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RESEARCH ARTICLE

Foliar phosphorus concentration modulates the defensive mutualism of an endophytic fungus in a perennial host grass

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Abstract

1. Grasses hosting *Epichloë* endophytes are protected against herbivores due to the production of various fungal alkaloids. Previous research has found that high foliar phosphorus concentrations reduce the level of the alkaloid ergovaline, thereby reducing the endophyte-mediated herbivore resistance. Yet, the impact of phosphorus on ergovaline biosynthesis versus its influence on endophyte growth and synthesis of other fungal alkaloids remains unresolved. Our objective was to elucidate these relationships.
2. We grew endophyte-symbiotic and non-symbiotic *Festuca arundinacea* plants and fertilised them with different doses of phosphorus. Later, half of the plants from each treatment were challenged with larvae of the generalist chewing insect *Spodoptera frugiperda*. We assessed the relationships between foliar phosphorus levels, fungal mycelium and alkaloid concentrations, as well as their impacts on larvae performance, herbivore-caused damage and plant biomass.
3. Endophyte mycelial biomass in plant tissue was found to be independent of foliar phosphorus concentration. The alkaloids lolines and peramine showed a linear relationship with mycelial biomass but no correlation with foliar phosphorus. Surprisingly, high ergovaline concentrations were positively associated with an interaction between endophyte mycelial biomass and foliar phosphorus concentration. Although herbivory increased loline concentration, only high concentrations of ergovaline and peramine were related to reduced *S. frugiperda* larvae weight gain. However, endophyte presence did not reduce herbivory damage on plants.
4. Contrary to expectation, we did not find a negative but a positive association between concentrations of foliar phosphorus and ergovaline alkaloid, through its interaction with endophyte mycelial biomass. Alternatively, our findings suggest that phosphorus plays a crucial role in modulating the *Epichloë*-mediated defensive mutualism, primarily through its effects on ergovaline rather than on endophyte concentration or production of other alkaloids.

KEYWORDS
Epichloë, fungal alkaloids, fungal endophytes, herbivory, *Spodoptera frugiperda*, tall fescue

1 | INTRODUCTION

Plants are permanently threatened by insect herbivores that can reduce their fitness (Agrawal et al., 2012). The impact of insect herbivory on plant communities can be significant, as these herbivores can reduce the population size of target plant species (Agrawal & Maron, 2022). However, plant abundance can persist or even increase in the face of herbivory if the plant evolves resistance-conferring traits, such as chemical defences, or forms symbiotic associations with microorganisms that provide defensive compounds (Agrawal et al., 2012; Clay et al., 2005). Particularly in the latter case, the effectiveness of microorganism-conferred resistance against herbivores can be modulated by nutrients availability. Nutrients play a crucial role not only by altering the quality of plant tissues for herbivore consumption but also by influencing the persistence of symbiotic microorganisms and the production of microorganism-derived defensive compounds (Ebeling et al., 2022; Faeth & Fagan, 2002; Graff et al., 2020; Rasmussen et al., 2007). Although humans enrich ecosystems primarily with phosphorus and nitrogen through agricultural practices such as fertilisation (Steffen et al., 2015), our understanding of how phosphorus modulates these complex interactions is less developed compared to that of nitrogen, particularly for grasslands (Ebeling et al., 2022). As humans continue to enrich those ecosystems with phosphorus and insect herbivory is expected to increase due human-induced climate change (Hamann et al., 2021; Peñuelas et al., 2013), it is important to increase our understanding of how this element modulates the complex interaction between plants, symbiotic microorganisms, and insect herbivores.

Certain plant species can enhance their defences against insect herbivores through associations with beneficial microorganisms, forming what is known as defensive mutualism (Clay, 2014; Panaccione et al., 2014; Pineda et al., 2010). In a defensive mutualism, a symbiotic microorganism benefits its host by protecting it against natural enemies with physical or chemical defences (Clay, 2014; Panaccione et al., 2014). Among the plants that benefit from these types of associations are certain cool-season grass species of the subfamily Pooideae (Poaceae), which establish symbiosis with *Epichloë* fungal endophytes (family Clavicipitaceae) and are found in natural and productive temperate grasslands worldwide (Rudgers et al., 2009; Semmartin et al., 2015; Vikuk et al., 2019). The association with *Epichloë* fungal endophytes has allowed these grass species to expand their defences against a broad spectrum of vertebrate and invertebrate herbivore species, a characteristic that is clearly observable when compared to plant species that do not form this association (Bastías et al., 2017; Saikkonen et al., 2010; Vicari & Bazely, 1993). The *Epichloë* endophyte-mediated resistance against herbivores in these grass species is attributed to the presence of various chemical classes of fungal alkaloids (Realini et al., 2024; Schardl et al., 2023).

Epichloë fungal endophytes are obligate symbionts, with some species being vertically transmitted from plant to seeds, a characteristic that allows both partners' fitness to align and can strengthen the mutualism (Gundel et al., 2011; Schardl et al., 2023). In addition to alkaloid-mediated herbivory resistance, symbiotic plants usually exhibit benefits such as improved resistance through silicon accumulation, increased growth and competitive abilities, and greater tolerance to abiotic stressors and pathogens (Bastías et al., 2022; Cibils-Stewart et al., 2023; Johnson et al., 2023; Malinowski & Belesky, 2000; Pérez et al., 2013; Vázquez-de-Aldana et al., 2013). The association with *Epichloë* endophytes provides a significant advantage to host grasses, particularly in environments with high herbivory pressure, leading to a rapid increase in symbiosis prevalence over time (Clay et al., 2005; Rudgers & Clay, 2007). Nevertheless, these advantages can lead to the dominance of *Epichloë*-associated plants in grasslands, posing a potential threat to biodiversity in local communities and altering ecosystem processes (Graff et al., 2020; Minás et al., 2021; Omacini et al., 2004; Rudgers & Clay, 2007). Therefore, while *Epichloë* endophytes play a crucial role in shaping the structure and functioning of grassland communities, their impact may depend on how soil nutrient availability modulates the defensive mutualism effectiveness.

