plants decreased by approximately 4% when subjected to aphids. The symbiosis with *Epichloë* fungal endophytes did not affect plant shoot weight (Tables 1 and 2). The effect of aphids on seed production was dependent on the symbiotic status of the grass plants where symbiotic plants almost double the production of the non-symbiotic counterparts (Tables 1 and 2). Seed production was similar among endophyte-symbiotic and non-symbiotic plants when

Table 1 Parameters of performance [shoot biomass, seed production, seed mass (1000-seed weight) and reproductive effort] of *Lolium multiflorum* mother plants symbiotic (E+) and non-symbiotic (E-) with the fungal endophyte *Epichloë occultans* challenged (Aphids) or not (Control) with the aphid *Rhopalosiphum padi*

Response variable	Herbivory treatment			
	Control		Aphids	
Plant	E-	E+	E-	E+
Shoot biomass (gDW.plant ⁻¹)	12.08 ± 0.48 b	13.01 ± 0.51 ab	10.96 ± 0.56 a	12.91 ± 0.46 ab
Seed production (gDW.plant ⁻¹)	2.71 ± 0.15 b	3.22 ± 0.32 b	1.63 ± 0.14 a	3.21 ± 0.28 b
Seed mass (gFW.plant ⁻¹)	5.04 ± 0.44 b	2.72 ± 0.12 a	2.61 ± 0.15 a	3.04 ± 0.16 a
Reproductive effort (unitless)	0.18 ± 0.01 b	0.19 ± 0.01 b	0.12 ± 0.01 a	0.20 ± 0.02 b
Fungal endophyte (in seed)	E-	E+	E-	E+
NAL (µg.g ⁻¹ DW)	n.d.	11 ± 2 a	n.d.	8 ± 3 a
NANL (µg.g ⁻¹ DW)	n.d.	75 ± 9 a	n.d.	78 ± 14 a
NFL (µg.g ⁻¹ DW)	n.d.	712 ± 81 a	n.d.	627 ± 127 a
Total lolines (µg.g ⁻¹ DW)	n.d.	798 ± 89 a	n.d.	713 ± 143 a
Mycelia (µg.mg ⁻¹ DW)	n.d.	0.80 ± 0.06 a	n.d.	0.91 ± 0.12 a

Reproductive effort was calculated as seed production/shoot biomass. Concentration of endophyte-derived alkaloids [NAL (*N*-acetyllooline), NANL (*N*-acetylnorlooline) and NFL (*N*-formyllooline)] and the biomass of endophyte mycelia are shown for seed only. Values are means ± SE ($n=7$). n.d. means no detected. Letters indicate significant pairwise differences between means (*Tukey HSD*, $P < 0.05$)

Table 2 Linear mixed-effects model results of performance parameters [shoot biomass, seed production, seed mass (1000-seed weight) and reproductive effort] of *Lolium multiflorum* mother plants symbiotic and non-symbiotic with the fungal endophyte *Epichloë occultans* challenged (Aphids) or not (Control) with the aphid *Rhopalosiphum padi*

Response variable	Source	df	X ²	<i>P</i> -value	
Plant	Shoot biomass (gDW.Plant ⁻¹)	Maternal Herbivory treatment (MH)	1	5.08	0.024
		Endophyte (E)	1	2.36	0.123
		MH x E	1	3.66	0.055
	Seed production (gDW.Plant ⁻¹)	Maternal Herbivory treatment (MH)	1	6.32	0.011
		Endophyte (E)	1	11.86	< 0.01
		MH x E	1	5.23	0.022
	Seed mass (gFW.Plant ⁻¹)	Maternal Herbivory treatment (MH)	1	38.3	< 0.001
		Endophyte (E)	1	5.45	0.019
		MH x E	1	58.52	< 0.001
Reproductive effort (unitless)	Maternal Herbivory treatment (MH)	1	8.15	< 0.01	
	Endophyte (E)	1	7.74	< 0.01	
	MH x E	1	9.56	< 0.01	
Fungal endophyte (in seed)	NAL (µg.g ⁻¹ DW)	Maternal Herbivory treatment (MH)	1	0.49	0.483
	NANL (µg.g ⁻¹ DW)	Maternal Herbivory treatment (MH)	1	0.68	0.408
	NFL (µg.g ⁻¹ DW)	Maternal Herbivory treatment (MH)	1	0.13	0.712
	Total lolines (µg.g ⁻¹ DW)	Maternal Herbivory treatment (MH)	1	0.08	0.775
	Mycelia (µg.mg ⁻¹ DW)	Maternal Herbivory treatment (MH)	1	1.00	0.316

