







RESEARCH ARTICLE

The Influence of Beneficial Fungi on Plant-Enemy Interactions and Plant Community Structure

Mycorrhizal fungi compromise production of endophytic alkaloids, increasing plant susceptibility to an aphid herbivore

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Handling Editor: Robert Bagchi**Abstract**

1. Symbiosis plays a critical role in plant biology. Temperate grasses often associate with several symbiotic fungi simultaneously, including *Epichloë* endophytes and arbuscular mycorrhizal (AM) fungi, in shoots and roots, respectively. These symbionts often modulate plant-herbivore interactions by influencing nutritional traits (i.e. AM fungi-mediated nutrient uptake) and/or the secondary chemistry (i.e. endophytic alkaloids) of their host plant. Moreover, such grasses also accumulate large amounts of silicon (Si) from the soil, which can be deposited in tissues to act as a physical anti-herbivore defence.
2. Recent evidence suggests that both endophytes and AM fungi independently facilitate Si uptake. However, the consequences of their interactions with piercing-sucking insects (i.e. aphids), or whether Si supply, endophytes, and AM fungi interact in this regard, are currently unknown. While Si deposition may be less effective against aphids than other herbivores (i.e. chewing caterpillars), Si supply can also alter plant secondary metabolite defences, which could affect sucking insects.
3. In a factorial greenhouse experiment, we evaluated whether these components, acting alone or in combination, altered (1) foliar primary chemistry, (2) Si and symbiont-chemical (endophytic alkaloids) defences, as well as (3) performance of the bird cherry-oat aphid (*Rhopalosiphum padi*) feeding on tall fescue (*Festuca arundinacea*).
4. Endophytes decreased all aphid performance parameters, including population growth and reproduction by 40%, but their impact was reversed by the presence of AM fungi, leading to a 52% increase in aphid performance compared with plants solely hosting endophytes. This improvement in performance was associated with reduced loline alkaloid levels and higher shoot nitrogen in AM-endophytic plants. Endophytes and AM fungi exhibited antagonism, with endophytes reducing AM colonization by 34% and AM presence decreasing endophyte loline alkaloids by 44%. While both fungi jointly increased Si accumulation by 39% under Si-supplied conditions, Si had no noticeable effects on aphids. Moreover, although Si supply

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had no identifiable effects on AM colonization, it reduced endophyte peramine alkaloids by 24%.

5. **Synthesis.** Our findings indicate that symbiotic fungal partnerships and silicon provision may benefit plants but could weaken anti-herbivore defences when combined. Revealing the complex interactions among diverse fungal symbionts and showcasing their effects on different anti-herbivore defences (chemical and physical) and herbivore performance for the first time.

KEYWORDS

AM fungi, aphids, endophytes, multitrophic interactions, silicon, symbiosis

1 | INTRODUCTION

Symbiosis plays a key role in plant biology affecting growth, adaptation, and speciation (Uroz et al., 2019). The plant and fungal kingdoms engage in symbiotic relationships such as mycorrhizae and endophytic associations, supporting nutrient uptake and stress tolerance. Fungi also play key roles in decomposition, nutrient cycling, and disease dynamics, underscoring their importance in ecosystem health and agriculture. For example, some temperate grasses within the Poaceae family, establish symbiotic associations with a myriad of microbes including asexual *Epichloë* endophytes (Ascomycota: Clavicipitaceae) and arbuscular mycorrhizal (AM) fungi (Glomeromycotina) in shoots and roots, respectively. These symbionts often occur simultaneously and influence enemies of the plant, including herbivorous animals, via changes in plant nutritional and defensive traits (Casas et al., 2022; Omacini et al., 2006; Perez et al., 2021). However, predicting the impact of simultaneous symbioses on plant–herbivore interactions remains challenging since they are usually studied separately.

As protective mutualists, endophyte anti-herbivore defences mainly operate directly via the production of toxic alkaloids (Bastías et al., 2017). In terms of alkaloids, four major groups of endophyte alkaloids have been reported including ergot alkaloids, indole-diterpenes, pyrrolizidines (e.g. loline) and pyrrolopyrazines (e.g. peramine) (Berry et al., 2019; Schardl, Florea, et al., 2013; Schardl, Young, et al., 2013). Nonetheless, only grasses harbouring endophytes that putatively produce peramine (e.g. anti-chewing insect defences) and loline (e.g. anti-aphid and anti-chewing defences) are desirable in pastures because they confer herbivorous insect resistance without affecting grazing mammals (Young et al., 2013). While lolines are strongly insecticidal (i.e. toxic), peramine often acts as a potent insect-feeding deterrent (Saikkonen et al., 2016). Additionally, endophytes can have a detrimental effect on herbivores by stimulating the increased production of host plant defences (Bastías et al., 2017).

As nutritional mutualists, AM fungi often enhance plant nutrient supply (mainly phosphorous and nitrogen) through their highly specialized intracellular structures (i.e. arbuscules and vesicles) and their extensive hyphal networks that capture soil nutrients otherwise unavailable for plants (Lanfranco et al., 2018). In

addition to improving plant nutritional quality, a growing number of reports suggest that AM fungi also improve host resistance to both herbivorous insects (Koricheva et al., 2009) and pathogens (Gernns et al., 2001). Specifically, for piercing-sucking aphids, AM fungi have been reported to have positive (Hartley & Gange, 2009; Simon et al., 2017), negative (Guerrieri et al., 2004) and neutral (Williams et al., 2014) impacts on aphid performance. Positive effects of AM fungi on aphid performance can be mediated by their effects on plant N uptake and the increase of tissue quality (Wilkinson et al., 2019). Nitrogen is often a limiting factor in insect herbivore diets, thus, nitrogen acquisition by insects is strongly correlated with higher food utilization (Mattson, 1980). AM fungi-mediated increases in plant growth may result in increased plant tolerance to herbivorous pests (Bennett et al., 2006) or AM fungi may negatively impact herbivores via increased production of host plant defences (e.g. defence priming, Pozo & Azcón-Aguilar, 2007). However, the exact mechanism for the influence of AM fungi on anti-herbivore defence still remains highly speculative (Biere & Bennett, 2013; Pozo & Azcón-Aguilar, 2007).

Both symbionts act as carbon sinks thus, dual infection, at least in some environments, may result in competition for photosynthates. This competition may result in excessive costs to the plant (e.g. non-additive dynamics), potentially negating any benefits derived from the symbiosis (Omacini et al., 2006). In this sense, endophytes might have both spatial and temporal priority over AM fungi with respect to plant carbon, since they are located within the leaf sheaths where carbon is fixed; thus, presumably they have greater access to carbon compared with fungi in roots. with regards to temporal advantages, endophytes are vertically transmitted through the host seed, therefore, they are associated with the host even before germination (Mack & Rudgers, 2008). Previous studies show that endophytes and AM fungi affect one another in terms of colonization and functionality, ranging from being (antagonistic Li, Guo, et al., 2018; Müller, 2003; Omacini et al., 2006) to mutually beneficial (Novas et al., 2010; Vignale et al., 2018). The underlying mechanism for antagonism includes the indirect effects of endophytes on the soil environment that reduce sporulation and colonization of AM fungi, mainly via the release of endophyte alkaloids into the soil (Omacini et al., 2006; Vignale et al., 2016). In cases of facilitation, endophytic exudates

in the soil have been proven to stimulate AM colonization and even lengthen extraradical mycelia (Novas et al., 2010; Vignale et al., 2018).