The ability of *Epichloë* endophytes to enhance the resistance of their host plant is primarily ascribed to the synthesis of antiherbivore alkaloids. There are four well-described alkaloids that exhibit different effects on herbivores, and the composition of alkaloids within a plant depends on the species of fungus present (Realini et al., 2024; Schardl et al., 2012, 2023). These alkaloids are ergovaline (ergots), lolitrem B (indole-diterpenes), lolines (1-aminopyrrolizidines) and peramine (pyrrolopyrazines) (Schardl et al., 2012, 2023). Ergovaline and lolitrem B are recognised for their detrimental effects on mammals, leading to livestock production losses, while lolines and peramine are toxic for insect herbivores (Johnson et al., 2013; Saikkonen et al., 2013; Schardl et al., 2012). Although ergovaline and lolitrem B have primarily been studied for their harmful effects on livestock, there is significant evidence suggesting their adverse impacts on insect herbivores as well (Graff et al., 2020; Potter et al., 2008; Realini et al., 2024). But the effectiveness of the *Epichloë*-based defensive mutualism can vary depending on herbivore species as well as the environmental conditions (Bastías et al., 2017; Bastías & Gundel, 2023; Saikkonen et al., 2010). For example, there are reports that lolines and peramine concentrations increases after herbivory (Bultman et al., 2004; Cibils-Stewart et al., 2023; Fuchs et al., 2017a; Sullivan et al., 2007), and the level of soil nutrients, particularly nitrogen, could influence the biomass of endophyte mycelium, thereby controlling alkaloid concentration (Faeth & Fagan, 2002; Rasmussen et al., 2007). As alkaloids are nitrogen-rich compounds, previous studies have mainly focused on the role of nitrogen as a modulator of *Epichloë*-based defensive mutualism, while other macronutrients, such as phosphorus, have received less attention.

The availability of phosphorus (P) is proposed to influence the production of ergot alkaloids (e.g. ergovaline) in *Epichloë*-symbiotic plants, which in turn modulates the effectiveness of the defensive mutualism (Malinowski & Belesky, 2000). However, few studies have investigated the relationship between P and ergot alkaloid production in plant-*Epichloë* associations. In *Festuca arundinacea* Schreb., commonly known as tall fescue (syn. *Lolium arundinaceum* and *Schedonorus arundinaceus*), fertilisation with P reduced the concentration of ergot alkaloids; nonetheless, the effects of P varied among plant genotypes (Malinowski et al., 1998). In a field experiment, *F. arundinacea* plants fertilised with high levels of P showed reduced ergovaline concentrations and were highly consumed by chewing insects (larvae of *Paracles vulpina*) (Graff et al., 2020). Although this latter paper showed a clear relationship between foliar P, ergovaline concentration, and its effects on plant–herbivore interaction, it remains unresolved whether phosphorus solely affects ergovaline production or also influences the synthesis of other alkaloids or endophyte colonisation. In addition, P levels can also affect plant-herbivore interaction through changes in plant tissue quality. Foliar P concentration and its stoichiometric relationships with other macronutrients, such as the nitrogen-to-phosphorus (N:P) ratio, can influence herbivores' consumption. For example, higher P concentration tends to correlate with increased herbivore consumption, particularly among chewing insects (Butler et al., 2012; Perkins et al., 2004). Hence, it is challenging to separate the hypothesised P-related inhibitory effect on ergot alkaloids from its potential impact on other fungal alkaloids, as well as on the growth of the host and symbiont, and the quality of plant tissue.

In this study, we examined the influence of foliar phosphorus levels on the resistance against insect herbivores conferred by *Epichloë* endophytes. Our general hypothesis posits that fungal alkaloid production, specifically ergovaline, declines as foliar P levels increase. Consequently, resistance against insect herbivores decreases and the overall plant performance declines. This pattern produced by high P concentrations may arise from a decrease in the abundance of *E. coenophiala* mycelium, a decrease in the production of *Epichloë*-derived alkaloids, particularly ergovaline, or an alteration in plant tissue quality. We cultivated both endophyte-symbiotic and non-symbiotic *F. arundinacea* plants under different P levels. Then, half of the plants were challenged with the generalist chewing insect *Spodoptera frugiperda* (Lepidoptera Noctuidae). We quantified *Epichloë* mycelia biomass and *Epichloë*-derived alkaloids, as well as the concentration of quality-associated nutrients in the plant tissue. Additionally, we assessed herbivore performance, herbivore-caused damage, and plant performance. We predicted that endophyte-symbiotic plants with higher foliar P levels will exhibit reduced concentrations of ergovaline compared to those with lower foliar P levels. Consequently, we predicted that the higher-P symbiotic plants will be more susceptible to consumption by chewing insects than their lower-P counterparts. Additionally, we anticipated that herbivore damage and larvae weight gain will be comparable between the higher-P symbiotic plants and the non-symbiotic plants.

2 | MATERIALS AND METHODS

2.1 | Biological material

2.1.1 | Plant material

Our study model was the symbiotic association between *F. arundinacea* and *E. coenophiala* (Leuchtmann et al., 2014). Due to its high agronomic value as a forage resource, *F. arundinacea* was introduced in the grasslands of the Pampa region, Argentina, and now is naturalised (Gibson & Newman, 2001; Scheneiter et al., 2016). Surveyed populations have shown that the prevalence of symbiosis with *E. coenophiala* is consistently near 100% (Graff et al., 2020; Gundel et al., 2009). This fungus is known to produce lolines, peramine and ergovaline (Schardl et al., 2012). However, there is also variability among fungus strains in the profile of alkaloids (Johnson et al., 2013).

