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## RESEARCH ARTICLE

# Silicon and *Epichloë*-endophyte defences in a model temperate grass diminish feeding efficiency and immunity of an insect folivore

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## Abstract

1. Plants deploy diverse anti-herbivore defences which reduce feeding and performance of herbivores. Temperate grasses use silicon (Si) accumulation and *Epichloë*-endophytes for physical and chemical (i.e. endophytic-alkaloids) defence against insect herbivores. Recent studies suggest that *Epichloë*-endophytes increase Si accumulation in their host grass. It is unknown, however, how this affects Si-deposition on the leaf surface, their impacts on insect herbivore feeding efficiency and their immunity to potential infection/parasitism.
2. To address this knowledge gap, we grew tall fescue (*Festuca arundinacea*) hydroponically with and without Si, in the absence or presence of the novel AR584 *Epichloë*-strain. We exposed plants to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in both in situ (intact leaves) and ex situ (excised leaves) feeding trials and determined the effects of Si and endophyte defences on herbivore feeding efficiency, growth rates and immunity against potential infection/parasitism.
3. Endophytic plants supplied with Si showed 110% and 143% increases in leaf silica density and leaf Si concentrations, respectively, when exposed to herbivory, compared to non-endophytic plants that were herbivore-free. Despite the endophyte-mediated increases in Si concentrations, *H. armigera* was only affected by Si supply; growth rates decreased by 87% and most feeding efficiency indices decreased by at least 30%. Si supply also increased mandibular wear by 16%, which was negatively correlated with *H. armigera* growth rates. Cellular and humoral immunity of *H. armigera* were negatively affected by both Si and endophytes. Endophytic-loline alkaloid concentrations were unaffected by Si supply or herbivory, whereas herbivory increased peramine concentrations by 290%.

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4. To our knowledge, this is the first report of Si defences and *Epichloë*-endophyte derived alkaloids compromising insect immunity via reduced melanisation response. Using tall fescue and *H. armigera*, our study suggests that deploying both physical (i.e. Si accumulation) and chemical (i.e. endophytic-alkaloids) defences acting against multiple insect herbivore traits, including feeding efficiency, growth and immunity, may be a successful defence strategy in temperate grasses. This multifaceted defence may be particularly difficult for insect herbivores to overcome.

#### KEYWORDS

antiherbivore alkaloids, endophytes, *Festuca arundinacea*, *Helicoverpa armigera*, insect immunity, physical defences

## 1 | INTRODUCTION

Plants and herbivorous insects have coexisted for at least 300 million years (Fürstenberg-Hägg et al., 2013) with herbivorous insects exerting strong selection pressures on plants to resist attack. Plant defences include a diverse array of chemical and physical traits that adversely affect herbivore performance including feeding, growth, reproduction and behaviour (Erb, 2018; Strauss & Agrawal, 1999). Plant defences may indirectly alter insect physiological performance by compromising insect immunity, however these physiological performance traits are less commonly reported (Smilanich et al., 2009).

Grasses (Poaceae) generally lack the diversity of defensive secondary metabolites found in other plant families (Vicari & Bazely, 1993) but often accumulate silicon (Si) in tissues as a form of physical defence against herbivory (Epstein, 1994). Si is present in most soils, taken up by plant roots, and subsequently deposited in plant tissues forming internal and external mechanical barriers (Debona et al., 2017). Notably, grasses possess specialised leaf epidermal cells known as 'silica cells' as well as spines and trichomes on the leaf surface that are mineralised via Si deposition (Hartley et al., 2015; Kumar et al., 2017). These depositions often increase plant tissue abrasiveness and rigidity, which can increase chewing insect mandibular wear, lacerate digestive tissues, and/or reduce their ability to acquire and metabolise macronutrients (e.g. nitrogen; Andama et al., 2020; Massey & Hartley, 2009).

An extra layer of chemical defence in many grasses is provided by the association with mutualistic *Epichloë* fungi (Ascomycota: Clavicipitaceae; Leuchtman, 1992). Asexual *Epichloë* are true obligate symbionts that exclusively infect temperate grasses (Christensen et al., 2008). There is evidence that *Epichloë*-endophytes often protect grasses from biotic and abiotic stresses, including herbivory (Dastogeer, 2018; Rho et al., 2018; but see Gundel et al., 2013). Endophyte-mediated resistance to herbivory largely derives from alkaloid production but can also include induction of the host plant's own defences (i.e. activation of the salicylic and jasmonic acid pathways; Bastías et al., 2018), promotion of host plant growth and vigour (Clay & Schardl, 2002); these can act solely or in combination. In terms of chemical defence, endophytes produce four major classes of antiherbivore alkaloids, including ergot

alkaloids (e.g. ergovaline), indole-diterpenes (e.g. lolitrem B and epoxyxanthitrem), pyrrolizidines (e.g. lolines) and pyrrolopyrazines (e.g. peramine; Berry et al., 2019; Schardl et al., 2013). However, only grasses harbouring endophytes that putatively produce peramine (i.e. anti-chewing insect defence) and lolines (i.e. anti-aphid and -chewing insect defence) are desirable in pastures because they confer insect resistance without affecting grazing mammals (i.e. cows; Young et al., 2013). These include the commercial 'novel animal-safe' strains such as the AR584 utilised in this study (Johnson et al., 2013).

Despite being recognised mostly as a physical defence, Si has been shown to influence plant secondary metabolite production (Hall et al., 2019), potentially through interference with herbivore suppression of plant defensive pathways, although this is highly speculative (Coskun et al., 2019). Alternatively, Si may act as a 'metabolically cheaper' herbivore defence than secondary metabolites such as phenolics and tannins (Cooke & Leishman, 2012; Frew et al., 2016; Johnson, Hartley, Ryalls, et al., 2020; Moles et al., 2013). However, studies considering whether interactions between Si and secondary metabolite defences interact to increase plant resistance to herbivory have conventionally only considered metabolites produced by the plant (e.g. Hall et al., 2020; Johnson, Hartley, & Moore, 2020; Reynolds et al., 2016). Whether Si has similar effects on *Epichloë*-alkaloids as those on plant-produced metabolites remains unclear. Despite the fact that alkaloids are produced by endophytes, they often come at an energetic cost to the plant (Bastías et al., 2017), so it may be beneficial for the plant to conserve alkaloid production if Si defences can be deployed at a lower cost (Johnson, Hartley, & Moore, 2020). Supporting this idea, Cibils-Stewart et al. (2021) reported that Si supply reduced *Helicoverpa armigera* (Lepidoptera: Noctuidae) relative growth rate, regardless of endophytic status, whereas endophyte-alkaloids played a secondary role by reducing herbivore relative growth rate only in the absence of Si supply.

While Si and endophytes are simultaneously present in temperate grasses in natural environments, only four studies have looked at the direct associations between them (Cibils-Stewart et al., 2020, 2021; Huitu et al., 2014; Johnson et al., 2023). Overall, endophytes increased foliar Si concentrations in their host grasses in both field (Huitu et al., 2014) and controlled-laboratory (Cibils-Stewart et al., 2020, 2021) settings. Despite endophytes increasing Si

concentrations in tall fescue, this did not further reduce the relative growth rate of *H. armigera* (Cibils-Stewart et al., 2021). Conversely, Si supply and *Epichloë*-endophytes could affect other herbivore performance traits, such as food utilisation and immunity but this remains to be addressed.

The impacts of plant antiherbivore defence on insect immunity have been much neglected (Islam et al., 2022). To our knowledge, there are no reports of the effects of *Epichloë* defences on herbivore immunity traits, and the effects of Si defences on herbivore immunity are poorly understood, with just two studies to date reporting that Si alone has no clear effects on chewing herbivore immunity (Frew et al., 2017; Islam et al., 2022). Frew et al. (2017) reported that Si defences did not affect the immunity of a root-feeding insect whereas Islam et al. (2022) found that immunity of *H. armigera* was not affected by Si alone.

