




## RESEARCH ARTICLE

# Elevated atmospheric CO<sub>2</sub> suppresses silicon accumulation and exacerbates endophyte reductions in plant phosphorus

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## Funding information

Australian Research Council, Grant/Award Number: FT170100342

Handling Editor: Julia Cooke

## Abstract

1. Many temperate grasses are both hyper-accumulators of silicon (Si) and hosts of *Epichloë* fungal endophytes, functional traits which may alleviate environmental stresses such as herbivore attack. Si accumulation and endophyte infection may operate synergistically, but this has not been tested in a field setting, nor in the context of changing environmental conditions. Predicted increases in atmospheric CO<sub>2</sub> concentrations can affect both Si accumulation and endophyte function, but these have not been studied in combination.
2. We investigated how elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>), Si supplementation, endophyte-presence and insect herbivory impacted plant growth, stoichiometry (C, N, P and Si), leaf gas exchange (rates of photosynthesis, stomatal conductance, transpiration rates) and endophyte production of anti-herbivore defences (alkaloids) of an important pasture grass (tall fescue; *Lolium arundinaceum*) in the field.
3. eCO<sub>2</sub> and Si supplementation increased shoot biomass (+52% and +31%, respectively), whereas herbivory reduced shoot biomass by at least 35% and induced Si accumulation by 24%. Shoot Si concentrations, in contrast, decreased by 17%–21% under eCO<sub>2</sub>. Si supplementation and herbivory reduced shoot C concentrations. eCO<sub>2</sub> reduced shoot N concentrations which led to increased shoot C:N ratios. Overall, shoot P concentrations were 26% lower in endophytic plants compared to non-endophytic plants, potentially due to decreased mass flow (i.e. observed reductions in stomatal conductance and transpiration). Alkaloid production was not discernibly affected by any experimental treatment. The negative impacts of endophytes on P uptake were particularly strong under eCO<sub>2</sub>.
4. We show that eCO<sub>2</sub> and insect herbivory reduce and promote Si accumulation, respectively, incorporating some field conditions for the first time. This indicates that these drivers operate in a more realistic ecological context than previously demonstrated. Reduced uptake of P in endophytic plants may adversely affect plant productivity in the future, particularly if increased demand for P due to improved plant growth under eCO<sub>2</sub> cannot be met.

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## KEYWORDS

alkaloids, atmospheric carbon dioxide, carbon, climate change, *Epichloë* endophyte, herbivory, phosphorus, silica, silicon

## 1 | INTRODUCTION

Grassland ecosystems account for 31%–43% of the Earth's terrestrial habitats, occurring on every continent except Antarctica, and store one third of global carbon stocks (Gibson & Newman, 2019; Schimel, 1995). Given the importance of these ecosystems, there is a crucial need to understand how these systems may respond to climate and atmospheric change, including unprecedented rises in atmospheric carbon dioxide (CO<sub>2</sub>; Gibson & Newman, 2019). Research from the past few decades has shed light on many functional responses of grasses to elevated CO<sub>2</sub> (eCO<sub>2</sub>; Gibson & Newman, 2019). For example, growth and photosynthesis generally increase in response to eCO<sub>2</sub> with carbon (C) concentrations often increasing at an accelerated rate relative to nitrogen (N) concentrations (Ainsworth & Rogers, 2007; Long et al., 2004; Wand et al., 1999). This can result in an increase in C:N ratios, with global averages ranging from 11% (Luo et al., 2006), 19% (Robinson et al., 2012) to 27% (Stilling & Cornelissen, 2007). eCO<sub>2</sub> also has the capacity to increase demand for other nutrients, such as phosphorus (P) via increased plant growth which could lead to declines in plant P where soil P is low or deficient (Jin et al., 2015).

Many grass species are hyper-accumulators of silicon (Si), up to 10% of their dry mass, more than any other inorganic constituent (Cooke & Leishman, 2011; Epstein, 1999). Si accumulation is now recognised as playing an important functional role in plant biology, particularly in terms of alleviating the adverse effects of biotic (e.g. herbivory) or abiotic (e.g. drought) environmental stresses (Debona et al., 2017). Herbivory, for example, is widely reported to rapidly increase Si uptake as a form of induced defence (Massey et al., 2007; Waterman et al., 2021). There is emerging evidence that eCO<sub>2</sub> reduces Si accumulation in many grass species due, in part, to concurrent increases in C concentrations (Biru et al., 2021; Johnson et al., 2022; Johnson & Hartley, 2018). The negative relationship between C and Si in plants may be due to “stoichiometric dilution” whereby an increase in one element, by definition, necessitates lower levels of other constituents (Hodson & Guppy, 2022; Quigley et al., 2020). Alternatively, there could be a trade-off between Si and C since Si may be used as a metabolically cheaper substitute for C constituents such as cellulose and phenolic defences (Hodson & Guppy, 2022; Raven, 1983), although this depends on environmental conditions (e.g. Si availability) and benefits may be reduced or negated when other costs of silicification are considered (de Tombeur et al., 2022). With greater C availability under eCO<sub>2</sub>, this may be less advantageous and plants may, for example, produce more C-based phenolic defences rather than accumulate Si (e.g. Johnson et al., 2022).