Concentration of endophyte-derived alkaloids [NAL (*N*-acetyllooline), NANL (*N*-acetylnorlooline) and NFL (*N*-formyllooline)] and the biomass of endophyte mycelia are shown for seed only. Significant ($P < 0.05$) results are displayed in bold and marginally significant trends ($0.05 < P < 0.10$) are presented in italic

plants were free of insect herbivores (≈ 3 g per plant). Seed mass was affected by aphid herbivory depending on the symbiotic status of plants. In the control condition, seed mass from endophyte-free plants was higher than symbiotic plants. When exposed to herbivory, seed mass from symbiotic and non-symbiotic plants were not significantly different. The proportion of biomass

allocated to reproduction of plants (i.e., reproductive effort) depended on the aphid treatment and symbiotic status (Tables 1 and 2). As with the observed pattern for seed production, symbiotic and non-symbiotic plants that were not challenged with aphids displayed the same reproductive effort (≈ 0.18). However, herbivory by aphids diminished the reproductive effort only in

non-symbiotic plants by $\approx 40\%$ (relative to endophyte-symbiotic plants).

In the symbiotic seeds, neither the concentration of loline alkaloids (NANL + NAL + NFL) nor the concentration of mycelial fungal biomass was affected by the herbivory treatment (Tables 1 and 2).

Consequences of the Maternal Plant Treatments on Progeny Plant Performance Maternal history of herbivory by aphids did not affect the shoot biomass in the grass progeny (Table 3). Shoot biomass was also not affected by aphid herbivory on progeny plants, but it was affected by the symbiosis with fungal endophytes. On average, symbiotic plants exhibited a 19% greater shoot biomass compared to non-symbiotic plants (Fig. 2).

Irrespective of the maternal history of herbivory, the aphid population sizes (i.e., the sum of the individuals in each category) on progeny plants were affected by the endophyte-symbiotic status (Table 3). The presence of fungal endophytes resulted in an average reduction of aphid population size by approximately 60% (data not shown). The same pattern was observed for the number of aphids in each instar (Table 3). The number of nymphs, apterous and winged adults were affected by the symbiotic status of the plants but not by the herbivory history of maternal plants (Fig. 3a, b, c). Overall, the number of aphid individuals in each instar was reduced by the presence of *E. occultans* by approximately 61% (Table 3).

The effects of either maternal herbivory or the endophyte were different depending on the instar considered (Table 3). The mean weight of apterous adults was negatively affected

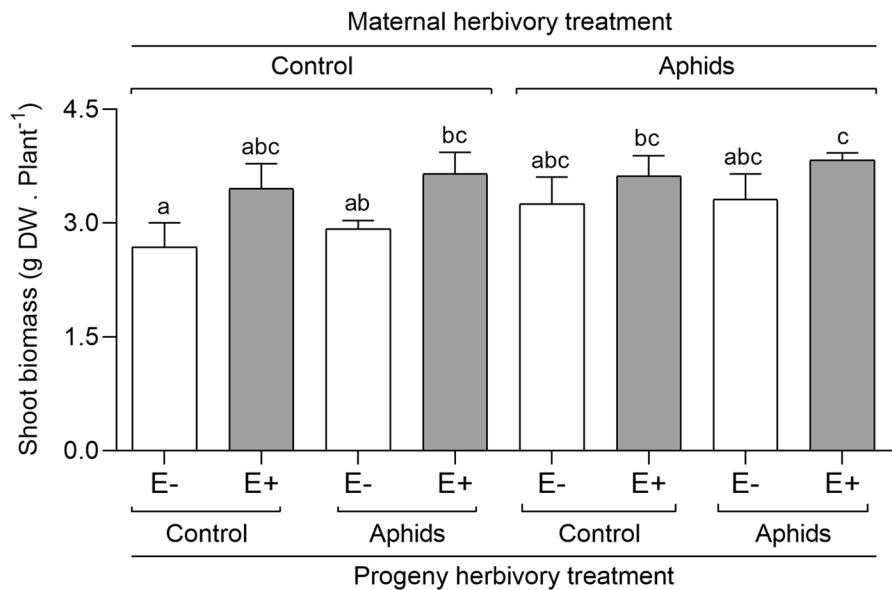
Table 3 Outputs from linear mixed models and generalized linear mixed models investigating variables of performance of the *Lolium multiflorum* progeny plants (shoot biomass), and of the aphid *Rhopa-*

losiphum padi (population structure and size, and individual weight) as affected by the symbiosis with the endophyte fungus *Epichloë occultans* and maternal history of herbivory with the same herbivore