In the initial cellular immune response in insects, stress signals (i.e. activated by wounds) attract haemocytes that encapsulate the foreign body (Krautz et al., 2014; Wang et al., 2010). Subsequently, a phenoloxidase cascade initiates a humoral response that leads to the melanisation of the haemocyte capsule, leading to suffocation of the foreign body (Krautz et al., 2014). An increase in phenoloxidase activity therefore usually indicates a stronger encapsulation response and thus, a stronger immune system (Bidla et al., 2009). Reduced digestibility (Benrey & Denno, 1997; Gherlenda et al., 2016) and signals from damaged organs (Krautz et al., 2014) have both been reported to activate insect immunity (Lampert, 2012). Feeding on Si-rich diets can impair digestion (Massey & Hartley, 2009) and lacerate organs (Andama et al., 2020) and therefore has the potential to impact immunity. Moreover, insect herbivores could potentially experience compromised immunity due to the allocation of energy towards detoxification or toxin storage (Moret & Schmid-Hempel, 2000). This phenomenon might be relevant considering the toxic antiherbivore properties associated with *Epichloë* derived alkaloids. It is plausible that such compromised immunity effects could arise following the ingestion of alkaloids from silicified plants colonised by endophytes: this immunocompromisation might be further exacerbated by laceration of tissue caused by silicified tissue. Alternatively, plant or endophyte-derived toxins may have a direct effect on specialised immune cells (haemocytes) of the insect's haemolymph (Barthel et al., 2014). Immunocompromised herbivores are potentially more susceptible to infection/parasitism (Lampert, 2012).

Both Si supply and endophytes increase constitutive and herbivore-induced Si-defence, at least for tall fescue, without affecting endophyte alkaloid production (Cibils-Stewart et al., 2021), but little is known about their combined effects on host leaf morphology and subsequent herbivore food utilisation and immunity. The present study therefore aimed to evaluate how Si supply, the AR584-endophyte (i.e. mammalian-safe) and *H. armigera* feeding, acting alone and in combination, affect tall fescue growth traits, primary chemistry, foliar Si concentrations and leaf morphology; the latter using scanning electron microscopy. Further, the effects of Si supply and endophyte infection on herbivore feeding efficiency,

mandibular wear, and cellular and humoral immune responses were quantified. To address these, the current study employed a hydroponic approach that enables uniform plant growth and control over Si supply (note that Si is present in virtually all soils); these issues may have been constraints in previous experiments (Cibils-Stewart et al., 2021).

We predicted that Si supply, endophytes and *H. armigera* feeding (H1) increase Si-defences (i.e. increase silicified phytoliths in the leaf surface) and that (H2) Si supply has no impact on endophyte-specific alkaloid production. As for the herbivore, we predicted that (H3) only Si supply negatively affects feeding efficiency indices including herbivore mandibular wear, whereas both Si and endophyte have (H4) negative effects on insect immunity. We expected negligible impacts of both Si supply and endophytes on plant growth and primary chemistry, based on previous observations (Cibils-Stewart et al., 2021).

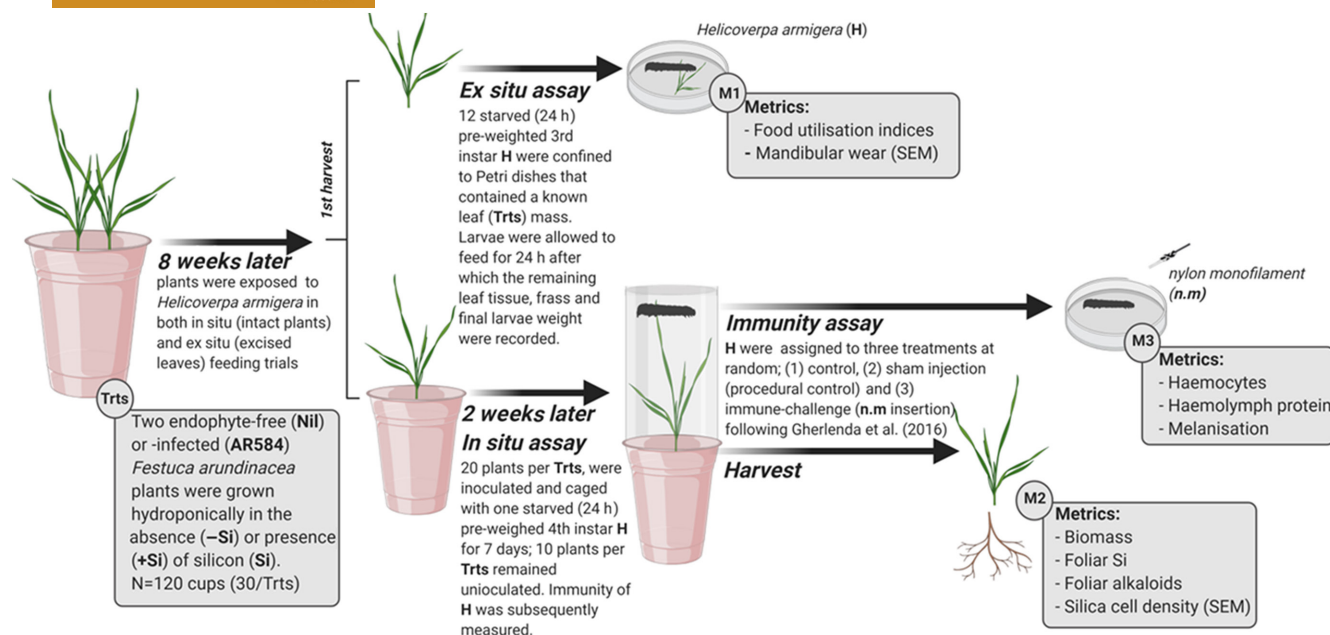
## 2 | MATERIALS AND METHODS

### 2.1 | Experimental procedure

Seeds of tall fescue (*Festuca arundinacea*) cv. INIA Fortuna harbouring the animal-safe endophyte strain AR584 of *Epichloë coenophiala* or as *Epichloë*-free (Nil) controls were surface sterilised and germinated in wet perlite growing media. Germination trays were placed in a naturally lit glasshouse under controlled environmental conditions of 22/18°C (day/night) and 50% relative humidity, at the Hawkesbury Institute for the Environment, Richmond, NSW, Australia the 9th of December of 2020.

Eleven days after germination (20 December), two uniformly sized plantlets were transferred to 0.52 L plastic cups (120 mm tall) fitted with a foam lid and filled with ~330 mL (90 mm depth) of nutrient solution with (+Si) or without (-Si) silicon (Si), as described by Hall et al. (2020). Cups were placed randomly and approximately 20 cm from each other and were set in the same greenhouse conditions as above. Briefly, to generate +Si treatments, liquid potassium silicate was added to the nutrient solution ( $K_2SiO_3$ ; Agsil32, PQ Australia, SA, Australia) at a concentration equivalent to 2 mM  $SiO_2$  (Ma & Yamaji, 2006). To balance the additional potassium ions in the +Si treatments, potassium chloride (KCl) was added to the control nutrient solution (-Si). Finally, the pH of both solutions (+Si/-Si) was adjusted to 5.6–6 using hydrochloric acid (2 M HCl) to reduce the polymerisation of silicates (Ma & Yamaji, 2006). A total of 120 cups, 60 for each germplasm (AR584 or Nil), were assigned to either the +Si or -Si hydroponic treatment, resulting in 30 replicate cups per treatment combination. The nutrient solution was refreshed three times a week and the cups were randomly shifted to minimise position bias on a weekly basis.

After 8 weeks (on 28 January), one plant per cup was selected at random and harvested (Figure 1). Immediately after harvest, two of the thickest tillers of each plant were blotted for *Epichloë* detection (Section 2.2) and foliar tissue was pooled by treatment and



**FIGURE 1** Schematic diagram summarising experimental design and procedures. Figure produced with Biorender (<http://biorender.com>); replication details also given in Table 1.

**TABLE 1** Details of replication: refer to Figure 1 for methodology.

	Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Ex situ assay	Larvae	Petri dish with excised plant material in 4 treatments (with and without endophyte, with and without Si-supply, and their combinations)	12 per treatment ( $n=4$ ; total 48)
In situ assay	Plant and Larvae	Plants growing in nutrient solution in 4 treatments (with and without endophyte, with and without Si-supply, and their combinations)	10 uninoculated plants and 20 inoculated plants per treatment ( $n=4$ ; total 120)
Immunity assay	Larvae	Larvae coming from the in situ assay (20 in 4 treatments) randomly assigned into 3 immunity treatments	6 per immunity treatment ( $n=12$ ; total 72)

stored in the fridge (4°C) to be utilised in the ex situ feeding assay (Section 2.4; Figure 1-M1).