Many cool-season C<sub>3</sub> grasses, including wild and domesticated species, associate asymptotically with *Epichloë* fungal

(Ascomycota: Clavicipitaceae) endophytes (Kauppinen et al., 2016; Leuchtman, 1992). *Epichloë* endophytes are obligate symbionts which occupy intercellular (apoplastic) spaces in shoots of their host grasses (Christensen et al., 2008; Leuchtman, 1992). Analogous to Si accumulation, associating with *Epichloë* endophyte may play a functional role for alleviating environmental stresses (Gundel et al., 2013), including herbivore resistance via production of defensive alkaloids (Bastías et al., 2017). Beneficial effects of endophytic association are widely reported (Kuldau & Bacon, 2008 and references therein), and strictly speaking vertically transmitted endophytes must be mutualistic (Newman et al., 2022). Nonetheless, some studies report antagonistic impacts on plants, particularly when resources are limited (Cheplick, 2007; Cheplick et al., 1989; Saikkonen et al., 2016).

Si accumulation and *Epichloë* endophytes have the capacity to impact each other, although this has been studied in just three grass species. In a field study, Huitu et al. (2014) reported that meadow fescue (*Festuca pratensis*) colonised with *Epichloë uncinata* contained 16% more Si compared to non-endophytic plants. In glasshouse studies, both AR584 and common-toxic (referred to as wild-type) endophyte strains of *Epichloë coenophiala* increased Si accumulation in tall fescue *Lolium arundinaceum* whereas only one strain (AR37) of *Epichloë festucae* var. *lolii* promoted Si accumulation in perennial ryegrass *Lolium perenne*; the other strains (AR1 and common-toxic) did not affect Si accumulation (Cibils-Stewart et al., 2020, 2022). Si supplementation generally did not affect endophyte production of alkaloids, except in the presence of insect herbivores when endophytic plants contained lower concentrations of some herbivore-induced alkaloids (Cibils-Stewart et al., 2022).

While receiving very limited attention, some studies have addressed the role of eCO<sub>2</sub> on *Epichloë* endophyte–grass functional responses (Chen et al., 2017; Hunt et al., 2005; Newman et al., 2003), including reductions in some, but not all, endophyte produced alkaloids (Brosi et al., 2011; Ryan et al., 2014). We do not yet understand the role of Si availability or the impacts of herbivory on endophyte–grass interactions under eCO<sub>2</sub>, however, even though these may be important moderating factors. The objective of this study was therefore to investigate how eCO<sub>2</sub>, endophyte-presence, Si supplementation and herbivory, acting alone and in combination, impacted plant growth, leaf stoichiometry (C, N, P and Si), leaf gas exchange (rates of photosynthesis, stomatal conductance, transpiration rates) and the production of endophyte-derived alkaloids. In addition to addressing how these complex interactions are affected by eCO<sub>2</sub> for the first time, we also aimed to establish whether predicted impacts of eCO<sub>2</sub> on suppression of Si are seen in more realistic field conditions. Previous reports showing eCO<sub>2</sub> suppression of Si accumulation come exclusively from controlled glasshouse or growth chamber experiments,

which do not incorporate stochastic climate variation (Lindroth & Raffa, 2016). Our main hypotheses are that:

1. eCO<sub>2</sub> promotes photosynthesis and plant growth, increasing shoot C concentrations while reducing shoot concentrations of N (increasing the shoot C:N ratio) and Si.
2. *Epichloë* endophytes and herbivores increase Si accumulation, which ameliorates the negative impacts of eCO<sub>2</sub> on Si accumulation.
3. Production of endophyte-derived alkaloids decreases under eCO<sub>2</sub> and with Si supplementation, but the latter only occurs when plants are under herbivore attack.