Another highly effective anti-herbivore mechanism in grasses is their ability to accumulate large amounts of silicon (Si) from the soil (Alhousari & Greger, 2018). Si anti-herbivore defences operate through several mechanisms, including strengthened plant tissues that can wear down herbivore mouthparts, impede tissue penetration, and interfere with digestion and nutrient acquisition (Andama et al., 2020; Cibils-Stewart et al., 2023; Massey & Hartley, 2009), or alter herbivore immunity (Cibils-Stewart et al., 2023). Si accumulation can also affect plant allocation to a range of secondary metabolites (Hall et al., 2019), including alkaloids (Hall et al., 2021). It has been suggested that Si may act as a metabolically cheaper herbivore defence (i.e. physical) than secondary metabolite production (i.e. carbon-based compounds), a hypothesis supported by the negative relationship between Si and both phenolics and tannins reported for many plant taxa (Cooke & Leishman, 2012; Frew et al., 2016; Moles et al., 2013).

There is evidence that Si accumulation in plants increases when colonized by either *Epichloë* endophytes (Cibils-Stewart et al., 2020, 2021, 2023; Huitu et al., 2014) or AM fungi (Frew et al., 2017), although not universal (AM: Johnson et al., 2022; Vega et al., 2021; endophytes: Johnson et al., 2023). However, despite their co-occurrence in nature, no studies have yet addressed whether the two symbionts interact to affect Si accumulation in plants and associated herbivore resistance, or whether Si supply affects symbiont-mediated herbivore defences. It is currently unknown how endophytes and AM fungi interact, and if any potential competition for carbon, affects the accumulation of Si and the production of alkaloid defences. Furthermore, there is still uncertainty about the effectiveness of Si defences against piercing-sucking insects such as aphids (Massey et al., 2006; Johnson et al., 2020; but see Reynolds et al., 2009). However, since symbiont-produced metabolites (i.e. endophytic alkaloids) generally come at an energetic cost (i.e. carbon) to the plant (Bastías et al., 2017), there is potential for Si supply to affect their production.

The objective of this study was to investigate the interactions between Si supply, multisymbiotic relationships (endophytes and AM fungi), and the presence/absence of aphid herbivory in the production and effectiveness of physical (Si) or symbiont-mediated (endophyte alkaloids) anti-aphid defences in grasses. For this, we determined whether Si supply, *Epichloë* endophyte, AM fungi, and herbivory by the global pest *Rhopalosiphum padi* altered (i) grass physical defences (i.e. foliar Si concentrations), as well as (ii) plant growth, primary chemistry and physiology. Moreover, we determined if these factors singly or in combination are additively or non-additively (i.e. antagonistically, additively, or synergistically) altered, (iii) symbiotic traits including AM fungi colonization and endophytic alkaloid production. While positive relationships between concentrations of hyphae and alkaloids have been demonstrated in some plant–endophyte symbioses, it is important to note that alkaloid production may not always be a performance variable for *Epichloë*

fungal endophytes. Finally, the consequence of the interactions between Si supply, *Epichloë* endophyte, and AM fungi presence was evaluated for (iv) population and individual performance of the aphid pest.

2 | MATERIALS AND METHODS

2.1 | Plants, insects, and experimental procedure

Two hundred tall fescue plants (*Festuca arundinacea* cv. INIA Fortuna) either *Epichloë*-free (Nil; $N=100$) or infected with the animal-safe AR584 novel *Epichloë coenophiala* (formerly *Neotyphodium coenophiala*) strain ($N=100$) were grown individually from seed in one-litre pots. Initially, these seeds were inoculated with the AR584 endophyte strain by AgResearch NZ, in collaboration with INIA under a work cooperation agreement. Following the endophyte incorporations, two distinct seed lines were maintained at the Margot Forde Forage Germplasm Centre (Palmerston North, NZ). Pots contained gamma-irradiated (50 kGy) 1:1 topsoil-sand mix that was homogenized with a soil mixer. Sand was used to reduce bioavailable phosphorus to $<16\text{ mg P kg}^{-1}$ and silicon to $<11\text{ mg Si kg}^{-1}$ (Table S1, Appendix S1).

The top $\frac{1}{4}$ of each pot received one of 2 AM fungi treatments: No AM (–AM) or Commercial AM (+AM). +AM was achieved by inoculating soil with Start-up Ultra© (Microbe Smart Pty. Ltd., South Australia) which contains spores from four isolates of *Rhizophagus irregularis* (formerly *Glomus intraradices*). Before inoculation, spores were extracted and separated from the inert substrate (calcined diatomaceous earth) using wet sieving and applied at a rate equivalent to the recommended rate of 250 g of inoculum per 2.5 L with hydrating water. To generate –AM treatment, the same steps were performed, except that the inoculant (extracted spores) was sterilized by autoclaving twice (121°C) to ensure spores were non-viable. To standardize the microbial community within each pot, the soil-sand mix in all treatments received a microbial filtrate (300 mL/10 L) a week after irradiation. This filtrate was created by using the extraneous extraction solution (without spores) from both the commercial AM fungi after wet sieving and the soil before irradiation following the procedure described in Frew et al. (2018). Only the top $\frac{1}{4}$ of the soil in each pot was inoculated with the –AM or the +AM solutions.

Plants were grown in a single, naturally lit glasshouse chamber at 22/18°C (day/night) and 60% relative humidity at the Hawksbury Institute for the Environment in Richmond, NSW, Australia. Pots were randomly shifted weekly to avoid position bias, and manually irrigated three times a week with 50 mL of either a solution with (+Si) or without (–Si) silicon (Si). Briefly, the +Si solution contained potassium silicate (K_2SiO_3 ; Agsil32, PQ Australia) at a concentration of 2 mM, whereas the –Si solution contained KCl to balance the addition of potassium in the +Si treatment. Further, using HCl, both solutions were adjusted to pH7 following procedures described in Hall et al. (2021). This resulted in a total of eight treatments that consisted of combinations of two different endophyte treatments (Nil

or AR584), 2AM fungi treatments (-AM or +AM), and two Si supply treatments (-Si or +Si). Each treatment combination contained 25 pots/replicates (200 plants in total; Figure 1 Symbiont treatments).

After 8 weeks, 10 plants per treatment combination were inoculated and caged with five adult (apterous) bird cherry-oat aphid *Rhopalosiphum padi* (Linnaeus, 1758). Transparent cylindrical Perspex cages with meshed air vents were fitted to the pots of all plants similar to those used by fig. 1S of Cibils-Stewart et al. (2021). Cultures of *R. padi* were established from a single parthenogenetic female obtained from a laboratory culture at Agriculture Victoria Research (Horsham, VIC, Australia) and reared on caged barley (*Hordeum vulgare* cultivar 'Hindmarsh'). Aphids reared on barley before the experiment prevent prior exposure to the plant species, standardize the setup, control the aphid population, and reduce variability in behaviour and responses. On a weekly basis, 10 teneral adult females were transferred to new caged barley for 24 h. Adults were then removed, leaving only same-age nymphs on plants. This procedure ensured same-aged aphids for experiment initiation.