Individual plants that remained in cups were grown in their original Si treatment for a further two-week period. At this point (on 10 February), intact plants were challenged with one *Helicoverpa armigera* (Lepidoptera: Noctuidae; +H) or remained herbivore-free (–H) for the in situ feeding assay (Section 2.5). One week after herbivore exposure, all plants were harvested and blotted for *Epichloë* detection (Section 2.2). Experimental design with details on replication are shown in Figure 1 and Table 1. Ten plants per treatment were randomly selected and individual shoots and roots were freeze-dried, weighed and stored at –20°C for further chemical analysis (Section 2.3), and five randomly selected samples in each treatment were utilised for morphological analysis (Figure 1-M2). For the morphological analysis, scanning electron microscopy (SEM) coupled with X-ray mapping (XRM) was deployed to determine Si deposition, following Vandegheer et al. (2021) (see Appendix S1 for further details on SEM leaf silica cell density and composition).

## 2.2 | *Epichloë* detection

*Epichloë* viability *in planta* was determined in all 240 experimental plants (originally 2 plants per cup) using tissue-print immunoblotting and histological detection immediately after each harvest following the procedures of di Menna et al. (2012).

## 2.3 | Foliar chemistry

### 2.3.1 | Si

Foliar Si concentration was measured utilising approximately 80mg of ground shoot tissue using X-ray fluorescence spectrometry (Malvern Panalytical) following the procedure of Reidinger et al. (2012). Si concentrations, expressed as % of dry mass, were measured in the presence of helium and calibrated against a certified control (Hiltbold et al., 2016).



### 2.3.2 | Primary chemistry

A subsample of approximately 6–7 mg of leaf tissue of five samples per treatment utilised for Si analysis was separated and weighed directly into a tared tin container using a microbalance. Carbon (C) and nitrogen (N) concentrations were further obtained using an elemental analyser (FLASH EA 1112 Series CHN analyser; ThermoFinnigan, Waltham, MA, USA).

### 2.3.3 | Endophyte alkaloids

Shoot peramine was analysed according to a slight modification of Popay et al. (2021). Briefly, samples (20 mg) were extracted with 500  $\mu$ L of 75% methanol (containing 1.7  $\mu$ g/mL homoperamine as an internal standard) for 1 h in the dark. Samples were then centrifuged (5000  $g$ <sup>1</sup>, 5 min) and the supernatant was transferred to 2 mL amber HPLC vials via a 0.45  $\mu$ m syringe filter (PVDF). Chromatographic separation was achieved using a Polar-RP column (100  $\times$  2.0 mm, 2.5  $\mu$ m, Phenomenex) and peramine quantified using a Thermo LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the parameters described in Rasmussen et al. (2012). 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*-acetyloline (NAL), *N*-acetylnoroline (NANL) and *N*-formyloline (NFL), was quantified following Bastias et al. (2018).

### 2.4 | Ex situ assay

Forty-eight late third instar larvae previously feeding on artificial diet (Teakle & Jensen, 1985) were starved for 24 h, weighed (0.094 mg  $\pm$  0.009 mean  $\pm$  standard deviation) and each provided with a known mass of tall fescue leaves from each treatment assigned at random (0.205 mg  $\pm$  0.032; mean  $\pm$  standard deviation). Individual larvae were confined to Petri dishes and kept in the glasshouse (original conditions). Larvae were allowed to feed for 24 h after which the remaining leaf tissue, frass and resulting larval weights were recorded. The exact procedure was repeated for a total of five consecutive days. New leaves (with known mass) of the original treatment were offered to each larva daily. The remaining leaf material and frass of each individual larva were collected daily in two separate Eppendorf tubes and oven-dried at 60  $^{\circ}$ C for 72 h. Subsequently, water content ((total fresh/total dry)  $\times$  100) was determined. Carbon (C) and nitrogen (N) concentrations of frass samples were then analysed following the exact procedures as depicted above (Section 2.3.2), except intact dried frass was utilised instead of milled leaf tissue. Si deposition in intact dried frass pellets was visualised using scanning electron microscopy (SEM) coupled with X-ray mapping (XRM; Appendix S1 for XRM details).

After the controlled ex situ feeding period, the final larval weight was recorded and larvae were decapitated. Mandibles were dissected to measure mandibular wear (degradation) according to Massey and

Hartley (2009) except utilising SEM (Appendix S1 Insect mandibles). Subsequently, six growth and food utilisation efficiency parameters were calculated according to Massey and Hartley (2009), including the relative growth rate, efficiency of conversion of ingested and digested food, approximate digestibility, relative consumption and proportion of nitrogen absorbed (Figure 1-M1; see Appendix S1 for details on formulas utilised).

### 2.5 | Herbivore in situ and immunity assays

#### 2.5.1 | In situ *H. armigera* assay

Of the 120 plants left, a total of 80 randomly selected plants, 20 per treatment, were challenged and caged at random with one starved (24 h) pre-weighed 4th instar *H. armigera* (0.184 mg  $\pm$  0.012 mean  $\pm$  standard deviation). The remaining 40 plants, 10 per treatment, were caged and left un-inoculated serving as herbivore-free-controls. After 1 week, larvae were carefully removed, starved for 24 h and utilised for the immunity assay (Section 2.5.2).

#### 2.5.2 | *H. armigera* immunity assay

Larvae were organised by previous treatment ( $N=4$ ; Nil or AR584 in the  $-Si$  or  $+Si$  treatments) and assigned to three immunity treatments at random: (1) control, (2) sham injection (procedural control) and (3) immune-challenge (nylon monofilament insertion) following procedures by Gherlenda et al. (2016). This approach enables evaluation of the immune response of the insect without the damaging effects of a sustained pathogen or parasitoid infection (Rantala & Roff, 2007) (see Appendix S1 for details of immunity treatments).

After immunity treatments were deployed, larvae were held at 24  $^{\circ}$ C in plastic containers with aeration, and after 72 h, monofilaments of the immune-challenged larvae were removed and stored in 70% ethanol until the melanisation response was measured as per Gherlenda et al. (2016) (Appendix S1 melanisation response). Immediately after monofilament extraction, live larvae were sedated (direct CO<sub>2</sub> emission), sterilised by dipping in 70% ethanol, and haemolymph was extracted by cutting a proleg (Gujar & Kalia, 2005). Pure haemolymph (40  $\mu$ L, unfixed/undiluted) was transferred to a haemocytometer to measure cellular immunity. Haemocytes were counted and classified into plasmatocytes, granulocytes and spherulocytes following Wang et al. (2010) (Appendix S1 cellular response). This classification at a cellular level is important because while plasmatocytes form the bulk of capsules around foreign bodies, granulocytes phagocytise foreign pathogens; conversely spherocyte function remains unknown for *H. armigera* (Wang et al., 2010; Zhai & Zhao, 2012). Subsequently, for humoral immunity, phenoloxidase (PO) activity and solubilised protein concentration (Bio-Rad protein assay kit) were assessed following Gherlenda et al. (2016) procedures (Figure 1-M3; Appendix S1 humoral response). While haemolymph protein concentration is a function of foliar protein

availability and thus might be more related to available N in tissue, PO increase usually indicates a stronger encapsulation response and thus a stronger immune system (Bidla et al., 2009).

## 2.6 | Statistical analysis

The statistical software R (R Core Team, 2015) was utilised for all statistical analyses. Assumptions of normality for residuals were verified for all models according to the inspection of quantile-quantile plots. All figures were produced using the 'ggplot2' package (Wickham, 2016).

To address hypothesis one (H1) that states that Si supply, endophytes and *H. armigera* feeding increased Si-defences (e.g. increase silicified phytoliths in the leaf surface), foliar Si concentration and resulting silica cell density (response variables) of +Si plants only were analysed using a two-way ANOVA (type III sum-of-squares) with endophyte and herbivory treatments as fixed effect factors. To address H2 that states that Si supply has no impact on endophyte-specific alkaloid production, using only endophytic plants, a multivariate analysis of variance (permutational MANOVA) with Si and herbivory treatments as fixed effects was utilised to determine differences in overall alkaloid profiles (response variables) with the 'vegan' package (Oksanen et al., 2019). The MANOVA function further provided results for univariate ANOVAs for treatment effects on individual alkaloids.

Moreover, to address H3 and H4 that state that only Si supply negatively affects feeding efficiency indices (H3), whereas both Si and endophyte have (H4) negative effects on insect immunity, all herbivore parameters including, relative growth rate, mandibular wear, food utilisation indices, and larval cellular and humoral immunity (response variables), were explored using a two-way ANOVA (type III) with endophyte and Si treatment as fixed factors. Additionally, a Pearson rank correlation coefficient test was used to analyse the relationship between relative growth rate and mandibular wear (Knowles et al., 1991). Finally, although not part of the hypothesis, for plant growth and primary chemistry, a three-way ANOVA (type III) with endophyte, Si supply and herbivory treatments as fixed effect factors was performed.