Festuca arundinacea seeds with (E+) and without (E−) endophyte were kindly provided by a working team settled at Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata (Ruta Nacional 226, km 73.5, Balcarce, Buenos Aires province, Argentina). They collected seeds from *Epichloë*-symbiotic plants in a grassland located in Mar Chiquita County (37°32′ S; 57°55′ W), Buenos Aires province, Argentina. They separated the seeds into two lots, one that was stored in dry conditions and at 5°C, and the other one that was stored at 20°C and 52% relative humidity. Storage under high temperatures and relative humidity increases the rate of endophyte loss, leading alive seeds to produce endophyte-free seedlings (Riveira Rubin et al., 2022; Welty et al., 1987). After a year of storage, they confirmed that seeds stored in dry and low temperatures produced endophyte-symbiotic seedlings (E+) while seeds stored at higher temperatures and humidity produced only endophyte-free seedlings (E−) (For more details see Petigrosso et al., 2013). Taking about 50 seeds from each group, stands of individual plants were established in the experimental field of the Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina, to produce fresh E+ and E− seeds for experiments. Endophyte presence in seeds was determined using a staining technique (Belanger, 1996; Card et al., 2011; Gundel et al., 2018). Seeds were immersed in NaOH (5%) for at least 12 h, stained with Rose Bengal, and observed under the microscope (100×). The seeds used in this work were produced a year before the present experiment.

2.1.2 | Insect herbivore

We used *S. frugiperda*, commonly known as fall armyworm, a chewing generalist herbivore that has been previously utilised in studies regarding endophyte-conferred resistance to herbivory (see e.g. Bastías et al., 2017; Saikkonen et al., 2010). Larvae were obtained from a colony that is permanently maintained in our laboratory (>85 generations). They are reared on a synthetic diet (composed of pinto beans, wheat germ, soybean protein, brewer yeast, ascorbic acid, tetracycline, methylparaben, sorbic acid and agar) and under controlled conditions (21°C, photoperiod L16:D8h and radiation 150 μmol/m^2/s).

2.2 | Soil origin and phosphorus treatments

The soil was collected from a field located in Gualeguaychú Department, Entre Rios province, Argentina (33°18′ S; 58°41′ W). It was analysed to determine the level of extractable phosphorus, total nitrogen, and carbon. For this, 10 samples of 3 g each were separately sieved with a 2 mm mesh net. The analysis was a standard one for nutrient contents in soils and was conducted by the Laboratory for Soil, Plant and Environment Analytical Services (LABSPA-CERZOS-CONICET, Bahia Blanca, Argentina). The extractable phosphorus concentration in the soil was 8.93 ± 0.40 mg P/kg soil (see Table S1 for the other elements).

Regarding the fertilisation treatments, we aimed to create four discrete levels of extractable P in the soil: (Control) 8.9 mg P/kg soil; (T1) 20 mg P/kg soil; (T2) 40 mg P/kg soil; and (T3) 80 mg P/kg soil. These levels were selected to create a wide range of soil P concentrations, ranging from nearly deficient to highly available and also to be comparable with previous studies (i.e. Graff et al., 2020; Malinowski et al., 1998). The amount of P applied to achieve those levels was estimated using the model presented by Rubio et al. (2008). This model predicts the rise in extractable P in the soil based on edaphic factors and the amount of P applied and was designed specifically for Argentinian soils from the Pampas region. The P was applied in the form of triple superphosphate [$Ca(H_2PO_4)$]. After the calculation, four P treatments were established with the addition of these quantities of triple superphosphate: (Control) 0 mg/kg soil, (T1) 55 mg/kg soil, (T2) 155 mg/kg soil and (T3) 355 mg/kg soil. The plants were fertilised at the beginning of spring (end of September, 2019).

2.3 | Experimental design

The fertilisation treatment resulted in a great variation in foliar P levels for each combination of treatments (Figure S2). Since the phosphorus fertilisation failed to produce four discrete groups of foliar P concentration, we used the actual foliar P concentration as an explanatory variable.

The seeds were sown in the middle of Autumn (May in the southern hemisphere) and the experiment finished with the plant harvest in late Spring (November in the southern hemisphere). At harvest, the plant stage was between tillering and the emergence of panicles. At the beginning, 60 E+ and 60 E− seeds were sown individually in 4-L pots. All the pots were filled to the top with the soil mentioned previously (see Section 2.2). The plants were grown in a common garden in natural conditions and watered regularly to avoid growth limitations and stress due to water deficit. Plants were arranged in a square grid with their positions randomly assigned. Two months after sowing, plants of similar sizes from each symbiotic type (40 E+ and 40 E−) were selected for the experiment.

The plants were kept under the same management until the end of winter (middle September) when they were randomly assigned to any of the fertilisation treatments. The different doses of the triple superphosphate fertiliser were applied following the instructions provided by the manufacturer (YPFagro®). To give the plants time to uptake the phosphorus, they were grown for 2 months before being challenged by herbivores.

The herbivory treatment was applied in mid-spring (November). Half of the plants from each treatment (combination of symbiotic status and fertilisation treatment) were challenged by a group of five *S. frugiperda* stage-3 larvae (replicates = 5, see Figure S1). Before being placed in the plants, each larva was weighed and the mean larvae weight per group was calculated. To prevent the herbivores from escaping, each plant was enclosed with a cylinder made of plastic 1-cm^2 mesh that was additionally surrounded by white voile fabric. The fabric allows the exchange of air and light, while the plastic cylinder prevents the fabric from collapsing on the plant. The herbivores were kept on the plants for 1 week, when the plants were harvested and the herbivores recovered for evaluation. No ethical approval was required for this research.

2.4 | Leaf nutrient content

Before placing the herbivores in the plants, 10 leaves from each plant were randomly selected and harvested. The leaves were dried in an oven at 60°C for 72 h, grounded and analysed. Foliar phosphorus concentration was determined by the spectrophotometric molybdenum blue phosphorus method (Murphy & Riley, 1962), using a UV–visible spectrophotometer (Spectronic 601). Nitrogen concentration was determined by Kjeldahl digestion method.

2.5 | Endophyte mycelium and alkaloid concentrations

To assess the biomass of endophyte mycelia and alkaloid concentration in foliar tissue, 10 leaves were randomly selected from each endophyte-symbiotic plant. The samples of tissue were taken from the base of the leaves, where *Epichloë coenophiala* dwells. The samples were grounded with liquid nitrogen, lyophilised, and stored in 2 mL plastic vials. All these determinations were performed in AgResearch Ltd., Forage Improvement, Grasslands Research Center, New Zealand.