Aphid-inoculated plants were compared with 10 caged aphid-free plants, selected at random, allowing us to determine the impact of aphid infestation on plant parameters. Using these plants, treatment effects on aphid population performance parameters were determined (Section 2.2.1; Figure 1 Impact on aphid population). The remaining five plants per treatment (Table S2) were inoculated with three apterous adults *R. padi* each (120 aphids total). Each individual aphid was confined to a clip cage that followed Cibils-Stewart et al. (2015) design, featuring a 0.5-cm thick foam rectangle (6.2 × 3.6 cm outside, 5.1 × 2.5 cm inside) with adhesive on

tops and bottoms. Pre-applied adhesive secured no-see-um mesh on one side for leaf cages, preventing aphid escape while ensuring ventilation for aphids. The remaining adhesive was attached to the leaf surface. Following Rowe et al. (2020) procedures, each cage was placed on the youngest leaf of three different tillers per plant. Using these plants, treatment effects on individual aphid performance parameters were determined (Section 2.2.2; see Table S2 for treatment combinations; Figure 1 Impact on individual aphid performance).

To assess the effect of the symbionts and Si supply (alone and in combination) on plant physiological performance, net photosynthesis (Anet), stomatal conductance (gs), and water-use efficiency (WUE) were measured in aphid-free plants with an infrared gas analyser (IRGA, LI6400XT, Li-Cor, Lincoln, NE, USA) following Vandegeer et al. (2020) procedures. Additionally, using a Minolta chlorophyll SPAD 502 meter, chlorophyll content (i.e. greenness) was measured in the same leaves, and utilized as a proxy of plant vigour (Druille et al., 2013).

Immediately after harvest, the symbiotic status of plants was detected (Section 2.3); for this, a 1 g section of the root was preserved in ethanol to determine the AM status (Section 2.3.2). Roots were carefully washed, and a 1 g (wet weight) subsample was taken from the same area from all replicates per treatment combination. Subsamples were stored in tissue embedding cassettes in 70% ethanol. The remaining shoots and roots were snap frozen in liquid nitrogen, freeze-dried, weighed (MS-TA Analytical balances; Mettler Toledo), and ball-milled to fine powder (Mixer Mills MM 400; Retsch). Samples were stored at -20°C until further chemical analysis (Section 2.4). The removed root sample was not accounted for in the overall root weight (Figure 1).

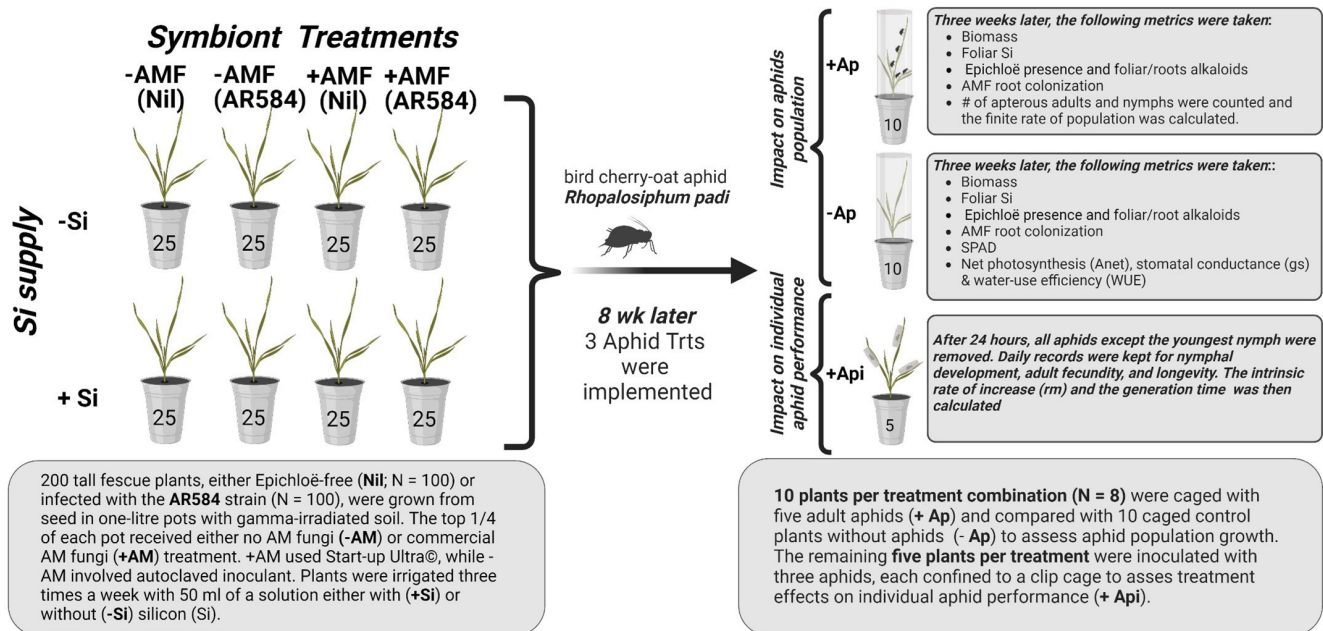


FIGURE 1 Schematic of experimental design. Numbers within the pots indicate replication within each treatment. Figure produced with Biorender (<http://biorender.com>).

2.2 | Aphid parameters

2.2.1 | Aphid population

Three weeks after aphid inoculation, aphid populations were categorized (apterous, adults, and nymphs) and counted. The finite rate of population change was then calculated for the entire 21-day trial (λ_{21}) as a ratio of change in aphid densities at the start (N_0) and end (N_{21}) of assay, where $\lambda_{21} = N_{21}/N_0$.

2.2.2 | Individual aphid performance

After 24 h, the original aphid and all but the youngest nymph (founder nymphs) were removed from the cage. Nymphal development (Pr; pre-reproductive period), adult fecundity (F; number of born nymphs daily), and longevity (L) of the founder nymphs were recorded daily for the entire lifespan of each aphid. Newly born nymphs were removed daily from clip cages after daily adult fecundity recordings. Using the above parameters, the intrinsic rate of increase (r_m) and the generation time (GT) were calculated as $r_m = 0.74 (\ln F/L)$ and $GT = 4Pr/3$, respectively (Wyatt & White, 1977).

2.3 | Symbiont detection

2.3.1 | Endophyte in shoots

The fresh cut end of one tiller of each plant was pressed onto a nitrocellulose membrane (tissue-print immunoblotting) to confirm the absence (Nil) or presence (AR584) of *Epichloë* in planta following di Menna et al. (2012) procedures; this was further confirmed with histological staining following Cibils-Stewart et al. (2020) procedures. No colonization was detected in the endophyte-free (Nil) plants.

2.3.2 | AM fungi in roots

Mycorrhizal colonization was scored following Frew et al. (2018) procedures with minor modifications. Briefly, samples within cassettes were rinsed with cold water and cleared with 10% KOH at room temperature for five consecutive days. Samples were then water-rinsed and stained with 5% ink-vinegar in a 60°C water bath for 10 mins (Vierheilig et al., 2005). Finally, samples were rinsed until water ran clear and were submerged in lacto glycerol (de-staining solution) overnight. Ten 1 cm segments per sample were mounted on glass slides with glycerine under a cover slip and a minimum of 25 intersects were scored for the presence of AM fungi using the intersect method (McGonigle et al., 1990). Only hyphae for which there was a visible connection to AM fungal structures (arbuscules, vesicles, spores) were counted. No colonization was detected in the -AM plants.