For the data analysed, we employed Type III ANOVAs and reported all interaction terms in the tables: we did not conduct model simplification analyses to assess the impact of non-significant interaction terms on the overall model because removing non-significant interactions can lead to an increase in the apparent sample size and may affect the power to detect main effects. Note that some individuals escaped our in situ trial, and thus, the data for this are unbalanced.

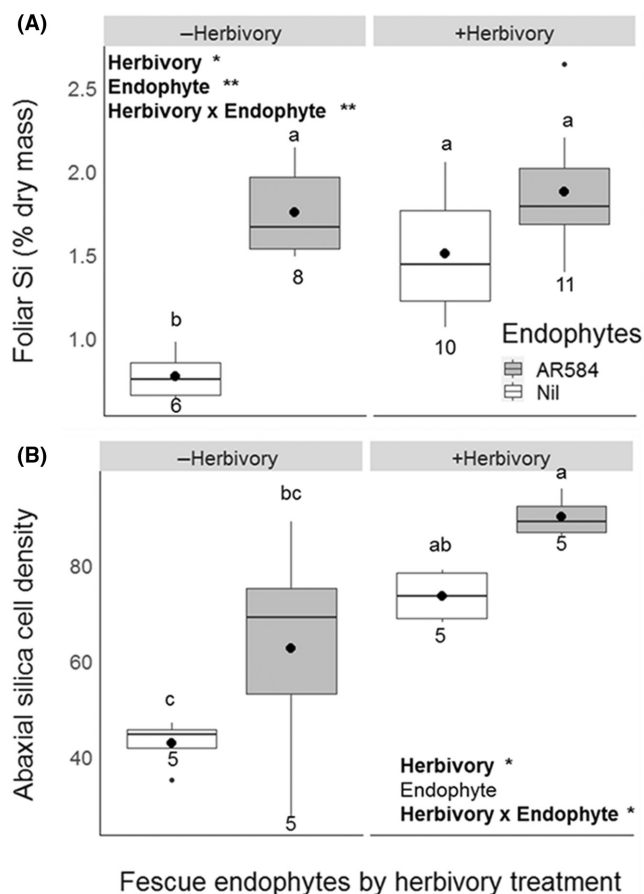
## 3 | RESULTS

### 3.1 | All factors increased leaf Si-physical defences but only herbivory affected endophytic-alkaloids

There was a significant interaction between endophyte presence and herbivory treatment for resulting foliar Si concentrations under Si supply. Specifically, Si concentration increased by 95% with

endophyte presence in the absence of herbivory; however, Si concentrations increased in all plants exposed to *H. armigera* herbivory (by 127% in Nil and 143% in endophytic plants), regardless of the endophytic status (Figure 2A; Table 2a). Silica cell density along the abaxial side of the midrib was lower on non-endophytic plants that were not challenged by herbivores, whereas both herbivory and endophytes solely and in combination increased silica cell density by 72%, 46% and 110% respectively, in a synergistic manner (Figure 2B, Table 2a). Silica cell density for the adaxial leaf side was not affected by any of the treatments (Table 2a; data not shown).

The combined effect of Si supply and herbivory did not affect total alkaloids produced by endophytic plants overall (MANOVA, Table 2b). However, foliar peramine concentration increased by 290% with herbivory, regardless of Si supply (Figure S1A), while total

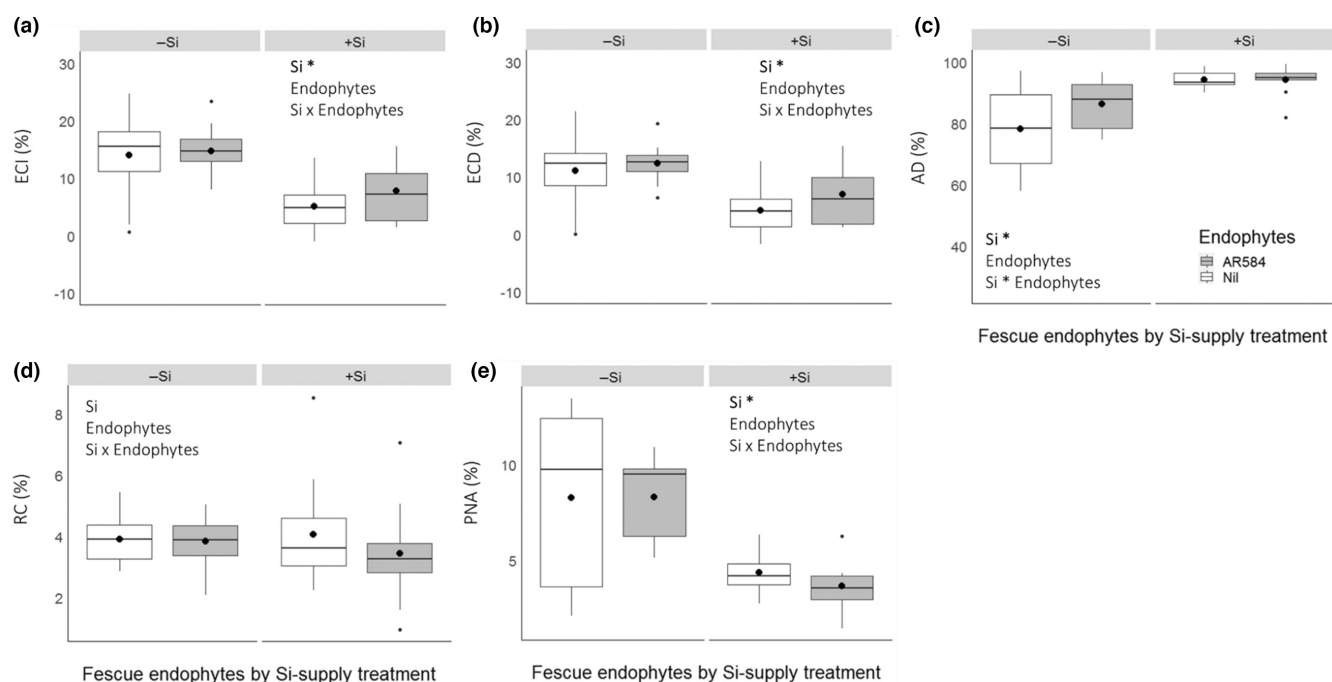


**FIGURE 2** Foliar (A) Si concentration and (B) silica cell density of endophyte-free (white, Nil) versus endophyte-infected (grey, AR584) *Festuca arundinacea* plants with and without herbivory growing hydroponically with silicon (+Si). Mean values are indicated with black circles, and inclusive median and interquartile ranges are indicated with lines. We started with 30 plants per treatment combination, 20 were inoculated with herbivores and 10 were left uninoculated; some plants in the uninoculated treatment died, as well as herbivores were not found (escaped), thus resulting N is indicated below each box plot. Asterisks and bold indicate significant differences in the model (\* $p < 0.05$ , \*\* $p < 0.001$ ). Different letters indicate significant differences; statistical analysis is given in Table 2a.

**TABLE 2** Results from multiple comparison tests for changes in (a) shoot Silicon (Si) and silica cell density for Si supplied plants, and (b) endophyte-alkaloid concentration for endophytic plants, including the three loline derivatives N-acetylloine (NAL), N-acetylornoline (NANL) and N-formylloine (NFL).

(a) Silicon		Foliar Si				Abaxial silica cell density				Adaxial silica cell density					
Factors		df	F	p		df	F	p		df	F	p			
Endophyte		1.31	39.95	<0.001		1.13	4.55	0.052		1.13	1.98	0.182			
Herbivory		1.31	0.85	0.361		1.13	8.67	0.011		1.13	0.06	0.809			
Endophyte×Herbivory		1.31	8.55	0.006		1.13	0.06	0.808		1.13	0.04	0.841			
ANOVAs															
(b) Alkaloids		MANOVA				Peramines		Lolines		NAL		NANL		NFL	
Factors		df	Pillai	p	F	p	F	p	F	p	F	p	F	p	
Si		1.38	0.74	0.615	0.22	0.639	2.10	0.155	2.10	0.283	2.94	0.094	1.12	0.297	
Herbivory		1.38	0.16	0.281	4.66	0.037	1.08	0.304	0.03	0.866	0.89	0.351	1.37	0.249	
Si×Herbivory		1.38	0.04	0.829	0.22	0.642	1.28	0.263	0.93	0.340	1.01	0.321	1.21	0.277	

Note: Models with significant main effects and/or interactions are noted in bold.