## 2 | MATERIALS AND METHODS

### 2.1 | Open top chambers

Ten cylindrical Open Top Chambers (OTCs) (2.1 m<sup>3</sup>, 1.5 m diameter, 1.2 m height), located in grassland within the Hawkesbury campus of Western Sydney University (150°44'28" E, 33°36'41" S) were constructed using six supporting posts and transparent corrugated plastic sheeting (Figure 1a). Five OTCs were maintained at ambient CO<sub>2</sub> (aCO<sub>2</sub>) levels; the remaining five were assigned to elevated CO<sub>2</sub> (eCO<sub>2</sub>) (Figure 1b). Each of the eCO<sub>2</sub> OTCs was supplied with CO<sub>2</sub> via underground tubing from a central bank of CO<sub>2</sub> cylinders. CO<sub>2</sub> concentration was manipulated by mixing a slow bleed of CO<sub>2</sub> into the stream of a 2 m<sup>3</sup>/min air delivered into chambers with fans and distributed throughout the chamber via circular tubing (6 cm diameter) at the base of the OTC. CO<sub>2</sub> was turned on during daylight hours (06:00–20:00 AEST) when plants were photosynthesising (and

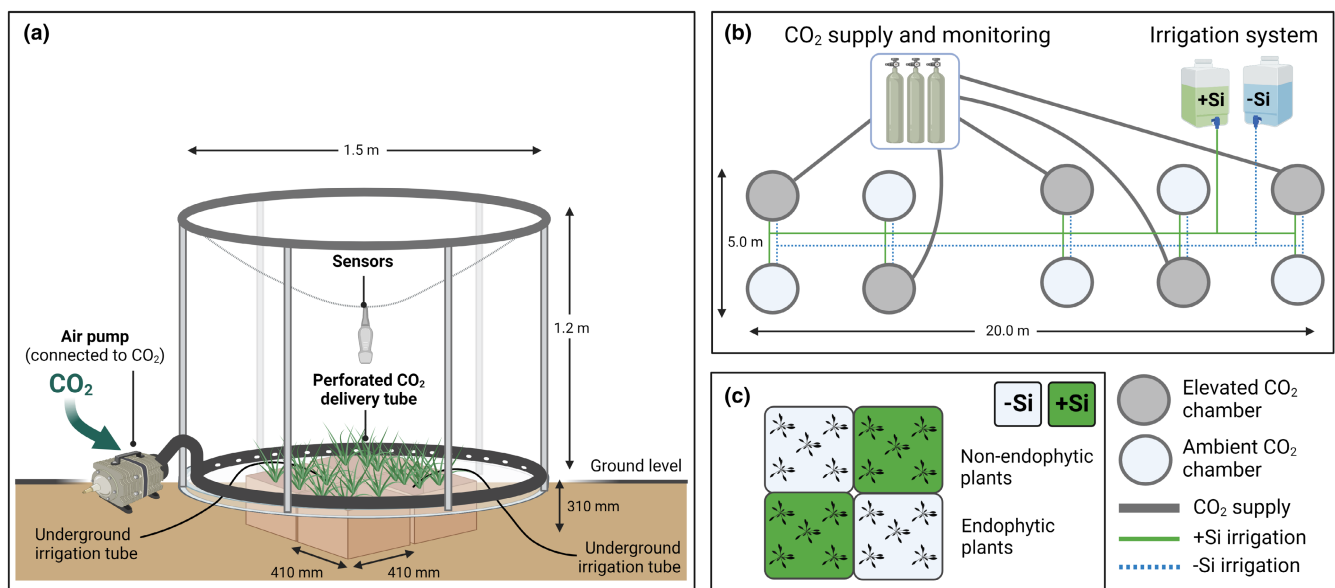
absorbing CO<sub>2</sub>). The aCO<sub>2</sub> OTCs had the same arrangement except that ambient air was pumped through the tube to ensure the same air flow as the eCO<sub>2</sub> OTCs.

CO<sub>2</sub> concentrations, air temperature and relative humidity within the OTCs were monitored using Dwyer CDTR sensors (Dwyer Instruments Pty Ltd) mounted in radiation shields hanging 30 cm from the ground and recorded every minute by a central CR1000 logger. Ambient CO<sub>2</sub> was recorded at the site using a Vaisala GMP343 CO<sub>2</sub> probe (IRGAS) at a height of 1.2 m. We applied eCO<sub>2</sub> concentration that was representative of the concentration expected in 2100 under the RCP6.0 scenario outlined by the IPCC, 2014. Ambient air temperature and RH outside the OTCs were monitored using a Dwyer RHP-203B sensor mounted in a ventilated radiation shield.