2.4 | Chemical analyses

2.4.1 | Shoot silicon, carbon, and nitrogen

Eighty milligrams of ground leaf material were analysed to measure Si concentration using an X-ray fluorescence spectrometer (Epsilon 3 x; PANalytical, EA Almelo, The Netherlands) following the method of Reidinger et al. (2012) and using certified plant material known Si concentrations (see Hiltbold et al., 2016 for full details). A subsample of 6–7 mg of ground tissue of aphid-free plants used for Si analysis was further utilized to measure carbon and nitrogen concentrations with an elemental analyser (FLASH EA 1112 Series CHN analyser, ThermoFinnigan, Waltham, MA, USA).

2.4.2 | Epichloë alkaloid concentrations

Shoot peramine production was analysed according to Berry et al. (2019) using a Thermo LTQxl linear ion-trap mass spectrometer equipped with an Accela 1250 HPLC in endophytic plants only. Chromatography was achieved using a SeQuant ZIC-HILIC column (150×2.1 mm, 5 μ, Merck KGaA, Darmstadt, Germany). Samples were extracted with 500 μL of 50% methanol (containing 1.7 μg/mL homoperamine as an internal standard) for 1 h in the dark. Samples (20 mg) were then centrifuged (5000 g, 5 min) and the supernatant was transferred to 2 mL amber HPLC vials via a 0.45 μm syringe filter (PVDF). Peak integration was conducted using LCQuan 2.7 (Thermo Fisher Scientific Inc., San Jose, CA, USA); alkaloid concentrations were determined from peak areas and calculated standard curves. Production of the three loline derivatives *N*-acetyllooline (NAL), *N*-acetyl norloline (NANL), and *N*-formyllooline (NFL) (added to afford the total lolines) was quantified following Bastías et al. (2018a).

2.5 | Statistical analysis

R was utilized for all statistical analyses (R Core Team, 2015) and all figures were produced using the 'ggplot2' package (Wickham, 2016). Assumptions of normality for residuals for all models were verified according to the inspection of quantile-quantile plots, and variables log-transformed in case of lack of normality (aphid λ).

Shoot and root biomass of freeze-dried tissue, along with foliar Si concentration were analysed with a four-way analysis of variance (ANOVA) using Si supply, AM fungi, endophyte, and herbivory as fixed effects. AM fungi colonization, alternatively, was analysed with a three-way ANOVA using Si supply, endophyte, and herbivory as fixed effects; only +AM plants were utilized for this analysis.

Aphid parameters were analysed using a three-way ANOVA, with Si supply, AM fungi, and endophyte as fixed effects using aphid-inoculated plants. Likewise, plant physiological and nutritional traits were analysed using a three-way ANOVA, with Si supply, AM fungi, and endophyte as fixed effects, aphid-free plants only. For all models described above, differences between treatment means were determined by Tukey's HSD

test using the 'emmeans' package (Lenth et al., 2018). A multivariate analysis of variance (MANOVA) using Si supply, AM fungi, and herbivory as fixed effect factors was utilized to determine differences in overall alkaloid profiles (sum of all alkaloids produced by the AR584-strain) and to assess the treatment effects on individual alkaloids at the strain-specific level. Finally, linear regressions were used to determine the relationship between aphid parameters and specific alkaloid expressions.

3 | RESULTS

3.1 | Symbionts increase foliar Si concentrations in the presence of Si supply

There was a significant interaction between Si supply and both symbionts (Table 1a). Specifically, the presence of both symbionts additively increased foliar Si concentration by 39% (endophyte alone: 16%, AM fungi alone: 18%) compared with plants with no symbionts in Si-supplied (+Si) conditions. Symbiont effects on foliar Si, however, were not observed in -Si conditions (Figure 2, Table 1a). Herbivory by aphids did not induce changes to Si concentrations in any of the treatment combinations tested (not shown in Figure 2 for clarity).

3.2 | Endophytes and AM fungi affected each other antagonistically

Endophyte presence reduced AM fungi colonization by 34% when compared with endophyte-free plants (Nil) regardless of Si supply, or aphid herbivory treatments (Figure 3, Table 1b). Similarly, AM fungi

reduced concentrations of all alkaloid production in endophytic plants (Table 2, MANOVA). However, when individual alkaloid concentrations were considered, AM fungi presence reduced loline alkaloids by 44% (Figure 4a) and Si supply reduced peramine by 24% (Figure 4b; Table 2 univariate ANOVAs).

3.3 | Endophytes alkaloids were the dominant anti-aphid defence

We observed strong endophyte effects on aphid population and performance. Specifically, a significant interaction effect between endophyte and AM fungi treatments was observed such that the aphid finite rate of population change (λ) was reduced by 40% in the presence of the AR584 endophytes. However, AM fungi positively impacted λ such that aphid abundance increased by 52% when endophytic plants were associated with both symbionts (Figure 5, Table 1c). AM fungi effects on λ were not observed in the absence of endophytes. Increased aphid λ within endophytic plants was negatively associated with loline concentrations (Figure 6a) and had no direct associations with peramine (Figure 6b). As anticipated, the interactions discussed above had a more substantial impact on nymph (immature stage) production than the survival of other life stages, which represents the growing category of the population (Table S3, Figure S1).

In terms of aphid performance, the intrinsic rate of increase (r_m) was significantly reduced with the presence of endophyte in interaction with the presence of AM fungi, in a similar manner to population λ , whereby, AM fungi presence increased r_m only when aphids were restricted to endophytic plants (Table 3 and Table S4). Generation time, however, was only increased by 25% with endophyte presence,

Factors	(a) Foliar Si			(b) AM colonization			(c) λ		
	df	F	p	df	F	p	df	F	p
Aphid	1110	1.82	0.181	1,32	3.76	0.061			
Endo	1110	9.31	<0.001	1,32	13.07	0.001	1,72	101.8	<0.001
AM	1110	20.32	<0.001				1,72	4.49	0.037
Si	1110	120.27	<0.001	1,32	0.128	0.722	1,72	0.04	0.841
Aphid×Endo	1110	1.69	0.195	1,32	1.02	0.318			
Aphid×AM	1110	0.27	0.598						
Endo×AM	1110	0.57	0.449				1,72	6.95	0.010
Aphid×Si	1110	0.02	0.891	1,32	0.01	0.959			
Endo×Si	1110	18.32	<0.001	1,32	0.09	0.759	1,72	1.66	0.201
AM×Si	1110	11.77	<0.001				1,72	1.09	0.299
Aphid×Endo×AM	1110	1.50	0.223						
Aphid×Endo×Si	1110	0.01	0.946	1,32	0.19	0.659			
Aphid×AM×Si	1110	0.25	0.621						
Endo×AM×Si	1110	0.58	0.210				1,72	1.93	0.168
Aphid×Endo×AM×Si	1110	0.082	0.365						

Note: Models with significant ($p < 0.01$, $p < 0.001$) main effects and/or interactions are noted in bold.