Response variable	Source	df	X ²	P-value	
Plant performance	Shoot biomass (g DW. Plant ⁻¹)	Maternal Herbivory treatment (MH)	1	2.58	0.107
		Endophyte (E)	1	8.50	< 0.01
		Progeny herbivory treatment (PH)	1	0.71	0.400
		MH x E	1	0.56	0.453
		MH x PH	1	0.04	0.823
		E x PH	1	0.01	0.905
		MH x E X PH	1	0.06	0.805
Population structure and size	Nymph (#)	Maternal Herbivory treatment (MH)	1	0.09	0.757
		Endophyte (E)	1	11.34	< 0.001
		MH x E	1	1.07	0.299
	Apterous adult (#)	Maternal Herbivory treatment (MH)	1	0.28	0.595
		Endophyte (E)	1	8.70	< 0.01
		MH x E	1	0.48	0.484
	Winged adult (#)	Maternal Herbivory treatment (MH)	1	1.61	0.203
		Endophyte (E)	1	12.33	< 0.001
		MH x E	1	3.48	0.062
	Total aphids (#)	Maternal Herbivory treatment (MH)	1	0.10	0.743
		Endophyte (E)	1	13.27	< 0.001
		MH x E	1	1.34	0.246
	Individual performance	Nymph weight (µg)	Maternal Herbivory treatment (MH)	1	0.07
Endophyte (E)			1	0.98	0.321
MH x E			1	0.29	0.587
Apterous weight (µg)		Maternal Herbivory treatment (MH)	1	0.17	0.676
		Endophyte (E)	1	16.76	< 0.001
		MH x E	1	0.98	0.320
Winged adult weight (µg)		Maternal Herbivory treatment (MH)	1	1.60	0.205
		Endophyte (E)	1	0.91	0.338
		MH x E	1	2.30	0.128

Significant ($P < 0.05$) effects are displayed in bold while marginally significant trends ($0.05 < P < 0.10$) are presented in italic

Fig. 2 Shoot biomass of *Lolium multiflorum* progeny plants with (E+) and without (E-) the fungal endophyte *Epichloë occultans* challenged (Aphids) or not (control) with the aphid *Rhopalosiphum padi*. Mothers of these plants had been challenged (Aphids) or not (Control) with the same herbivore. Shoot biomass was measured right after finishing the herbivory treatment. Values are means \pm SE ($n=7$). Letters indicate significant pairwise differences between means (Tukey HSD, $P < 0.05$)



(33% lower) by endophyte presence but not by the herbivory history of their maternal plants (Fig. 3e). Mean weights of nymphs and winged adults were not affected by the maternal history of herbivory nor by the symbiosis with endophytes (Fig. 3d, f).

With a significant effect of the symbiosis with fungal endophytes, the total biomass of aphid herbivores per plant was marginally affected by the maternal history of herbivory (Table 4). With no history of herbivory, the biomass of herbivores was 54% lower in symbiotic plants compared with that in non-symbiotic ones (Fig. 4a). However, the legacy of maternal herbivory resulted in a greater difference (87% lower) in the total herbivore biomass supported by endophyte-symbiotic versus non-symbiotic plants (Fig. 4a).

The concentration of loline alkaloids in shoot tissues of progeny plants did not depend on the history of herbivory of the maternal plants. Instead, the alkaloid concentration was greater within endophyte-symbiotic progeny plants challenged with aphids (Table 4, and Fig. 4b). Compared to plants not challenged with aphids, progeny plants challenged with aphids showed a 1.5-fold greater concentration of loline alkaloids (Fig. 4b).