**FIGURE 3** *Helicoverpa armigera* efficiency of conversion of (a) ingested (ECI) and (b) digested (ECD) food, (c) approximate digestibility (AD), as well as (d) relative consumption (RC) and (e) proportion of nitrogen absorbed (PNA) when feeding on endophyte-free (white, Nil) or endophyte-infected (grey, AR584) *Festuca arundinacea* plants growing hydroponically in the absence (-Si) or presence (+Si) of silicon (Si) in an ex situ (excised tissue) feeding assay. Details are as per Figure 1; statistical analysis is given in Table 3; N = 12 per treatment.

loline concentration (Figure S1B) and individual loline derivatives remained unaffected by any of the treatments (ANOVAs, Table 2b).

### 3.2 | Si supply affected *H. armigera* feeding efficiencies and increased mandibular wear

Despite endophyte-mediated increases in foliar Si, most *H. armigera* food utilisation indices were only significantly affected by direct Si supplementation in the ex situ assay. Specifically, the efficiency of conversion

of ingested and digested food, and the proportion of nitrogen absorbed decreased by 32%, 13% and 51%, respectively, and approximate digestibility increased by 15% with Si supply, whereas relative consumption remained unaffected (Figure 3; Table 3). Accordingly, the relative growth rate reduced by 45% (Figure 4a) and mandibular wear significantly increased with Si supply (Figure 4b; Table 3), resulting in a significant negative correlation between relative growth rate and mandible wear irrespective of endophytic status of the grass offered (Figure 4c).

SEM images revealed the presence of leaf surface silica cells in both -Si and +Si plants; however, when the elemental composition



**TABLE 3** Results from multiple comparison tests for changes in *Helicoverpa armigera* relative growth rate (RGR) and the five food utilisation parameters calculated: efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), approximate digestibility (AD), relative consumption (RC) and proportion of nitrogen absorbed (PNA), and mandibular wear (M. wear).

Factors	df	RGR		ECI		ECD		AD		RC		PNA		M. wear	
		F	p	F	p	F	p	F	p	F	p	F	p	F	p
Si	1.43	36.0	<b>&lt;0.001</b>	8.46	<b>&lt;0.001</b>	4.11	<b>&lt;0.001</b>	25.7	<b>&lt;0.001</b>	1.01	0.321	9.86	<b>0.006</b>	98.65	<b>&lt;0.001</b>
Endophyte	1.43	1.1	0.298	0.86	0.358	1.27	0.265	2.98	0.091	1.19	0.281	0.05	0.819	1.03	0.315
Si × Endophyte	1.43	0.30	0.582	0.13	0.721	0.57	0.453	3.12	0.084	0.78	0.381	0.07	0.789	0.24	0.626

Note: Models with significant main effects and/or interactions are noted in bold.

of these Si cells was investigated using XRM mapping, only silica cells in the +Si treatment were mineralised with Si (Figure 5a,b; Figure S2); this had clear effects on mandible incisor degradation (Figure 5c,d). Frass pellets from larvae that fed on +Si plants contained mineralised silica cells that appeared to be intact after their passage through the gut, irrespective of plant endophytic status (Figure 5f): elemental Si was not detected in frass pellets of larvae that fed on -Si plants (Figure 5e; Figure S3).

### 3.3 | Si supply and endophyte presence compromised *H. armigera* immunity

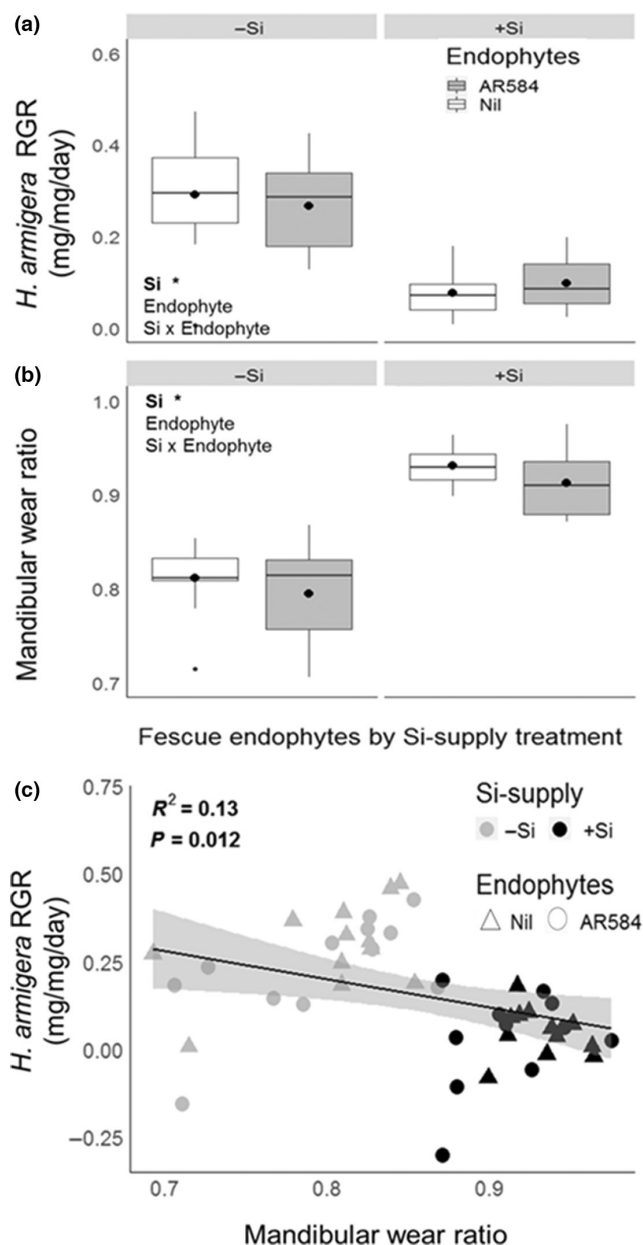
When herbivore cellular responses were evaluated, only the number of spherulocytes was affected by the treatments (Figure 6). Spherulocytes were higher in unchallenged larvae that fed on endophytic Si supplied plants and challenged larvae that were fed on control plants (Nil -Si), whereas the lowest spherulocytes were observed for the unchallenged larvae that fed on control plants (Nil -Si) (Table 4; Table S1). An unknown haemocyte was detected in samples, presumably a prohaemocyte (uk in Figure 6; personal communication with Dr. Xiao-Fan Zhao). For humoral immunity, Si supply significantly reduced haemolymph protein concentrations with no effects of endophyte or immunity treatment, whereas no treatment effects were observed for phenoloxidase activity (Figure 7a; Table 4; Table S1). Melanisation response of the immune-challenged larvae was significantly reduced by both endophyte and Si supply in an additive manner, with no interaction (Figure 7b; Table 4; Table S1).