The biomass of endophyte mycelia was assessed using an enzyme-linked immunosorbent assay (ELISA) developed by AgResearch NZ (Faville et al., 2015). The ELISA plates were coated with *E. coenophiala* antigen. Additionally, a 1% bovine serum albumin was used as a blocking agent. The mycelia biomass of each sample was quantified using standard curves prepared with *E. coenophiala* endophyte standard. The detection of the endophyte in the seed sample extracts was determined by observing the reduced binding of specific *E. coenophiala* antibodies to the coating antigen. This detection method involved using a commercially available anti-rabbit-horseradish peroxidase (HRP) conjugate and 3,3',5,5'-Tetramethylbenzidine substrate for HRP to generate a visible signal. The relationship between the mean absorbance and the logarithm of the analyte concentration was determined through curve fitting using a four-parameter logistic regression. The results were expressed as *E. coenophiala* immunoreactive equivalents per microgram of dry weight.

The concentrations of the three alkaloids in foliar tissues were quantified using slightly modified protocols as described by Moore et al. (2015). For loline alkaloids, 50 mg were extracted from each sample, for 1 h with 50 μL of 40% methanol/5% ammonia and 1 mL of 1,2-dichloroethane (containing 54.8 ng/mL of 4-phenylmopholine as an internal standard). The samples were centrifuged and analysed with a gas chromatography flame ionisation detector (GC2010Plus; Shimadzu Corporation, Japan). For ergovaline, each sample received 1 mL of prepared extraction solvent, a mixture of methanol and water (50/50, V/V) with 0.54 μg/mL ergotamine tartrate (Sigma Chemical Co., St. Louis, MO, USA) as an internal standard. Ergovaline content was determined using high-performance liquid chromatography with a Gemini-NX C18 150 × 2.0 mm (3 μm) column (Phenomenex, Torrance, CA, USA) and detected with a Shimadzu RF-10Axl fluorescence detector (excitation at 310 nm, emission detected at 410 nm). To separate targeted analytes, a linear gradient profile (eluent A = 10 mM ammonium carbonate with 20% acetonitrile [v/v], eluent B = acetonitrile; T0 = 8% B, T9 = 25% B, T7.5 = 60% B, T8 = 83.3% B, T9 = 83.3% B followed by equilibration to primary conditions over the following 9 min) was used. For peramine, the analysis involved extraction with a solvent mixture (methanol/water in a 50/50, V/V ratio) containing 1.70 ng/mL homoperamine nitrate (BDG Synthesis, Lower Hutt, New Zealand) as an internal standard. Targeted analytes were separated using a Synergy Polar-RP 100 × 2.00 mm (2.5 μm) column (Phenomenex, Torrance, CA, USA) with a linear gradient profile (eluent A = aqueous 0.1% formic acid, eluent B = acetonitrile; T0 = 5% B, T9 = 40% B, T11 = 90% B, T12 = 90% B, followed by equilibration to initial conditions over the next 8 min). Quantification was performed using mass spectroscopy (MSQ; Thermo Scientific) based on the parameters described in Rasmussen et al. (2012). The limit of quantification was established at 0.1 μg/g DM (0.1 ppm).

2.6 | Performance of *S. frugiperda* larvae

We assessed the performance of the herbivore by measuring the weight gain observed during the period they were feeding on the plants. At the end of the herbivore treatment (7 days), larvae were recovered and weighed again with a precision scale (±0.001 g) to estimate the mean larva weight per plant. Values of larvae weight are expressed relative to the initial mean weight and on a fresh basis.

2.7 | Herbivore damage estimation

We used a herbivory index to estimate the damage caused by herbivores to plants (Boege & Dirzo, 2004; Salgado-Luarte et al., 2023). Plants not exposed to herbivores were not included in this calculation as they did not show signs of damage (personal observation). For each estimation, five leaves were randomly selected and photographed. Then, the damaged area was estimated using the software ImageJ (Abràmoff et al., 2004). As in Boege and Dirzo (2004), each leaf was scored according to the level of damage: 0 = no damage, 1 = 1%–6% damage, 2 = 6%–12% damage, 3 = 12%–25% damage, 4 = 25%–50% damage, and 5 = more than 75% of damage. These categories were selected because low levels of damage were most frequent and high levels of damage were less common. Thus, categories with narrower ranges were needed in the lower part of the range to capture differences in plant damage (Boege & Dirzo, 2004). Adapted from Boege and Dirzo (2004), the herbivory index for each plant was calculated as: $\textit{Herbivory index} = d / N \times \sum n_i \times C_i$, where d is the number of leaves with damage, N the total number of evaluated leaves, i is the ith damage score, n_i the number of leaves with the ith damage score, C_i is the midpoint of the ith damage score (i.e. $C_0 = 0$, $C_1 = 3.5$, $C_2 = 9.0$, $C_3 = 18.5$, $C_4 = 37.5$ and $C_5 = 87.5$).

2.8 | *F. arundinacea* biomass

After herbivores were recovered, all plants were harvested. The number of leaves was counted in all the plants. In herbivore-challenged plants, the number of damaged leaves was counted and assessed to determine the herbivory index (see above). After that, all fractions of the above-ground biomass were placed in an oven at 60°C for 72 h to obtain dry weight. Plant biomass was weighed with a scale to the nearest 0.01 g.

2.9 | Replication statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Symbiotic-status	Plant	5 endophyte-symbiotic, 5 endophyte-free (combinations with fertilisation and herbivory treatments)

2.10 | Statistical analysis

All the analyses were made with the R programming language (v4.2.2; R Core Team, 2022), using base R functions and some additional packages (MASS package, Venables & Ripley, 2002; car package, Fox & Weisberg, 2019; nlme package, Pinheiro & Bates, 2023). In all the cases, the assumptions of each model were checked using graphical and analytical methods (Zuur et al., 2009). Full tables of statistical results are in Data S2.