Discussion

Herbivory by aphids did not lead to an increase in either *Epichloë* mycelial biomass or loline alkaloid concentration in seed produced by *L. multiflorum* mother plants symbiotic with *E. occultans* (question i). As expected, the level of plant resistance was increased by the fungal endophyte. Additionally, there was a tendency for this resistance to be increased

in progeny plants whose mothers had been exposed to the same insect herbivore. Although the current exposure of progeny plants to aphids induced the concentration of loline alkaloids, it was independent to the history of the mother plants regarding the exposure to the same herbivore. Compared to non-symbiotic plants, endophyte-symbiotic plants exhibited a higher performance in terms of shoot biomass that was irrespective of the maternal or current herbivory treatment. Therefore, question ii, posed at the beginning of our work, was positively but partially supported by the results.

Results suggest that there is certain independence between the status of the endophyte-produced defences in seeds and the resistance response of the next generation plants to insect herbivores. For example, maternal herbivory in *Arabidopsis thaliana* and *Solanum lycopersicum* plants were not associated with changes in the biochemistry or size of seeds but with a greater resistance to herbivores in the progeny (Rasmann et al. 2012). These latter results are similar to ours despite being different systems (different plants species and lacking the involvement of endophytes). Herbivory by *R. padi* on *L. multiflorum* mother plants caused no effect on either seed mass (i.e., weight) or alkaloid concentration, but it modulated the extent of herbivory resistance exhibited by progeny plants. A positive relationship has been observed between *Epichloë* mycelial biomass and alkaloid concentration in seed as well as in vegetative tissues (Freitas et al. 2020; Ryan et al. 2015; Ueno et al. 2020). The accumulation of lolines in grass seed could have been synthesised in vegetative tissues and mobilized to the reproductive units during the filling stage (Ueno et al. 2020). Here, herbivory by *R. padi* induced the synthesis of loline