### 3.4 | Plant growth and primary chemistry

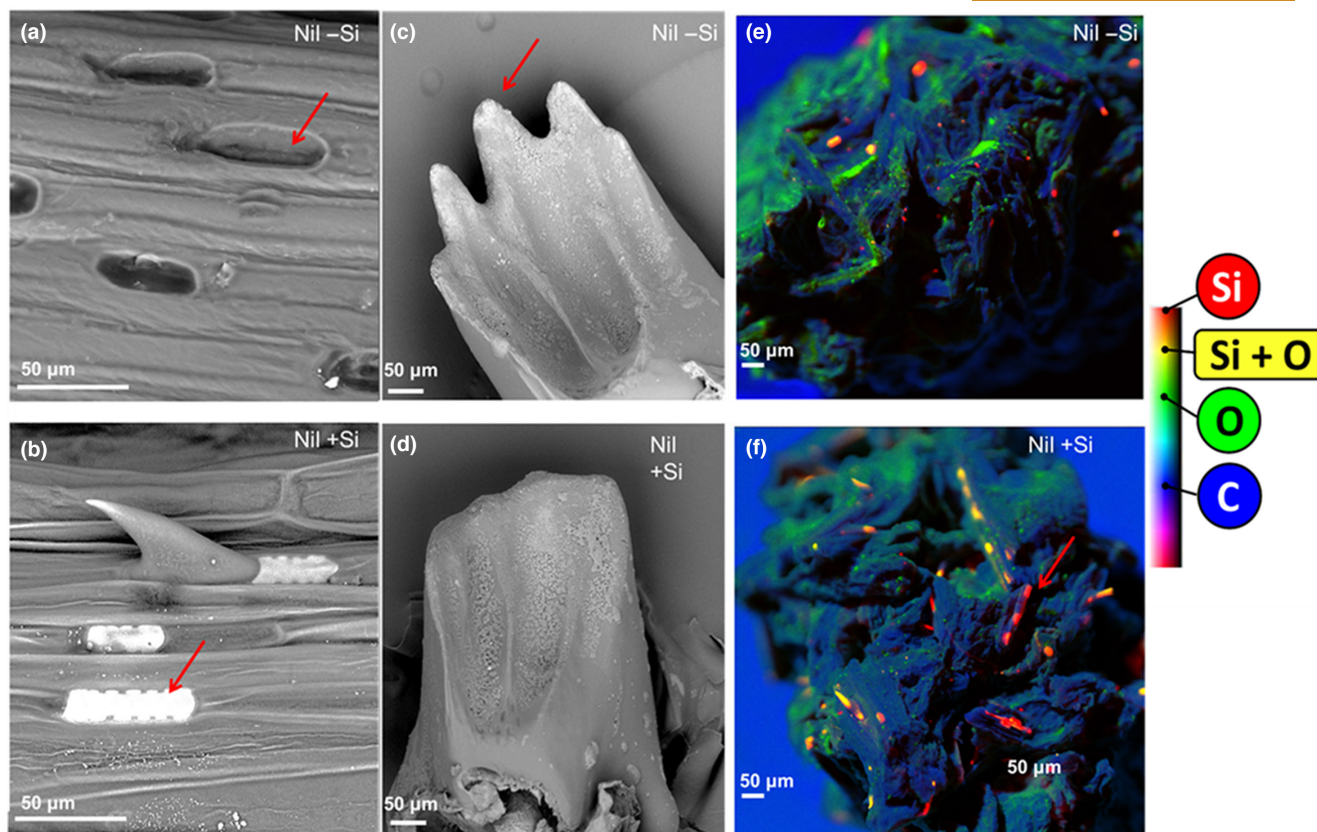
In the intact plant feeding trials (in situ assays), +Si plants exposed to herbivory displayed higher root, shoot and total biomass, compared to their non-supplemented counterparts, with no clear endophyte effects (Figure S4A-C; Table S2A). Herbivory increased plant carbon content and Si supply alone reduced shoot nitrogen, increasing the C:N ratio (Figure S4D-F; Table S2B).

## 4 | DISCUSSION

To our knowledge, this is the first study to assess the impacts of Si supply and endophytes on insect immunity, an often-neglected



**FIGURE 4** *Helicoverpa armigera* (a) relative growth rate (RGR), (b) mandibular wear (1/incisor: mandible length ratios) and (c) their correlation in the in situ (intact tissue) feeding assay with triangles representing non-endophytic (Nil) plants, circles representing endophytic (AR584) plants, and colour fill representing plants growing hydroponically in the absence (-Si: grey) and presence (+Si: black) of silicon (Si). Details are as per Figure 1; statistical analysis is given in Table 3; N = 20 per treatment.



**FIGURE 5** Backscatter electron (BSE) SEM micrographs (500 $\times$ , scale bar = 50  $\mu$ m) of the (a, b) adaxial leaf surface with red arrows indicating (a) empty or (b) filled (mineralised) Si cells, along with *Helicoverpa armigera* (c, d) mandibles (red arrows indicating incisors), and (e, f) the corresponding pseudo-coloured elemental X-ray maps of frass pellets highlighting elemental abundance of Si (red), O (green) and C (blue). Yellow regions indicate presence of both Si and O, typical of silica ( $\text{SiO}_2$ ) deposition in leaf surface (red arrows indicating Si depositions in frass). Refer to [Figures S3](#) and [S4](#) for full spectra comparisons of leaf surface and frass pellets, respectively.

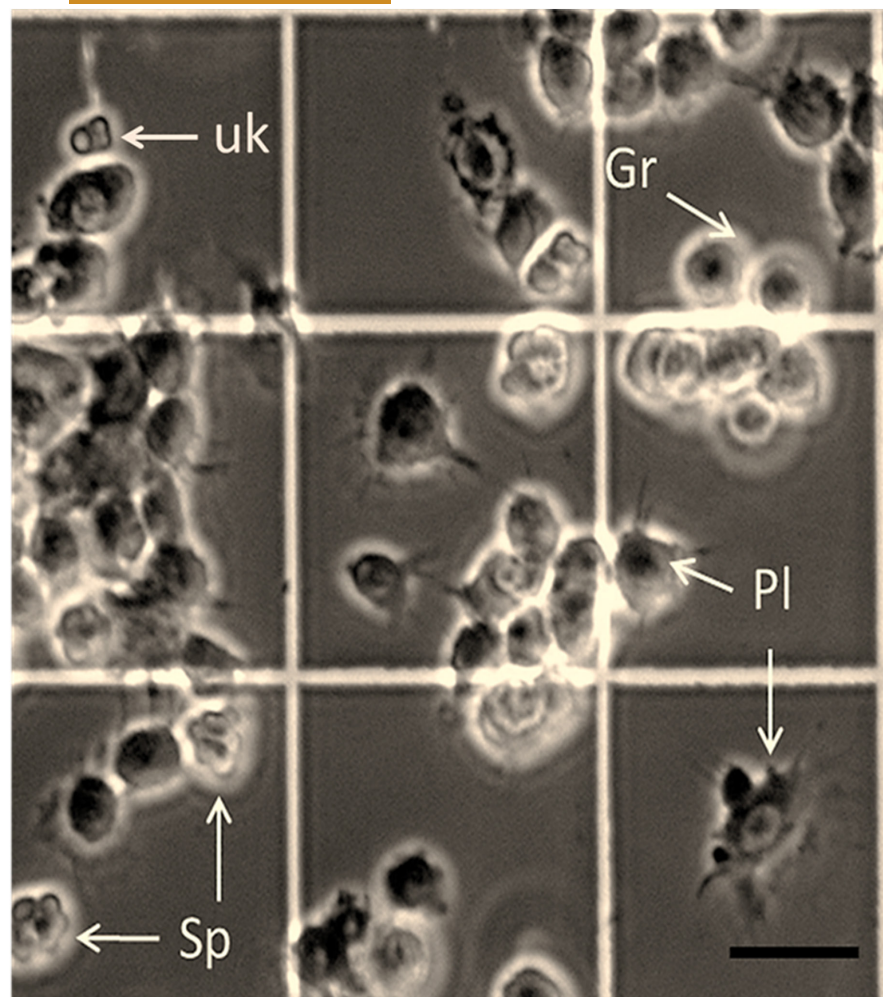
performance parameter in plant defence studies. Our results provide evidence that both Si supply and endophytes can work in a complementary way to increase resistance in a model grass species to the ubiquitous herbivorous pest *H. armigera* by reducing its immune response via reduced melanisation. This supports our last hypothesis (H4) that herbivore immunity would be decreased because of both Si supply and endophyte presence ([Figure 8R4](#)). As predicted by H2, Si supply did not interfere with the production of endophytic alkaloids ([Figure 8R2](#)), but reduced feeding efficiency in several important ways supporting H3 ([Figure 8R3](#)): this reduced feeding possibly contributed to reduced melanisation (i.e. immunity).

Finally, in contrast to our predictions in H1, foliar Si concentrations only increased by endophytes in the absence of herbivory ([Figure 8R1](#)). It is possible that herbivory induced such large increases in foliar Si that any endophyte-mediated increases in the presence of herbivory could have been masked. This supports the idea that plants might have a limit in terms of the amount of Si they can accumulate. Si defences can be induced following herbivory (Johnson, Rowe, & Hall, 2020; Massey et al., 2007) but beyond a certain level silicification of tissue might become too costly in terms of transporter energy requirements and structural demands due to

the higher density of Si compared to other plant components (i.e. lignin; Raven, 1983; Simpson et al., 2017). Although these results are from a hydroponic system, allowing us to control Si in the most effective way, we suggest our findings are likely to hold up in a more natural setting (e.g. pastures) because *H. armigera* performance was impacted similarly by Si supply in other studies, which used soil systems (Cibils-Stewart et al., 2021; Frew et al., 2019; Johnson et al., 2023).

#### 4.1 | Si supply alone reduced *H. armigera* performance and only herbivory affected endophytic alkaloid production

Si resistance was the predominant anti-herbivore mechanism operating in our system: Si supply reduced *H. armigera* growth by 87%, most feeding efficiency indices by at least 30% and increased mandibular wear by 16%. Si supply also reduced the proportion of nitrogen absorbed and the efficiency of conversion of ingested and digested food indicating that herbivores were able to assimilate less nutrients when feeding on Si rich (+Si) diets. These phenomenon was previously reported by Massey et al. (2006) and Massey and



**FIGURE 6** Haemocytes in *Helicoverpa armigera* haemocoel seen under the haemocytometer: plasmatocytes (Pl), granulocytes (Gr), spherulocytes (Sp), unknown (uk—presumably prohaemocyte). Scale: 5  $\mu$ m.