Foliar phosphorus concentration, endophyte mycelia and alkaloid concentrations were analysed using multiple regression models. For the analysis of lolines, we found one outlier with three times higher lolines content than expected. We confirmed that it was an influential observation in the model estimations by analysing the model's standardise residuals and Cook's distances (Greco et al., 2019). Then, we analyse the data with robust regression model using the *MASS* package (Greco et al., 2019; Venables & Ripley, 2002). Finally, the effect of herbivory by *S. frugiperda* over the concentration of each alkaloid was analysed with a linear model using generalised least squares, where errors were modelled as a function of herbivory using the *varIdent* function from the *nlme* package (Pinheiro & Bates, 2023; Zuur et al., 2009).

The herbivores' performance was estimated using the mean weight gained by *S. frugiperda* larvae. The effect of *E. coenophiala* on larvae weight gain was analysed with an ANOVA. The relationships between the weight gained and each alkaloid concentration and nutrient stoichiometry (i.e. C:N ratio and N:P ratio) were analysed using linear regression models. The plant damage was estimated using a herbivory index (see *Herbivory index* for details), and plant performance was assessed by measuring above-ground biomass. The relationships between the herbivory index and foliar P concentration, nutrient stoichiometry and alkaloid concentrations were analysed using linear models. The effect of herbivory and endophyte presence on plant's above-ground biomass was analysed with an ANOVA.

3 | RESULTS

3.1 | Foliar phosphorus, fungal mycelia and alkaloid concentrations

Endophyte mycelial biomass was neither affected by foliar phosphorus ($F_{1,30}=0.264$, $p=0.611$) nor by herbivory ($F_{1,30}=0.894$, $p=0.352$) (Figure 1). Ergovaline concentration was dependent on the interaction between mycelial biomass and foliar phosphorus. Ergovaline levels increases with mycelial biomass, and these increases were higher at high foliar P concentrations (Mycelia × Foliar P: $t=2.207$, df = 29, $p=0.035$; Figure 2). The alkaloids lolines and peramine were linearly related to endophyte mycelial biomass (Lolines: $t=5.288$, df = 31, $p<0.001$; Peramine: $t=3.892$, df = 31, $p<0.001$; Figure 3b,c), but foliar phosphorus has no effect over their concentrations (Lolines: $t=-1.605$, df = 30, $p=0.119$; Peramine: $t=-0.176$, df = 30, $p=0.861$).

The concentration of lolines alkaloids was higher in plants exposed to herbivory by *S. frugiperda* ($F_{1,31}=4.801$, $p=0.036$). Neither the concentration of ergovaline ($F_{1,31}=0.773$, $p=0.386$) nor that of peramine ($F_{1,31}=0.591$, $p=0.4478$) was altered by the presence of the chewing insects (Figure S3).

3.2 | Fungal alkaloids concentrations, nutrients stoichiometry and herbivore performance

Endophyte presence did not reduce larvae growth relative to non-symbiotic plants ($F_{1,30}=0.625$, $p=0.436$) (Figure S4). However, among endophyte-symbiotic plants, herbivore performance was negatively associated with ergovaline and peramine concentrations. Weight gain of *S. frugiperda* larvae was negatively related to concentrations of ergovaline ($t=-2.230$, df = 10, $p=0.049$; Figure 4a) and peramine ($t=-4.283$, df = 10, $p<0.001$; Figure 4c).

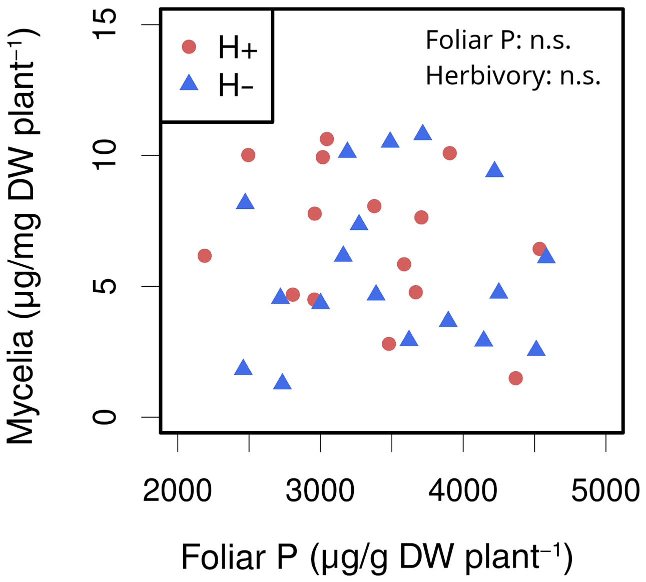

FIGURE 1 Relationship between *Epichloë* mycelial biomass and foliar phosphorus concentration in plants of *Festuca arundinacea* associated with *Epichloë coenophiala*. Each point corresponds to an individual plant that was either challenged (H+, red dots) or not (H−, blue triangles) by *Spodoptera frugiperda* larvae. The lack of a regression line indicates a non-significant relationship ($p>0.05$; n.s. not-significant).

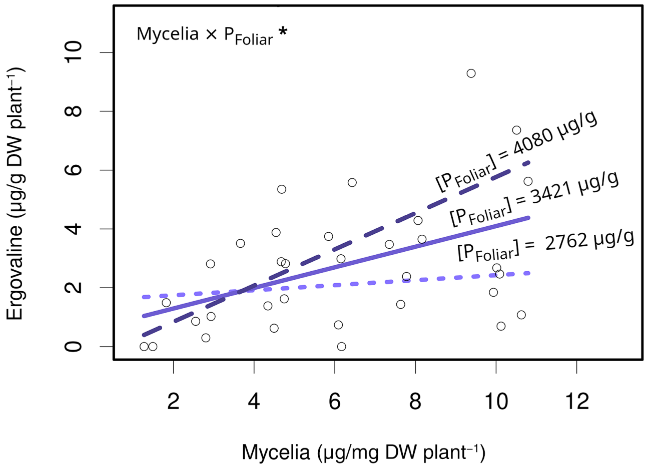

FIGURE 2 Relationships between the concentration of ergovaline alkaloid and mycelial biomass of *Epichloë coenophiala* for three different concentrations of foliar phosphorus in *Festuca arundinacea* plants (i.e. solid line represents the mean foliar phosphorus concentration [3421 µg/g DW plant^{-1}]; dashed line, one standard deviation above the mean [4080 µg/g DW plant^{-1}]; dotted line, one standard deviation below the mean [2762 µg/g DW plant^{-1}]). Asterisk indicate statistically significant effect (*$p<0.05$).