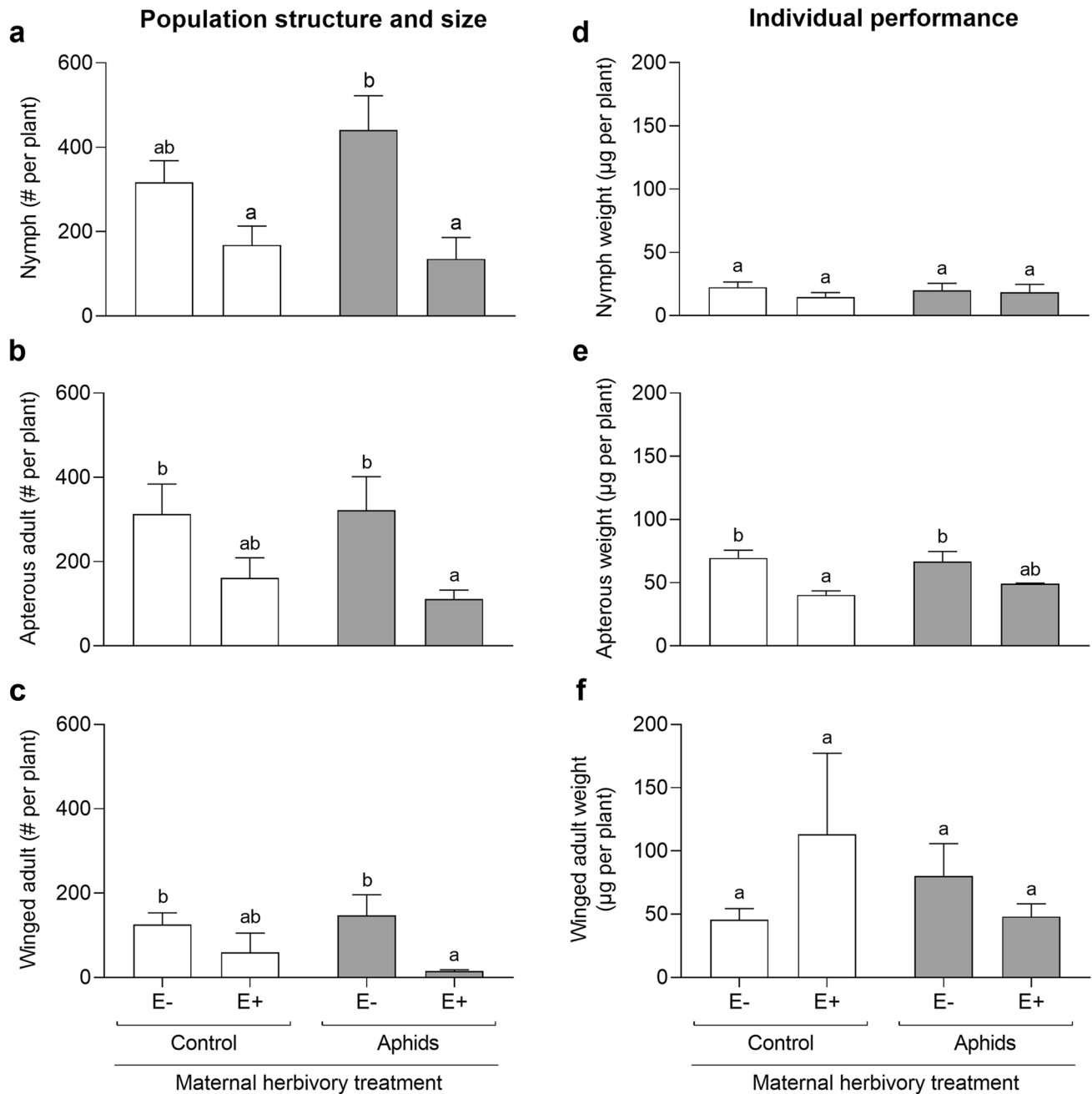


Fig. 3 Parameters of individual and population performance for the different instars (nymphs, apterous adults, and winged adults) of the aphid *Rhopalosiphum padi* reared for 21 days on *Lolium multiflorum* plants with (E+) and without (E-) the fungal endophyte *Epichloë occulta*. Plots (a) and (d) correspond to nymphs, (b) and (e) corre-

spond to apterous adults, and (c) and (f) winged adults. The mother of these plants had also been challenged (Aphid) or not (Control) with the same herbivorous insect. Values are means \pm SE ($n=7$). Letters indicate significant differences between means (Tukey HSD, $P < 0.05$)

alkaloids in *L. multiflorum* plants, which is added to the list of previous antecedents in other plant-endophyte symbiosis (*Festuca arundinacea* - *E. coenophiala*, *Lolium perenne* - *E. festucae* var. *loii*) and artificial or actual herbivory by biting or chewing insects (Bultman et al. 2004; Fuchs et al. 2017b;

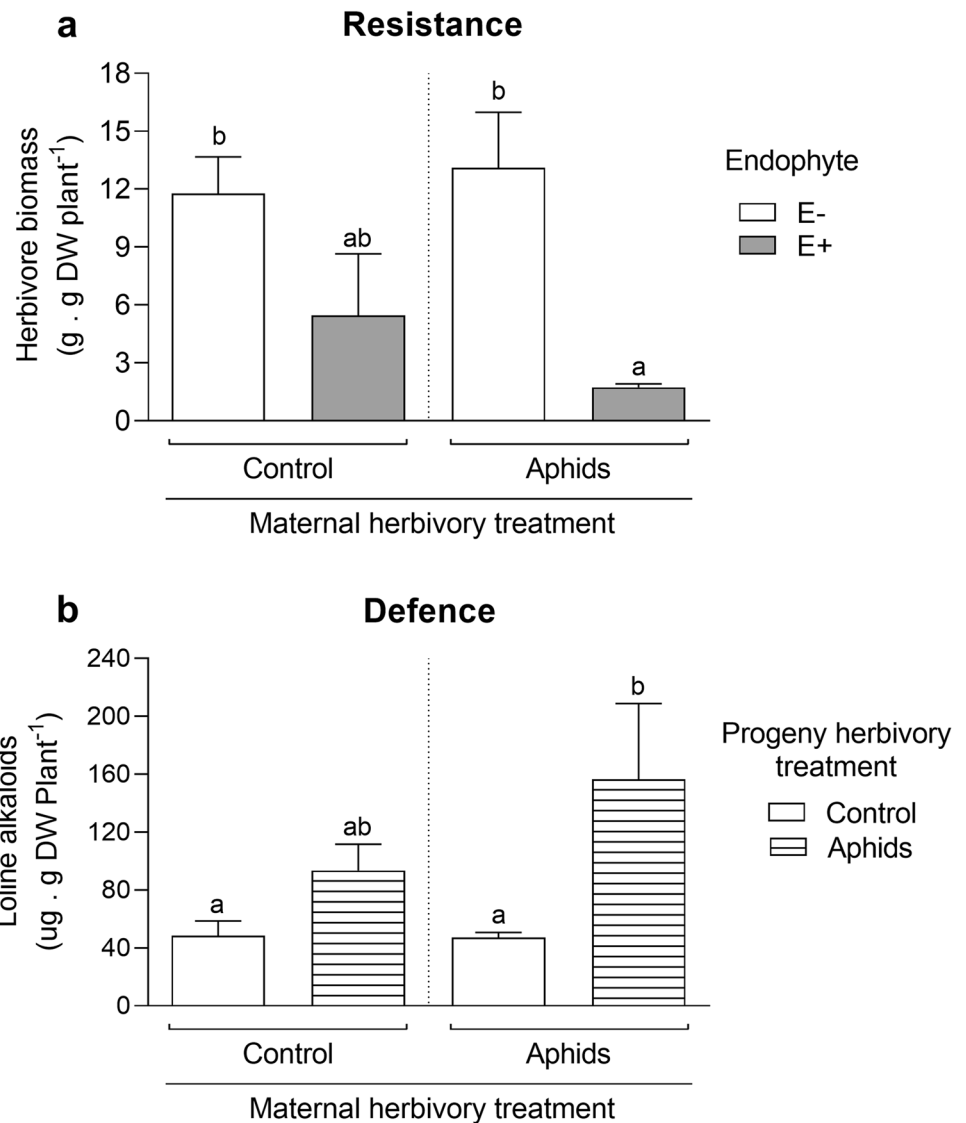
Sullivan et al. 2007). However, this aphid-mediated induction of alkaloids in plant did not translate into greater concentrations of alkaloids in seeds even though mother plants were starting to go reproductive (heading state – anthesis) when the herbivory treatment was terminated. To investigate