**TABLE 4** Results from multiple comparison tests for changes in *Helicoverpa armigera* humoral and cellular immunity as affected by Si-supply and *Epichloë*-endophytes in an in situ feeding assay.

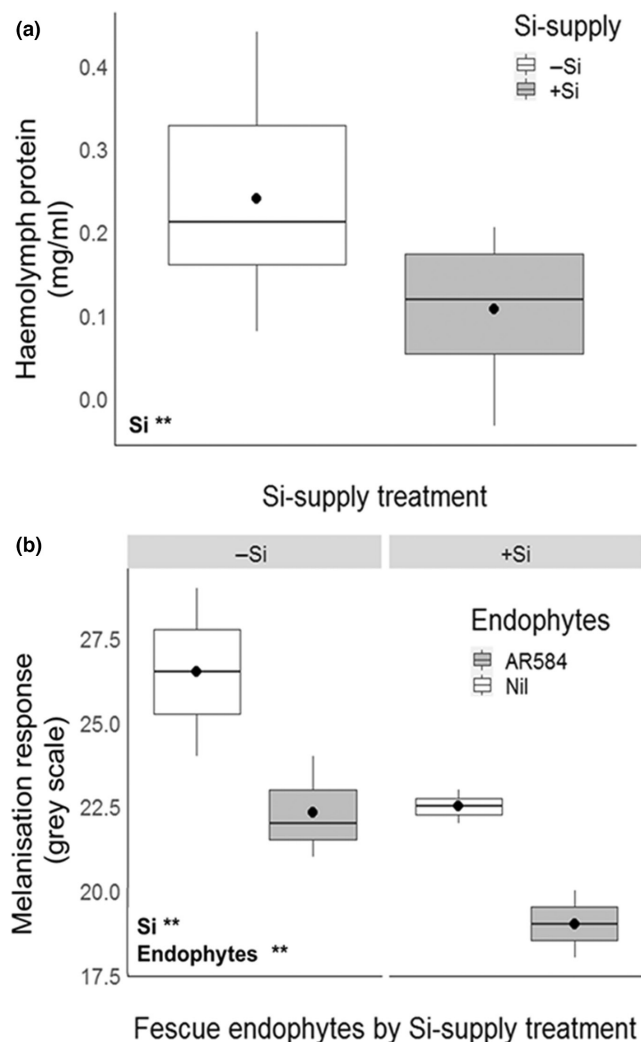
Factors	df	Humoral				Cellular						Melanisation response (Immuno-challenged treatment only)		
		PO		Protein		Gr		Pr		Sp		df	F	p
		F	p	F	p	F	p	F	p	F	p			
Si	1.44	0.86	0.371	12.19	<b>0.004</b>	0.15	0.703	2.85	0.117	2.25	0.159	1.13	16.35	<b>0.002</b>
Endophyte	1.44	0.83	0.378	1.11	0.312	0.02	0.912	0.77	0.397	11.53	<b>0.005</b>	1.13	19.90	<b>0.001</b>
Immunity	2.44	0.14	0.869	2.55	0.119	0.16	0.862	1.17	0.341	4.95	<b>0.026</b>			
Si×Endophyte	1.44	1.71	0.215	0.40	0.537	0.03	0.861	0.18	0.674	12.9	<b>0.003</b>	1.13	0.1351	0.721
Si×Immunity	2.44	1.95	0.185	2.57	0.117	2.49	0.125	1.53	0.254	1.43	<b>0.027</b>			
Endophyte×Immunity	2.44	2.72	0.106	3.81	0.052	1.03	0.383	0.05	0.994	9.21	<b>0.004</b>			
Si×Endophyte×Immunity	1.44	2.61	0.132	0.37	0.552	1.29	0.278	0.98	0.342	6.96	<b>0.022</b>			

Note: Models with significant main effects and/or interactions are noted in bold. Humoral immunity: phenoloxidase (PO) and haemolymph protein (Protein); cellular immunity: granulocytes (Gr), prohaemocytes (Pr) and spherulocytes (Sp); immunity treatments include control, sham injection and immuno-challenged. Refer to Table S1 for mean  $\pm$  standard errors of each individual treatment; Figure 7 only displays parameters that were statistically significant.

Hartley (2009) for other grasses, such as *Brachypodium pinnatum*, *Festuca ovina* and *Lolium perenne*, were they observed reductions of 84%, 68% and 51% in the efficiency by which ingested material

was converted to body mass (ECI), respectively. Si supply reduced foliar N in the current study, potentially exacerbating the reduction in proportion of nitrogen absorbed. Despite this reduced foliar





**FIGURE 7** Significant changes in *Helicoverpa armigera* (a) haemolymph protein (humoral immunity), and (b) melanisation response details as per Figure 1. Statistical analysis is given in Table S1. Refer to Table 4 for mean  $\pm$  standard errors of each individual treatment:  $N$  = at least four per treatment as some individuals were not found after the in situ assay.

quality, relative consumption was unaffected in the current study. Some studies have reported increased consumption to compensate for poor diet (Karban & Agrawal, 2002; Massey et al., 2006), but others (e.g. Moise et al., 2019) have found that some herbivores are able to maintain N assimilation efficiency on plants with Si added despite their decreased consumption levels through enhanced post-ingestion physiology. Values for approximate digestibility tend to decrease in relation to insect body size mainly due to increased feeding rate and gut size as larvae grow; thus, bigger larvae have greater food intake, but shorter retention times, making enzymatic degradation and nutrient absorption less efficient (Slansky & Scriber, 1985). In our study, the larvae fed on Si-rich (+Si) diets were small relative to those feeding on -Si diets, possibly explaining the higher approximate digestibility observed and lack of effect on consumption. This is supported by other recent studies that demonstrate that Si supply

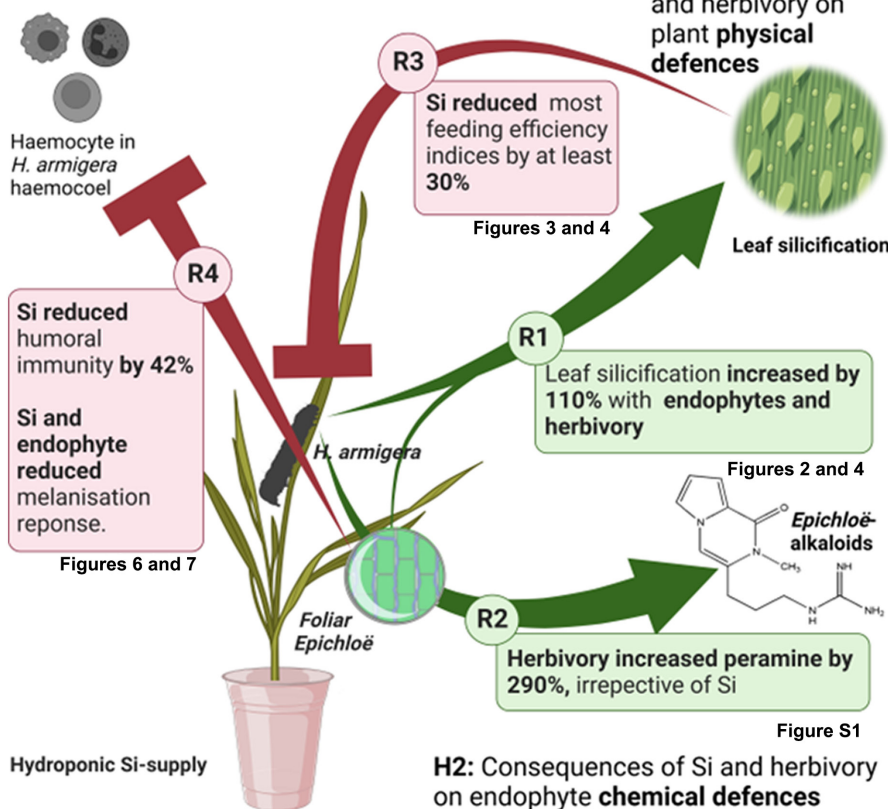
reduces relative growth rate of *H. armigera* even at the lower foliar Si concentrations found in plants as a result of water stress (Vandegheer et al., 2021), carbon dioxide increases (Biru et al., 2021), nitrogen limitation (Johnson et al., 2021) or short-term Si exposure (72-h) at the onset of herbivory (Waterman et al., 2021). Collectively, these studies indicate that the negative impact of silicification operates at a relatively low threshold, beyond which further silicification does not affect *H. armigera*.