The relationship was not significant between herbivore weight gain and concentration of loline alkaloids ($t=-1.536$, df = 10, $p=0.156$; Figure 4b).

Endophyte presence increased the N:P ratio by 15% ($F_{1,72}=7.22$, $p=0.009$) and did not affect C:N ratio ($F_{1,72}=0.967$, $p=0.329$).

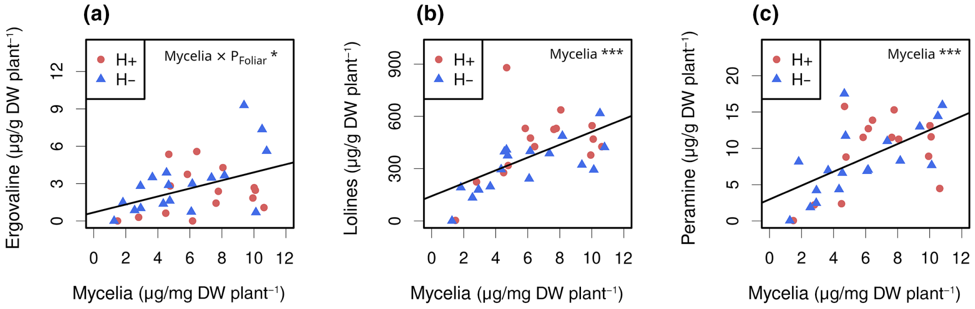

FIGURE 3 Relationships between concentrations of fungal alkaloids [Ergovaline (a), lolines (b) and peramine (c)] and mycelial biomass of *Epichloë coenophiala* in *Festuca arundinacea* plants. Each point corresponds to an individual plant that was either challenged (H+, red dots) or not (H−, blue triangles) by *Spodoptera frugiperda* larvae. Asterisks indicate statistically significant effects (*$p < 0.05$; ***$p < 0.001$).

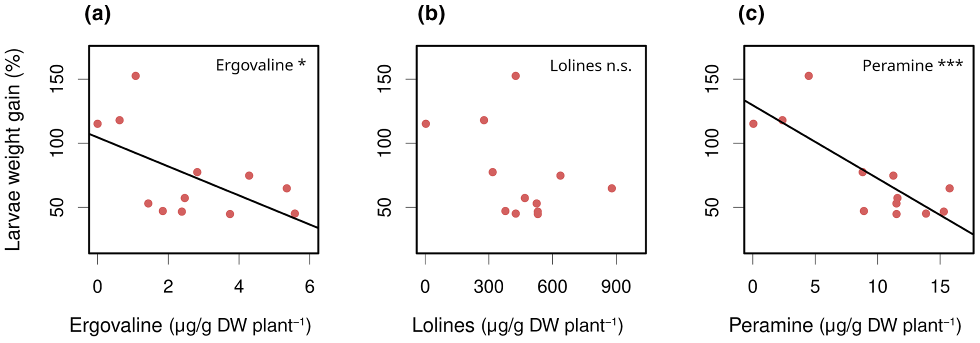

FIGURE 4 Relationships between weight gain by the *Spodoptera frugiperda* larvae and each fungal alkaloid concentration [Ergovaline (a), Lolines (b) and Peramine (c)]. Each point represents a *Festuca arundinacea* plant associated with the endophyte fungus *Epichloë coenophiala* and challenged by the herbivore. Asterisks indicate statistically significant effects (*$p < 0.05$; ***$p < 0.001$; n.s. not-significant).

However, herbivore weight gain was independent of N:P ratio ($t = -1.454$, df = 30, $p = 0.156$) and C:N ratio ($t = 1.144$, df = 30, $p = 0.262$) (Figure S5).

3.3 | Plant damage by *S. frugiperda*

The level of damage caused by the herbivores was not related to any of the analysed factors. The herbivory index was not influenced by any of the analysed alkaloids (Ergovaline: $t = 0.394$, df = 8, $p = 0.704$; Lolines: $t = 0.262$, df = 8, $p = 0.798$; Peramine: $t = 0.411$, $p = 0.692$; Figure S6), nor by the presence of *E. coenophiala* ($F_{1,28} = 0.406$, $p = 0.529$). Regarding plant nutrients, the herbivory index showed no significant relationships with either foliar P, C:N ratio, or N:P ratio (Foliar P: $t = -1.376$, df = 28, $p = 0.179$; C:N ratio: $t = 0.528$, df = 28, $p = 0.601$; N:P ratio: $t = 1.529$, df = 28, $p = 0.137$; Figure S7).

3.4 | Performance of *F. arundinacea* plants

Plant biomass was influenced by the symbiosis with *E. coenophiala* and the herbivory by *S. frugiperda* larvae. On average, endophyte-symbiotic plants have 12% more biomass than endophyte-free ones (Symbiotic status: $F_{1,71} = 5.524$, $p = 0.021$; Figure 5a). Overall, herbivory reduced plant above-ground biomass by 22% (Herbivory: $F_{1,71} = 31.479$, $p < 0.001$; Figure 5b). The effects of *Epichloë* presence and herbivory were independent of the other factor (Symbiotic status × Herbivory: $F_{1,70} = 0.542$, $p = 0.464$).