Table 4 Linear and generalized mixed models for total herbivore biomass in *Lolium multiflorum* progeny plants and the concentration of total loline alkaloids (NAL + NANL + NFL) (i.e. only reported for endophyte-symbiotic plants), respectively

Response variable	Source	df	X ²	P-value
Herbivore biomass per plant (g)	Maternal Herbivory treatment (MH)	1	0.01	0.917
	Endophyte (E)	1	3.60	0.050
	MH x E	1	2.86	<i>0.090</i>
Loline alkaloids ($\mu\text{g}\cdot\text{g}^{-1}\text{DW}$)	Maternal Herbivory treatment (MH)	1	0.50	0.477
	Progeny Herbivory treatment (PH)	1	10.99	<0.001
	MH x PH	1	0.404	0.524

Significant ($P < 0.05$) results are displayed in bold while marginally significant trends ($0.05 < P < 0.10$) are presented in italic

Fig. 4 a Total herbivore biomass in *Lolium multiflorum* progeny plants symbiotic with (E+) and without (E-) the fungal endophyte *Epichloë occultans* challenged (Aphids) or not (Control) with the aphid *Rhopalosiphum padi*. Mothers of these plants had been challenged (Aphids) or not (Control) with the same herbivorous insect. **b** The concentration of total loline alkaloids (NAL + NANL + NFL) was measured right after the herbivory treatment only in endophyte-symbiotic plants. Values are means \pm SE ($n = 7$). Letters indicate significant pairwise differences between means (Tukey HSD, $P < 0.05$)



this, the temporal dynamics of alkaloid concentration in response to herbivory requires further experimentation since most studies only take punctual measurements and failed to identify an aphid-mediated induction of fungal-derived

alkaloids in vegetative plant tissues (Ueno et al. 2016; Fuchs et al. 2017b). Therefore, despite a variation observed in alkaloid concentrations in seeds (see Gundel et al. 2018a; Ueno

et al. 2020), the factors controlling such variation are still unknown.