TABLE 1 Results from multiple comparison tests for changes in (a) shoot Si concentration, (b) AM fungi colonization, and (c) aphid finite rate of population change (λ), as affected by Si supply (Si), symbionts (AM fungi and endophytes; Endo), and aphid herbivory.

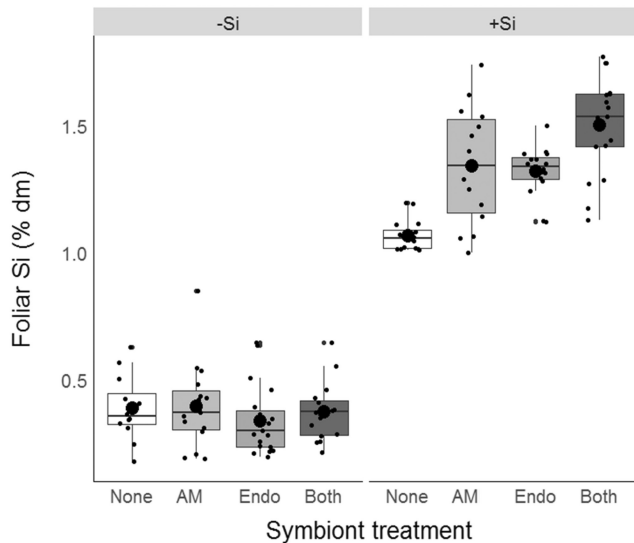


FIGURE 2 Foliar Si concentration for *Festuca arundinacea* plants grown in soil without (–Si) and with (+Si) silicon supply in the absence (None) or presence of single (AM or Endo) or dual (Both) symbiont infection; symbionts: AM=Arbuscular mycorrhiza fungi or Endo: AR84 novel *Epichloë* endophyte. Mean values are indicated with big black circles, and inclusive median and interquartile ranges are indicated with lines. Asterisks and bold.

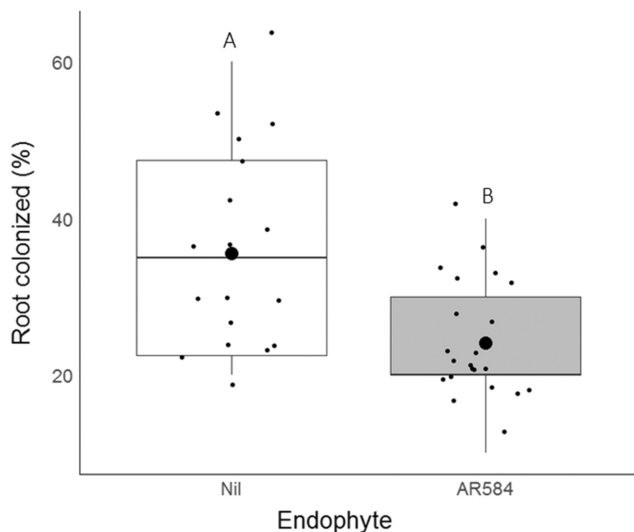


FIGURE 3 AM root colonization for *Festuca arundinacea* plants grown in the absence (Nil) or presence (AR584) of a foliar *Epichloë* endophyte; details as per Figure 2 with statistical analysis given in Table 1b.

with no AM fungi or Si supply effects observed (Table 3 and Table S4); indicating that aphid developmental time was compromised by the presence of endophytes alone.

3.4 | Plant growth, vigour, and forage quality

Sustained herbivory by aphids over 3 weeks reduced, and AM fungi presence increased, shoot and root biomass; the latter, in interaction

with endophyte presence for shoot mass (Figure S2, Table S5). AM fungi reduce nutrient limitation (Table S6), leading to increased biomass production (Figure S2). When physiological parameters (Anet, gs, or WUE) were measured for herbivore-free plants only, including vigour (greenness), none of our treatment combinations affected these parameters (Table S6A–D). However, when foliar nitrogen and carbon were measured as a proxy of foliage quality, AM fungi increased nitrogen, whereas Si supply reduced carbon (Figure S3). Phosphorus concentrations and C:N ratios, however, remained unaffected by our treatments (Table S6E–H).

4 | DISCUSSION

To the best of our knowledge this is the first study to investigate the interactions between Si supply, multisymbiotic relationships (endophytes and AM fungi), and the consequences for an insect herbivore. We provide novel evidence that the presence of both types of fungi additively increased silicon accumulation in Si-supplemented conditions (Figure 7 R1). Despite the positive increases in Si defences, endophytes, AM fungi and Si supply were antagonistic to each other. Specifically, endophyte reduced AM fungi colonization (Figure 7 R2), AM fungi reduced loline alkaloids (Figure 7 R3) and Si supply reduced peramine (Figure 7 R3); herbivory by aphids, however, had no effects either on Si defences or on symbiotic traits.

Furthermore, in terms of anti-aphid defences, we report that endophyte effects dominated (also see Hartley & Gange, 2009). In fact, the presence of endophytes reduced aphid performance parameters to such an extent that plants associated with endophytes alone had a greater anti-aphid effect than those associated with both symbionts (Figure 7 R4). This suggests that symbiotic fungal associations may be beneficial to plants, but they might have deleterious effects on herbivore defences when acting in combination.

4.1 | AM fungi and endophytes were antagonistic

Endophyte presence reduced AM fungi colonization by 34%, whereas AM fungi presence reduced endophyte loline alkaloids by 44%. Previous studies have reported both positive and negative effects of symbiont co-occurrence in similar grass systems. In the case of negative effects, it can be envisaged that plants preferentially allocate resources to the most efficient symbiont (Bever, 2015). Interestingly, *Epichloë* endophytes associated with wild grasses promote AM fungi colonization (Casas et al., 2022; Novas et al., 2010; Vignale et al., 2018), whereas the opposite has been reported for domesticated grass species (Li, Guo, et al., 2018; Müller, 2003; Omacini et al., 2006), such as the tall fescue used in this study. Due to the contrasting results between agronomic grasses and wild native grasses, Novas et al. (2009) argued that more native grass-endophyte-mycorrhiza systems should be evaluated to determine the directionality of interactions in nature; this is also supported by Perez et al., 2021.

TABLE 2 Multivariate analysis of variance (MANOVA) and univariate ANOVAs of specific alkaloids (individual predictors) for changes in endophyte alkaloid concentrations of endophytic plants as affected by Si supply (Si), AM fungi (AM), and aphid herbivory.

Endophyte alkaloids	MANOVA			ANOVAs									
	Overall alkaloids			Peramine		Total lolines		NAL		NANL		NFL	
Factors	df	Pillai	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Si	1,70	0.06	0.093	4.96	0.029	1.54	0.218	0.26	0.612	0.04	0.946	1.83	0.185
Aphid	1,70	0.50	0.165	1.70	0.195	0.19	0.663	0.61	0.440	0.11	0.739	1.91	0.176
AM	1,70	0.09	0.032	2.62	0.109	7.33	0.008	3.46	0.001	1.59	0.016	1.19	0.004
Si×Aphid	1,70	0.03	0.302	0.07	0.787	2.03	0.157	0.45	0.505	0.07	0.782	1.55	0.222
Si×AM	1,70	0.02	0.538	0.26	0.606	1.24	0.267	0.01	0.973	0.04	0.844	0.05	0.808
Aphid×AM	1,70	0.05	0.142	0.81	0.368	0.95	0.334	1.05	0.312	0.29	0.591	2.85	0.101
Si×Aphid×AM	1,70	0.01	0.967	0.01	0.922	0.20	0.886	0.21	0.643	0.02	0.959	1.49	0.231

Note: Models with significant ($p < 0.05$, $p < 0.001$) main effects and/or interactions are noted in bold.