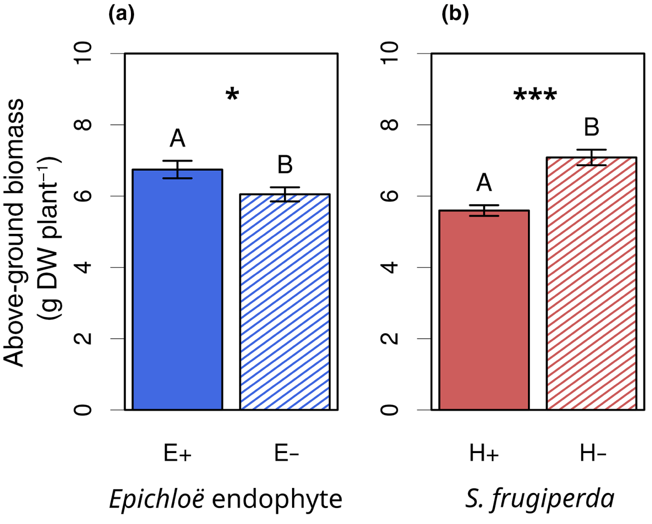

FIGURE 5 Above-ground biomass of *Festuca arundinacea* plants as affected by (a) the presence (E+) or absence (E−) of the fungal endophyte *Epichloë coenophiala*, and by (b) the presence (H+) or absence (H−) of *Spodoptera frugiperda* larvae. Values are average ± SE ($n = 33–40$). Different letters indicate statistically significant differences and asterisks indicate the significance (*$p < 0.05$; ***$p < 0.001$).

4 | DISCUSSION

We hypothesised that high foliar phosphorus levels would reduce the production of *Epichloë*-derived alkaloids, particularly ergovaline. We expected this reduction to be associated with reduced *Epichloë*-mediated resistance against herbivores and diminished plant performance. Our results indicate that high foliar phosphorus levels were not associated with low alkaloid levels. Mycelial biomass was independent of foliar phosphorus, and lolines and peramine were linearly related to mycelial biomass. Surprisingly, high ergovaline concentrations were related to a positive interaction between endophyte mycelial biomass and foliar phosphorus concentration. While loline alkaloids were the only ones increasing in concentration after plant exposure to herbivores, they did not influence larvae growth. Conversely, elevated levels of ergovaline and peramine were related to reduced weight gain in *S. frugiperda* larvae. However, endophyte presence did not reduce herbivory damage. Herbivory reduced the above-ground biomass of plants but, on average, plants associated with *Epichloë* endophytes had greater biomass. Our findings suggest that phosphorus plays a crucial role in modulating the *Epichloë*-mediated defensive mutualism, primarily through its effects on ergovaline production rather than on endophyte biomass, other alkaloids production or tissue quality.

Endophytic mycelial biomass was neither affected by foliar phosphorus nor by herbivorous larvae. Previous studies have reported positive correlations between alkaloid concentration and endophyte mycelial biomass (Fuchs et al., 2017b; Rasmussen et al., 2007; Ueno et al., 2020). Thus, the previous reports showing that low concentrations of ergot alkaloids were associated with high phosphorus levels (i.e. Graff et al., 2020; Malinowski et al., 1998) could be explained not only by the hypothesised reduction in ergot biosynthesis (Malinowski & Belesky, 2000), but for an effect of phosphorus impairing the fungal endophyte growth within the plant (Bastías et al., 2018; Ryan et al., 2014). At least within the range of P concentration in leaf tissue we were able to achieve in our study, we did not detect any results suggesting support for this hypothesis. We observed that endophyte mycelial biomass remained independent of foliar P concentration, as well as other manipulated or measured variables such herbivory, carbon and nitrogen concentrations that potentially could have been affected by this macronutrient. Further investigations encompassing a broader range of phosphorus concentrations are required to elucidate the intricate relationship between ergot alkaloid concentrations, phosphorus levels, and fungal endophyte growth within plants.

We observed a positive correlation between the concentration of all alkaloids and increasing endophyte mycelia biomass. Notably, the correlation was weaker for ergovaline compared to lolines and peramine. The weaker association with ergovaline can be attributed to its dependence not only on *Epichloë* mycelial biomass but also on its interaction with foliar phosphorus concentration. This is in line with previous reports, some of which found ergovaline to have the weakest relationship with endophyte mycelia (Fuchs et al., 2017b), or not a discernible relationship at all (Rasmussen et al., 2007). Unlike lolines and peramine, the hydrophobic nature of ergovaline, as noted by Spiering et al. (2005), can explain part of its weaker correlation with mycelial biomass. The relationship can vary significantly based on the specific plant part sampled and the species-specific endophyte growth pattern, which ultimately determines the presence of fungal mycelium in the plant (Realini et al., 2024). Furthermore, our results suggests that the weak association between ergovaline and mycelial biomass could be explained by its interactive effect with macronutrients.

The damage caused by the chewing larvae of the insect *S. frugiperda* resulted in an increase in loline concentration but did not induce peramine concentration. Plants activate induced defences in response to signals associated with the risk of herbivory (Karban, 2020). These signals can originate from herbivores, such as insect oral saliva, or from plant damage, such as damage-associated molecular patterns resulting from wounding (Karban, 2020; Waterman et al., 2019). Previous studies have reported the induction of lolines after simulated herbivory or attack by chewing herbivores (Bultman et al., 2004; Patchett et al., 2008; Sullivan et al., 2007), and the specific induction of peramine in response to chewing insects (Cibils-Stewart et al., 2023; Fuchs et al., 2017a). This suggests that plant-*Epichloë* systems can recognise and respond in a herbivore-specific manner, activating the production of specific alkaloids. Considering this, we expected peramine to be induced by the chewing larvae. However, in our experiment, chewing by larvae of *S. frugiperda* did not induce peramine. We did find an induction of lolines, which is consistent with Sullivan et al. (2007), who reported that herbivory by the chewing insect *S. frugiperda* up-regulated genes involved in the biosynthesis of these alkaloids in *Epichloë*-associated *F. arundinace* plants. However, larvae of another Lepidoptera Noctuidae, *Helicoverpa armigera*, did not induce the concentration of loline alkaloids in plants of the same host species (Cibils-Stewart et al., 2023). This highlights a complex interplay within plant-*Epichloë* systems, where they not only perceive and discriminate herbivore-specific stimuli but also exhibit diverse responses to the same stimuli across different endophyte strains and species.