The performance of individuals and aphid populations were scrutinized in search for evidence of an altered plant resistance level due to the maternal history of herbivory and symbiosis with fungal endophytes. The number of aphids in each instar as well as the entire population (the sum of the three instars) were only affected negatively by the fungal endophytes but not by the mother history of herbivory (Fig. 3; Table 3). Weight of apterous adults was the only impaired variable of aphid individual performance by the presence of fungal endophytes. However, the variable of aphid performance that integrates the individual and population levels (biomass of herbivores) showed a marginal effect of interaction between the endophyte presence and the history of mother plant exposure to herbivores (Fig. 4; Table 4). Therefore, in our study, the herbivory experienced by *L. multiflorum* mother plants with or without *Epichloë* endophytes had a weak legacy effect on the overall progeny performance and level of herbivory resistance. Not all studies have found increased resistance in progeny from mother plants subjected to herbivory (e.g., Agrawal 2001; Holeski et al. 2013). Interestingly, most *Epichloë* fungal endophytes such as the one we used here '*E. occultans*', are maintained through maternal lineages by means of vertical transmission and are the symbiotic microorganisms that endow grasses with an effective anti-herbivory mechanism (Bastías et al. 2017; Panaccione et al. 2014; Saikkonen et al. 2013; Scharld et al. 2013). Therefore, despite this weak effect of herbivory in inducing intergenerational resistance, as long as the endophyte symbiont is transmitted through the seeds, progeny plants are protected against herbivores (Gundel et al. 2017, 2020). A role of *Epichloë* fungal endophytes in intergenerational effects has been reported in response to ozone and water availability (Bubica Bustos et al. 2020; Ueno et al. 2021; Xia et al. 2018). All this insipient evidence reinforces the idea that vertically transmitted *Epichloë* fungal endophytes would modulate the environmental factor mediated induction of intergenerational effects in plants.

The expression of defences may be costly for plant growth but ultimately, it may result in increased plant fitness (Garcia et al. 2021). Here, after 21 days of being challenged by aphids, endophyte-symbiotic progeny plants exhibited a significantly greater concentration of loline alkaloids compared with plants that were not exposed to the insect herbivore. The general relationship was that the greater the alkaloid concentration, the lower the aphid population size (a higher the level of resistance). This effect was not linked with a variation in plant shoot biomass. Furthermore, endophyte-symbiotic progeny plants were larger (higher shoot biomass) than their endophyte-free counterparts, regardless of the maternal, or current, history of herbivory. Previous experimentation has shown

that endophyte symbiosis can hinder plant recovery from defoliation (e.g., Saari et al. 2010; García Parisi et al. 2012), which may result from plant strategies evolved to cope with insect herbivory (e.g., a resistance/tolerance trade-off) (Partida-Martinez and Heil 2011). However, a study of *Poa autumnalis*, indicated that plants symbiotic with *Epichloë* exhibited increased plant tolerance to folivory without any compromise to plant growth compared to their non-symbiotic counterparts (Gundel et al. 2020). Discrepancies between studies may be due to differences in plant and fungal species, herbivore guild (folivores, chewers or sapsuckers), the magnitude of tissue eaten by the herbivore and the availability of soil nutrients (Saikkonen et al. 2010). Even though scarcity of resources can limit the endophyte-derived benefits to their host plants (Faeth and Fagan 2002), evidence shows that *Epichloë* can confer resistance and boost plant growth under a wide range of environmental conditions (Bastías et al. 2021). Beside the resource-based trade-off, recent advances show that trade-offs in plants are controlled by hormone signalling pathways (Erb and Reymond 2019; Robert-Seilaniantz et al. 2011). Synthesis of auxin has been indicated as a putative mechanisms underlying endophyte-mediated plant growth improvement (De Battista et al. 1990). By endowing plants with alternative circuits of defences (alkaloids), and synthesizing hormone-promoting plant growth, *Epichloë* have been suggested to break down growth-defense trade-off (Saikkonen et al. 2013; Bastías et al. 2017). Therefore, if soil resources are not limiting, *Epichloë* can protect their host plants against herbivores without compromising plant growth.

In summary, our work suggests that maternal herbivory by aphids could increase the level of the endophyte-conferred defences and resistance in the progeny plants. Since we did not detect any intergenerational effect of the herbivory by aphids on endophyte-free plants, we propose that the differential fitness between symbiotic and non-symbiotic plants could increase by means of the intergenerational reinforcement of the resistance in presence of herbivores. Thus, besides selecting favourably for an inherited protective symbiont, high pressure of herbivory could favour endophyte-symbiotic progeny plants from herbivore attacked mother plants which would likely be primed through a mechanism of epigenetic regulation. However, future experiments should be designed to unveil the underlying molecular mechanisms of such herbivore-induced intergenerational resistance in symbiotic plants with *Epichloë* fungal endophytes.

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Author Contributions LMBB, MAMG and PEG conceived the ideas and designed the study. LMBB, SDC, WJM, collected the data. ACU and FB analysed the data. LMBB, ACU, SDC and PEG wrote the first draft of the paper. All authors provided substantial feedback and revisions.

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Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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