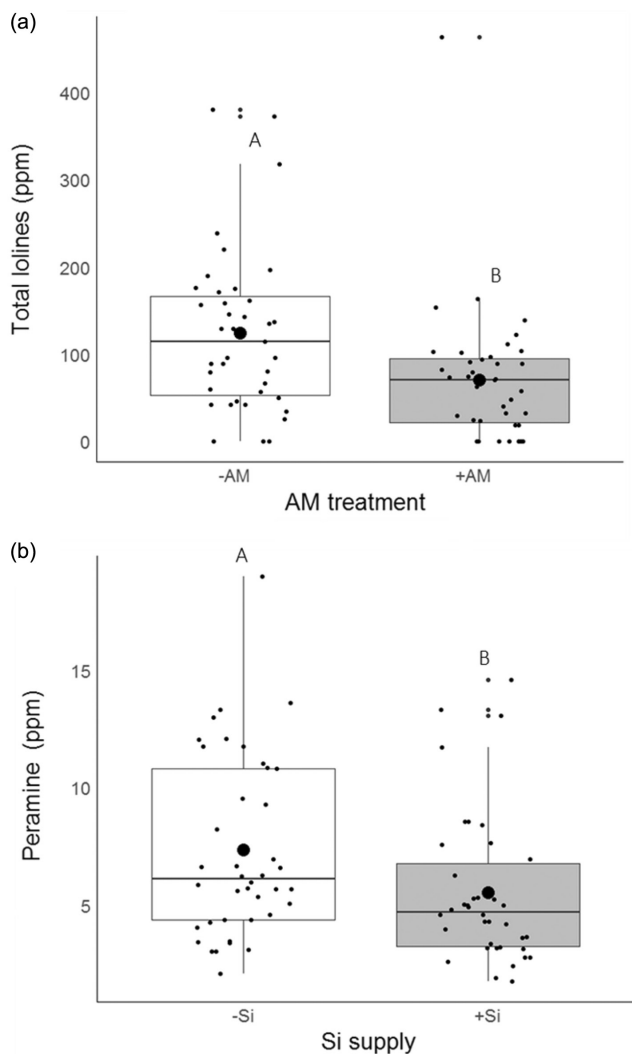


FIGURE 4 Foliar concentrations of (a) total lolines in -AM (white bars) and +AM (grey bars) plants and (b) peramine in -Si (white bars) and +Si (grey bars) plants. All plants were infected with the AR584 novel *Epichloë* endophyte; details as per Figure 2 with statistical analysis given in Table 2.

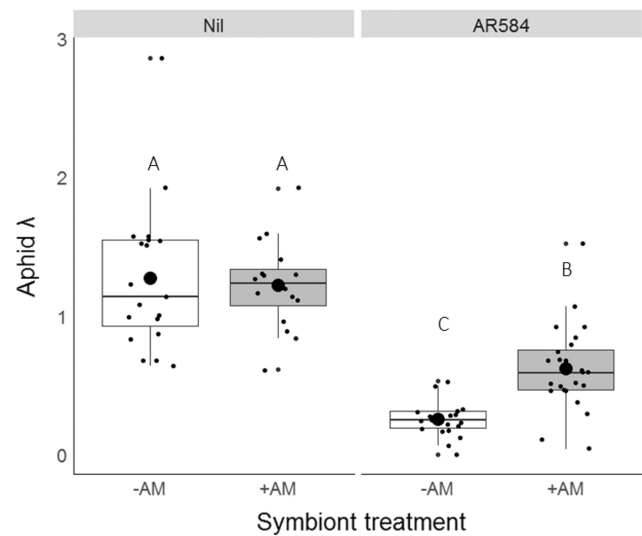


FIGURE 5 Finite rate of population change (λ) for *Rhopalosiphum padi* aphids restricted to *Festuca arundinacea* grown in soil in the absence (Nil or -AM) or presence (AR584 or +AM) of foliar *Epichloë* endophyte or root arbuscular mycorrhiza fungi; details as per Figure 2 with statistical analysis given in Table 1c.

In an evolutionary context, most plants interact with both endophytes and AM fungi, symbiotic partners linked to beneficial co-adaptation and rapid co-evolution with their hosts (Sachs & Simms, 2006). However, artificial selection through breeding often prioritizes enhancing plant productivity, tolerance, and forage quality for livestock, overlooking symbiont needs and benefits to their host. Symbionts demonstrate their greatest benefits during stressful conditions; for example, endophytes in pest outbreaks (Gundel et al., 2013) and nutrient deficiencies for AM fungi (Gehring & Bennett, 2009). Hence, plants hosting both symbionts likely regulate these interactions to maintain mutualism and optimize individual fitness (Sachs & Simms, 2006). In nutrient-poor environments, for instance, AM fungi might take precedence over endophytes. The nature of these symbiont interactions can also vary based on fungal

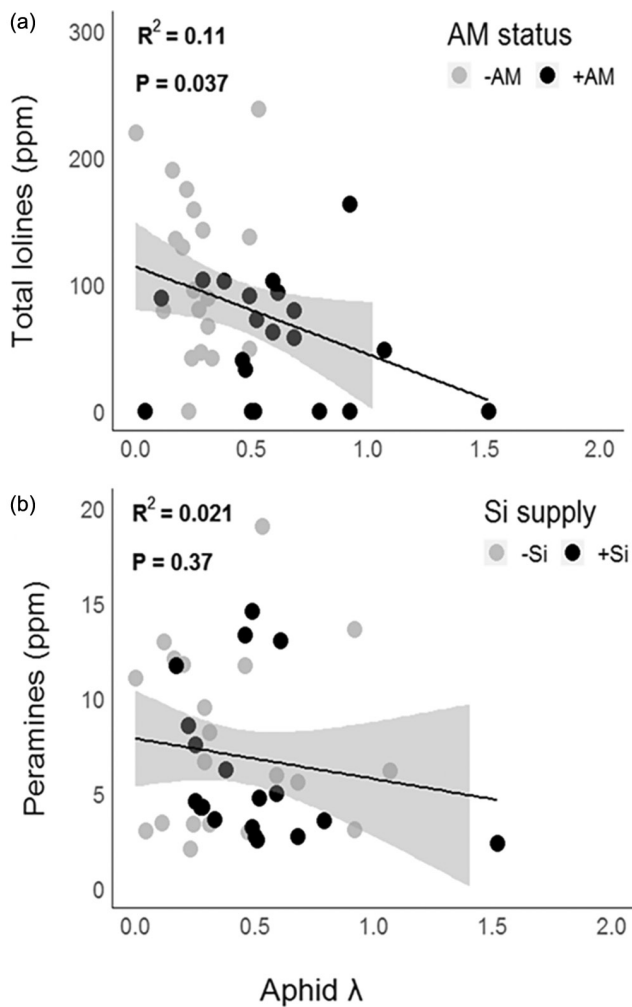


FIGURE 6 Relationships between foliar concentrations of (a) total lolines and (b) peramine with finite rate of population change (λ) for *Rhopalosiphum padi* aphids restricted to *Festuca arundinacea* associated with the AR584 novel *Epichloë* endophyte.

and host genotypes, soil nutrients, and environmental conditions (Novas et al., 2011). For instance, the relatively high soil phosphorus content in our experiment (16 mg/kg) might have influenced interactions between AM fungi colonization and endophytes, as previously noted by Li, Guo, et al. (2018).