We found that higher ergovaline concentrations were associated with a positive interaction between endophyte mycelial biomass and foliar phosphorus. This finding directly contradicted our hypothesis and previous research, as we expected high phosphorus levels to reduce ergovaline concentration (Graff et al., 2020; Malinowski et al., 1998). However, other ecological factors may provide an explanation for this phenomenon. The symbiosis outcome can be altered by different combinations of plant genotype and *Epichloë* strain, including changes in alkaloid production (Malinowski et al., 1998; Müller & Krauss, 2005; Young et al., 2014). For example, *F. arundinacea* plants associated with different *E. coenophiala* strains have been observed to produce varying amounts of ergot alkaloids (Dinkins et al., 2023). Moreover, different combinations of plant genotype and endophyte strain show varying responses to changes in soil nutrient levels. For instance, certain *F. arundinacea* genotypes increased the ergovaline production in response to P fertilisation, while others showed the opposite response (Malinowski et al., 1998).

Similar variable effects have also been reported in response to the nitrogen, which has been found to either increase or reduce alkaloid concentration (Belesky et al., 1988; Buckley et al., 2019; Hunt et al., 2005; Rasmussen et al., 2007). The impact of nutrient availability on alkaloid production appears to be complex and contingent upon interactions with other factors.

Among endophyte-symbiotic plants, high concentrations of ergovaline and peramine were associated with reduced growth of *S. frugiperda* larvae. However, contrary to expectations, the presence of endophytes did not lead to a reduction in larvae growth or plant tissue consumption compared to endophyte-free plants. *Epichloë*-mediated resistance to *S. frugiperda* larvae has been extensively investigated, although results have been inconsistent. Some studies have reported a reduction in *S. frugiperda* larval weight in the presence of endophytes (Ball et al., 2006; Bastías et al., 2018; Bultman & Conard, 1998; Clay & Cheplick, 1989; Salminen et al., 2005), with two of these studies attributing the weight reduction to ergot alkaloids (i.e. Clay & Cheplick, 1989; Salminen et al., 2005). Conversely, other studies have reported an increase in larval weight when feeding on endophyte-symbiotic plants (Bultman & Bell, 2003), while others have found no significant difference in the response of the herbivore between endophyte-symbiotic and endophyte-free plants (Afkhami & Rudgers, 2009). Although high alkaloid concentrations often correlate with reduced performance of *S. frugiperda* larvae on endophyte-symbiotic plants, it is not uncommon in the literature to find instances where the defensive effect of *Epichloë* endophytes is negligible.

The lack of effect of endophyte presence on *S. frugiperda* larvae could be explained by the larvae instar and the use of a no-choice experimental design. Evidence suggests that older instars of *S. frugiperda* are less sensitive to endophyte-mediated resistance (Bastías et al., 2018; Bultman & Conard, 1998). Since nearly all *S. frugiperda* larvae had reached the fifth instar by the end of the experiment, they might have been able to cope successfully with the endophyte-defended tissues and continue feeding despite the toxicity. However, there is a potential for a carryover effect on the development of subsequent stages, such as the pupa, as observed by Graff et al. (2020), where higher ergovaline concentrations were associated with a longer time required for moth emergence. Additionally, *Epichloë* endophytes can influence plant–herbivore interactions by acting as a deterrent to herbivores. For instance, Afkhami and Rudgers (2009) found that while *S. frugiperda* performance was not affected by endophyte presence in a no-choice experiment, larvae preferred to feed on endophyte-free tissue in a choice experiment. In our experiment, *S. frugiperda* larvae were confined to either endophyte-symbiotic or endophyte-free plants with no opportunity to switch between alternative plants. As a result, the larvae inability to feed based on their preference could have prevented a potential endophyte-mediated deterrence effect.

The role of phosphorus as a positive modulator of the defensive mutualism between grasses and *Epichloë* endophytes could significantly impact grassland structure and functioning, potentially favouring the dominance of *Epichloë*-associated grasses. The addition of phosphorus to grasslands through fertilisation is a common practice, as this nutrient is often a limitation for plants' primary production (Peñuelas et al., 2013; Vitousek et al., 2010). Moreover, human-driven climate change is anticipated to increase herbivory by insects (Hamann et al., 2021). The combination of these factors could lead to an increase in the prevalence of grass-endophyte symbiosis (Clay et al., 2005; Clay & Holah, 1999; Vázquez-de-Aldana et al., 2013). This could pose a threat to native species, especially considering that some native grasslands are already facing invasion by exotic grasses, and the symbiosis with *Epichloë* can further increase their invasive potential (Moore et al., 2019; Rudgers & Clay, 2007). For instance, in native grasslands of the Inland Pampa region in Argentina, exotic grasses dominate communities in long-term succession experiments (Tognetti et al., 2010), and their infection rates by *Epichloë* are notably high (Graff et al., 2020; Gundel et al., 2009). This shift not only alters the composition of plant communities but also impacts the functioning of these ecosystems (Yahdjian et al., 2017).

AUTHOR CONTRIBUTIONS

All the authors conceived the ideas, designed the methodology, and collected and analysed the data. All the authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.c2fqz61jd (Decunta et al., 2024).

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SUPPORTING INFORMATION

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

Table S1. Results from soil analysis before the fertilization treatment.

Figure S1. The experimental design was a full factorial, with *Festuca arundinacea* plants associated and not with *Epichloë coenophiala* (E+, E−), four levels of phosphorus fertilization (Control, T1, T2 and T3) and herbivory by *Spodoptera frugiperda* larvae (H+, H−).

Figure S2. Foliar phosphorus concentration in endophyte-associated (E+) and endophyte-free (E−) *Festuca arundinacea* plants.

Figure S3. Concentrations of fungal alkaloids [(a) Ergovaline, (b) Lolines, (c) Peramine] in Festuca arundinacea plants associated with *Epichloë coenophiala* and challenged by larvae of Spodoptera frugiperda (H+) and control plants (H−).

Figure S4. Relationships between weight gain of larvae of *Spodoptera frugiperda* in plants of *Festuca arundinacea* associated (E+) or not (E−) with the fungus *Epichloë coenophiala* ($F_{1,30}=0.625$, $p=0.436$).

Figure S5. Relationships between weight gain of larvae of *Spodoptera frugiperda* and nutrient stoichiometry in plant tissues.

Figure S6. Relationships between herbivory index and fungal alkaloid concentrations [(a) Ergovaline, (b) Lolines, (c) Peramine].

Figure S7. Relationships between herbivory index and nutrients stoichiometry.

Data S2. Statistical tables.

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