In addition to showing increased mandible wear, we provide novel evidence that mineralised silica cells in Si supplied plants are large relative to the incisors (the cutting edge) of *H. armigera* mandibles. Additionally, phytoliths in frass appeared intact when larvae fed on Si rich diets. Andama et al. (2020) also reported fully intact silicified macro-hairs in herbivore frass, which was linked to digestive tissue laceration. This underscores the importance of Si as an effective physical defence: Si-filled cells in the leaf surface increase abrasiveness (Vandegheer et al., 2021), which can reduce nutrient extraction (including nitrogen), as well as imposing physical damage on herbivore guts directly. This conclusion is supported by the findings of Massey and Hartley (2009), although 28% of the variation in N absorbed was explained by mandible wear, this can only impact within an instar as caterpillar shed their mandibles during moulting; despite this, long-term impacts of Si on performance were observed which likely resulted from damage to the mid-gut of the larvae, a part of the digestive tract which is not shed during moulting (Chapman, 1983). This was confirmed when larvae were switched to a low Si diet and performance did not improve (Massey & Hartley, 2009). Thus, the relative impacts of mandible wear and gut damage on herbivore growth rate will vary depending on time since the last moult compared with time exposed to abrasion due to Si addition; this could provide an explanation for why in this study, even though we report a significant negative correlation between relative growth rate and mandible wear irrespective of endophytic status of the grass, the absolute change was small and the variance high. Finally, we report here that Si supply did not affect endophytic alkaloid production; however, herbivory increased peramine levels by 290%. This is consistent with Fuchs et al. (2017) who reported that peramine concentrations increased in grasses following locust (another chewing insect herbivore) but not aphid (a sap feeder) feeding. This result suggests that insect cues (e.g. oral secretions, tissue removal) that trigger plant induced defences can also trigger the production of endophytic alkaloids that are toxic for insects (Fuchs et al., 2017), suggesting that endophyte grass symbioses involves a close chemical crosstalk between the interacting partners.

#### 4.2 | The combined effect of Si supply and endophytes negatively affected insect immunity

In our study, we observed that haemolymph protein concentration decreased with Si supply. Haemolymph protein is a function of foliar protein availability (Gherlenda et al., 2016), and since Si supply reduced tissue N, it seems likely that this contributed to reduced

## Consequences of Si and endophyte on herbivore feeding (H3) and immunity (H4)



**FIGURE 8** Overall effects and interactions (result: R). H1–H3: reminder of our objectives; refer to [Figure 1](#) for full details on each experimental stage. Negative and positive impacts depicted with inhibition lines and arrows, respectively. Figure produced with Biorender (<http://biorender.com>).

concentrations of haemolymph protein. Finally, both Si supply and endophytes suppressed the melanisation response. Previous research has shown that herbivores exposed to nutrient-limited diets are less able to encapsulate parasitoid larvae than herbivores provided with ad libitum nutritious diets (Benrey & Denno, 1997; Mattson, 1980; Salt, 1964). The negative impacts of Si on foliar N concentrations and nutrient extraction by *H. armigera* possibly explain this reduced capacity for melanisation. Endophytes also affected the melanisation response but to a lesser extent. This may be the consequence of alkaloid production since they have the capacity to deter feeding or reduce the efficiency of food utilisation (post ingestion; Hennessy et al., 2016).

At the cellular level, interactions between Si supply, endophyte and our immunity stimulation increased the density of spherulocytes in pure haemolymph, while densities of plasmatocytes and granulocytes, remained unchanged. While plasmatocytes form the bulk of capsules around foreign bodies and granulocytes phagocytise foreign pathogens, spherulocytes function remains unknown for *H. armigera* (Wang et al., 2010; Zhai & Zhao, 2012). However, increased spherulocytes numbers were observed in *H. armigera* exposed to pyrethroids (known to affect the neuroendocrine system), which may be analogous to the effects of alkaloids (neurotoxins; Gujar & Kalia, 2005).

Whether the decreased immunity reported here makes insects more vulnerable to their natural enemies in natural settings is unknown. Both Si and endophytes have independently been reported

to negatively affect natural enemies of aphids; either by Si supply reducing aphid body size, thereby changing the quality of the parasitised host (Hall et al., 2021), or by endophyte-alkaloids being sequestered by aphids, which then have cascading effects on natural enemies (Fuchs & Krauss, 2018).

Understanding the mechanisms of Si- and symbiont-based defences in grasses is crucial for enhancing herbivore resistance without resorting to pesticides. Our findings suggest that silicon (Si) supplementation does not hinder, and may even enhance, the symbiotic relationships that many cool-season grasses have with *Epichloë* endophytes. Importantly, this Si-endophyte interaction did not negatively affect any of the plant growth parameters we tested, regardless of the presence or absence of herbivory, a finding also supported in greenhouse (Cibils-Stewart et al., 2021) and field settings (Johnson et al., 2023). In agricultural soils, the bioavailable form of Si (monosilicic acid;  $\text{Si}(\text{OH})_4$ ) can become depleted (Epstein, 1999), but this can be managed through the use of manufactured or naturally occurring Si fertilisers (Haynes, 2014). Unlike chemical pesticides, Si intervention is unlikely to have widespread negative impacts on other parts of the ecosystem (Jeer et al., 2021; Ma & Yamaji, 2006) but may be a particularly effective alternative to pesticides, especially against insect pests prone to developing insecticide resistance (e.g. *H. armigera*; Anderson et al., 2018). Furthermore, Si silicification is unlikely to affect the quality of livestock feed, as animals like ruminants are well-adapted to high Si diets (Johnson, Hartley, Ryalls, et al., 2020). Similarly, the use



of commercially available mammalian-safe endophyte-infected seeds (novel endophytes) offers an alternative to reduce reliance on synthetic chemicals (Gundel et al., 2013).

Hydroponic studies indicate that supplying Si to plants results in a rapid (<72h) build-up of Si-based defences against herbivores (Waterman et al., 2021). If this rapid response is similar in field conditions, Si application could be used as a reactive measure during pest outbreaks, but further experimentation in real-world field conditions, including with different insect populations, is necessary to validate this conclusion. There are some studies, however, that point to this induction of Si defences in the field, as similar levels of induction were reported for *Deschampsia caespitosa* in the field (Hartley & DeGabriel, 2016) and induction has also been demonstrated at a landscape scale in an ecological study (Ruffino et al., 2018). Finally, using the same tall fescue cultivar by endophyte combination, Johnson et al. (2023) examined the effects of elevated atmospheric CO<sub>2</sub>, silicon supplementation, the presence of endophytes and insect herbivory on various aspects of plant growth and physiology using open-top chambers and provided evidence that *H. armigera* herbivory increased Si accumulation by 24%, with endophytes having neutral effects.

## 5 | CONCLUSIONS

The present study shows that Si and endophytic-alkaloid defences may be a highly complementary and effective defence strategy. Si supply did not discernibly impact endophytic-alkaloids, which are effective defences against piercing-sucking aphids (Bastías et al., 2017), a feeding guild which is generally less impacted by Si defences (Hartley & DeGabriel, 2016; Johnson, Rowe, & Hall, 2020). In short, possessing both Si and endophyte-derived defences may confer resistance to a broader range of insect herbivores. Moreover, combining physical and chemical defences may be a particularly difficult obstacle for herbivores to adapt to: each presents different selective pressures on herbivores. The fact that multiple fitness traits, including feeding efficiency, physiological performance, physical injury and immunity are affected by Si-endophyte defences potentially makes adaptation even more difficult. There is emerging evidence that diversified plant defences (Pearse et al., 2018; Thiel et al., 2020) are an important, but largely overlooked, factor that reduces herbivore performance. We suggest that the combination of Si and endophyte-derived defences represents a multi-faceted defence strategy for temperate grasses.

## 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. R. Putra, T. Islam and X. Cibils-Stewart performed the immunity assay. R. Wührer, D. J. Fanna and X. Cibils-Stewart performed SEM work. X. Cibils-Stewart and W. J. Mace performed the chemical analysis (alkaloids). X. Cibils-Stewart analysed the data with input from S. N. Johnson. 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.

## DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.0cfxpnw83> Cibils-Stewart et al. (2023).

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## SUPPORTING INFORMATION

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

**Appendix S1.** Methods.

**Appendix S2.** Tables and figures.

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