In the present study, AM fungi only reduced endophytic-derived lolines and not peramine. Despite endophyte hyphae not colonizing host roots, loline alkaloids have been shown to be transported into the roots of the fescue host. Furthermore, the presence of lolines in roots was associated with protection against root-feeding insects (Barker et al., 2015). Thus, it can be envisaged, that a similar mechanism might have operated here whereby lolines in roots hampered AM fungi colonization, as lolines were detected in roots in our study (Table S7).

On another note, while Si supply had neutral effects on AM fungi colonization, it reduced endophyte peramine levels by 24%. Cooke and Leishman (2011) suggested that Si could serve as a less versatile resource compared with carbon. Recent research indicates that Si can be effectively mobilized throughout the plant, offering a

TABLE 3 The intrinsic rate of increase (r_m) and the generation time (GT) of *Rhopalosiphum padi* aphids restricted to *Festuca arundinacea* grown in soil in the absence (Nil or -AM) or presence (AR584 or +AM) of foliar *Epichloë* endophyte and root arbuscular mycorrhiza fungi; statistical analysis given in Table S3.

Endophyte	AM	Si	Developmental parameters	
			r_m (\pm SEM)	GT (\pm SEM)
Nil	-AM	-Si	0.0342 (\pm 0.0022) a	0.1774 (\pm 0.0051) b
		+Si	0.0320 (\pm 0.0014) a	0.1684 (\pm 0.0046) b
	+AM	-Si	0.0259 (\pm 0.0007) ab	0.1750 (\pm 0.0057) b
		+Si	0.0258 (\pm 0.0018) ab	0.1859 (\pm 0.0091) b
AR584	-AM	-Si	0.0074 (\pm 0.0035) c	0.2236 (\pm 0.0096) a
		+Si	0.0129 (\pm 0.0007) c	0.2278 (\pm 0.0094) a
	+AM	-Si	0.0209 (\pm 0.0010) b	0.2205 (\pm 0.0072) a
		+Si	0.0192 (\pm 0.0021) b	0.2082 (\pm 0.0097) a

metabolically cheaper alternative to carbon. This mechanism promotes a more favourable leaf carbon balance over short periods. Given this, rapidly growing grasses with high carbon demands might utilize Si for defensive purposes while minimizing the production of secondary metabolites, specifically endophyte-derived peramine alkaloids. This strategic use of Si could liberate carbon resources for endophyte symbiosis. However, the specific mechanisms by which Si reduces symbiont-chemical defences remains highly speculative.

Endophyte antagonism might be driven by indirect effects on the soil environment, decreasing AM fungi sporulation and colonization through the release of endophyte alkaloids (Omacini et al., 2006; Vignale et al., 2016). Conversely, facilitation occurs when endophytic exudates in the soil stimulate AM colonization and extend extraradical mycelia (Novas et al., 2010; Vignale et al., 2018). Finally, our selection of specific AMF and endophyte commercial strains was based on their compatibility with the target grass species and their capacity to offer advantages like improved nutrient uptake, resilience to stress, and protection against herbivores. Although natural populations can have diverse microbial communities, using commercially available strains enables us to control variables and ensure reproducibility. The use of these well-studied microbial strains allows us to evaluate their influence on plant-microbe interactions and their potential significance in ecological and agricultural contexts.

4.2 | Both symbionts increased Si in an additive manner

Co-occurrence of endophytes and AM fungi additively increased silicon accumulation in Si-supplied conditions by 39%. Both AM

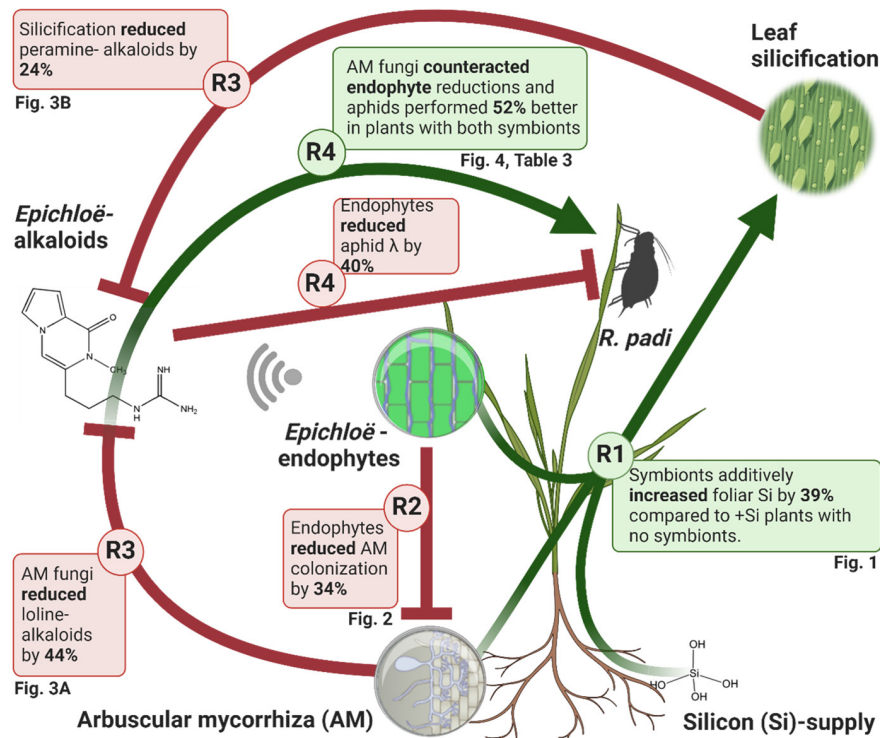


FIGURE 7 Summary of the impact of multisymbiotic associations (arbuscular mycorrhiza and *Epichloë* endophytes), Si supply and herbivory by aphids (*Rhopalosiphum padi*) on tall grass fescue (R1; R: Result 1) physical defences (tissue silicification), (R2) AM colonization, (R3) endophyte alkaloid concentrations and (R4) aphid performance. Negative and positive impacts depicted with inhibition lines and arrows respectively. Figure produced with Biorender (<http://biorender.com>).

fungi (e.g. Frew et al., 2017) and endophytes (e.g. Cibils-Stewart et al., 2020, 2021, 2023) have been reported to independently increase foliar Si, however, this is the first time to our knowledge of a combined effect. Cibils-Stewart et al. (2020) and Frew et al. (2017) attributed this to a possible increase in plant transpiration rates mediated by the symbionts supported by Perez et al. (2021) findings that would result in higher passive uptake of Si. However, present results do not support this as all physiological parameters measured remained unchanged either by symbionts or Si supply. Nevertheless, symbiont-alterations in the number and activity of plant aquaporins that might increase active Si uptake, or changes in internal morphology mediated by symbionts (Franco et al., 2020) still remain as plausible mechanisms.

Despite both types of fungi additively increasing foliar silicon, these Si defences had no direct effects on aphids in our study. This lack of direct impact on aphids aligns with findings reported by others (Massey et al., 2006; Rowe et al., 2020) However, the increase in Si concentrations mediated by both symbionts in our study could potentially make grasses more resistant to chewing herbivores. Previous research has shown that Si defences are highly effective against such herbivores (Hartley & DeGabriel, 2016).

4.3 | Endophytes alone were the dominant anti-aphid defence

Endophyte defences were the main source of resistance to aphids. The presence of AM fungi, however, had the opposite effect and caused aphid populations to increase although they remained suppressed compared with aphid populations on non-endophytic plants.

AM fungi also increased shoot nitrogen in the current study; aphids can acquire the nitrogen delivered to plants by AM fungi (Wilkinson et al., 2019), which may partly explain their improved performance on AM-endophytic plants. In addition to improved nitrogen availability, loline concentrations were lower in our AM fungi endophytic plants, relative to purely endophytic plants. Toxicity of loline alkaloids to aphids, and specifically to *R. padi*, has been extensively reported (Bastías et al., 2018b; Eichenseer et al., 1991; Wilkinson et al., 2000) and this is potentially the more impactful mechanism by which AM fungi compromise plant resistance to aphids in endophytic plants. In this regard, this is the first empirical evidence demonstrating AM fungi increasing suitability of the host plant to an aphid via reductions in endophytic alkaloids.

We observed that the positive impact of the foliar endophyte on the host plant's anti-aphid resistance was diminished, although not completely nullified, in the presence of AM fungi. To date only two studies investigated the interactions between endophytes and AM fungi and the consequences to insects; to our knowledge, this is the first to measure defences. These studies focused on ryegrass and chewing herbivores; specifically the noctuid moth *Phlogophora meticulosa* (Vicari et al., 2002) and the Argentine stem weevil *Listronotus bonariensis* (Barker, 1987). Despite the differences in systems, results from these studies point to the same direction whereby dual infection generally reduced the impact of endophytes on herbivore performance, though this effect seemed to vary with the performance parameter measured and with experimental conditions. Here we provide a clear mechanism for the interactive effects of AM fungi and endophytes on aphid performance, specifically novel evidence that AM fungi reduce the levels of loline alkaloids produced by the endophyte defence.

Alternative mechanisms must be responsible for the effects observed in the previous studies as loline alkaloids are not produced in endophyte-ryegrass associations.

When studying the effects of endophytic and AM fungi on insect herbivores, a trend emerges: generalist insects are usually harmed, while specialists benefit, as noted by Hartley and Gange (2009) and Koricheva et al. (2009). Including diverse feeding guilds in our research would deepen our understanding. Currently, only three studies have explored co-infection impacts on insects, involving just two plant species despite widespread symbiont occurrence. This study is unique in identifying a clear mechanism. To grasp these interactions' significance, more experiments are vital, given their roles in ecological communities. Understanding the collective impact of diverse symbiotic interactions involves unravelling complex mechanisms between microbial symbionts and their hosts, influenced by non-symbiotic microbial interactions and environmental factors (Tsiknia et al., 2020). This insight is crucial for developing integrated technologies and strategies for efficiently utilizing beneficial microbes in plants (Tsiknia et al., 2020).

AUTHOR CONTRIBUTIONS

X. Cibils-Stewart and S. N. Johnson planned and designed the research. X. Cibils-Stewart conducted all the experimental work and collected the data. X. Cibils-Stewart and R. K. Vandegeer performed the plant physiology measurements. X. Cibils-Stewart and W. J. Mace performed alkaloid analysis. X. Cibils-Stewart analysed the data with input from S. N. Johnson and J. R. Powell. X. Cibils-Stewart led the writing of the manuscript with significant input from all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14410>.

DATA AVAILABILITY STATEMENT

Data are available at Dryad: <https://doi.org/10.5061/dryad.6m905qg8c> (Cibils-Stewart 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. Characteristics of the soil used before and after sand addition (measured after soil irradiation); sand was used to lower phosphorus and bioavailable silicon.

Table S2. Treatment combinations (TRT) and final replication number for tall fescue plants (*Festuca arundinacea* cv. INIA Fortuna) either *Epichloë*-free (Nil) or infected with the animal safe AR584 novel *Epichloë* endophyte strain (Endophyte) growing in soil with (+Si, +AM) or without (–Si, –AM) silicon (Si) supply and arbuscular mycorrhiza (AM) fungi; plants were either inoculated with *Rhopalosiphum padi* aphid (*R. padi*) or remained aphid-free (Control) (Insect) to determine treatment effects on population (Pop) or individual (Ind) reproductive performance.

Table S3. Results from multiple comparison tests for changes in *Rhopalosiphum padi* population structure: (A) apterous and (B) alates adults, as well as (C) nymphs, as affected by endophyte (Endo), arbuscular mycorrhiza (AM), and Si supply (Si).

Table S4. Results from multiple comparison tests for changes in developmental (intrinsic rate of increase (r_m) and the generation time (GT)) for *Rhopalosiphum padi* as affected by endophyte (Endo), arbuscular mycorrhiza (AM), and Si supply (Si).

Table S5. Results from multiple comparison tests for changes in plant (A) shoot and (B) root mass, as affected by endophyte (Endo), arbuscular mycorrhiza (AM), Si supply (Si), and herbivory by aphids.

Table S6. Results from multiple comparison tests for changes physiological (A) greenness (SPAD), (B) Photosynthesis, (C) stomata

conductance (SC), (D) transpiration (Tr), as well as, nutritional quality (E) phosphorous, (F) Nitrogen (N), (G) Carbon (C), and (H) C:N ratio of *Festuca arundinacea* foliage as affected by endophyte (Endo), arbuscular mycorrhiza (AM), and Si supply (Si).

Table S7. Root concentrations of total lolines on plants without (AM–) and with (AM+) AM fungi, without (Si–) and with (Si+) Si supply, and with (Aphid) and with (NO Aphid) Aphid inoculation, along with the results from multiple comparison tests.

Figure S1. Population structure of *Rhopalosiphum padi*; Average number of (A) apterous and (B) alates adults, as well as (C) nymphs restricted to *Festuca arundinacea* grown in soil in the absence or presence of single (AM or Endo) or dual (Both) symbiont infection; AM, Arbuscular mycorrhiza fungi Endo: AR584 novel *Epichloë* endophyte; Asterisks and bold indicate significant differences in the model (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); results from multiple comparison tests in Table S3.

Figure S2. (A) Shoot and (B) root mass for *Festuca arundinacea* grown in the absence (None) or presence of single (AM or Endo) or dual (Both) symbiont infection; AM, Arbuscular mycorrhiza fungi Endo: AR584 novel *Epichloë* endophyte. Mean values indicated with big black circles, inclusive median and interquartile ranges indicated with lines. Asterisks and bold indicate significant differences in the model (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); results from multiple comparison tests in Table S5.

Figure S3. Foliar concentrations of (A) nitrogen and (B) carbon as affected by the absence (Nil or –AM) or presence (AR584 or +AM) of foliar *Epichloë* endophyte or root arbuscular mycorrhiza (AM) fungi; Asterisks and bold indicate significant differences in the model (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); results from multiple comparison tests in Table S6.

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