The concentration of CO<sub>2</sub> within the elevated chambers was relatively stable at 186 ± 5.65 ppm (mean standard ± standard error) above the ambient CO<sub>2</sub> concentration. Temperatures inside the chambers were between 0 and 3°C higher (1.63 ± 0.84°C; mean standard ± deviation) than the outside temperature, depending on time of day and cloud cover. The average diurnal (06:00–20:00) relative humidity was 53 ± 15%.

### 2.2 | Experimental design

The experiment was conducted September 1–December 16, 2020 during which there was an average air temperature of 19.0°C, with a minimum of 3.6°C and a maximum of 40.5°C, a daily average rainfall of 2.12 mm and cumulative rainfall of 226.6 mm. Relative humidity averaged at 69.6%. Four pots (410 × 410 mm and 310 mm deep) were embedded in an excavated trench (c. 850 × 850 mm and 400 mm deep) at the centre of each OTC (Figure 1a,c) replicating the design



**FIGURE 1** Schematic showing (a) main features of an OTC, (b) configuration of OTCs in the field in relation to CO<sub>2</sub> supply and irrigation system and (c) an example of the 2 × 2 factorial combination of Si supply and endophyte status in each OTC. The specific arrangement of pots within each OTC was randomised.

described by Johnson et al. (2016). Each pot was filled with c. 35 kg of air-dried soil that was low in bioavailable Si and total P (full details in Table S1), which had a field capacity of 13% soil water content (SWC). Each pot was fitted with an on line pressure-compensated "spider dripper" (PC High CNL Dripper, Netafim) comprising four spikes equidistantly embedded into the soil (Figure 1b). Each dripper was fed from underground tubes connected to one of two 200 L tanks that delivered Si supplemented (+Si) and non-supplemented (-Si) irrigation; two of the pots in each OTC received +Si irrigation with the other two receiving -Si irrigation, all assigned at random (Figure 1c). Irrigation was delivered at the rate of 9.3 L min<sup>-1</sup> via water pumps (Model-FL-43, Escaping Outdoors) operating at 20 psi connected to each tank. We confirmed irrigation was uniformly distributed to all the drippers. The +Si irrigation incorporated 2 mM potassium silicate (K<sub>2</sub>SiO<sub>3</sub>; Agsil32, PQ Australia), whereas controls (-Si) were watered with a solution containing KCl to balance the addition of potassium in the +Si treatment. Solutions were adjusted to pH 7 using HCl following Hall et al. (2020). Pots were maintained at 80% field capacity (c. 10%–11% soil water content); SWC was measured three times per week using a handheld theta probe (Delta T Devices, Cambridge, United Kingdom [UK]) to calculate necessary irrigation levels.

## 2.3 | Experimental procedure

Seeds from two accessions of tall fescue, *Lolium arundinaceum* (Schreb.) (syn., *Festuca arundinacea* syn., *Schedonorus arundinaceus* syn., *Schedonorus phoenix*) (cv. INIA Fortuna), one possessing the novel AR584 *Epichloë coenophiala* strain and one that was naturally *Epichloë*-free (Nil), were surface sterilised and planted in 8 cm coir bags filled with seed raising potting mix (Scott's Osmocote Seed & Cutting Premium Potting Mix, Bunnings Australia). AR584, sold as MaxQ II in the USA, is an animal safe endophyte strain that produces peramine (conferring resistance to some chewing insects) and lolines (conferring resistance to piercing-sucking and chewing insects) in the absence of any ergovaline production (a potent vascular constrictor and causative agent for fescue toxicosis; Young et al., 2014). Plants were maintained in the glasshouse 22–24°C, 60% relative humidity, until establishment then relocated outdoors for hardening 48 hours prior to transplanting to field pots. Five plants were transplanted into each of the four pots within each chamber (Figure 1c). Plants were transplanted at the beginning of September 2020 and maintained for a further 5 weeks, after which 1 L of aquasol fertiliser (45 g per 4.5 L) was applied to each pot. When plants were 2 months old, leaf gas exchange measurements (see below) were taken on one plant per pot at weekly intervals for 4 weeks.

When plants were 3 months old, three plants in each pot were selected at random and individually surrounded with transparent Perspex cylindrical sheaths (65 mm diameter, 305 mm tall). These sheaths were embedded into the soil to the depth of 20 mm, which was sufficient to prevent insect escape without damaging the roots. Sheaths had three mesh apertures (45 mm diameter): two

placed either side of the cylinder and one on the lid (see Figure S1 in Johnson et al., 2020). Two of the plants in each pot were subjected to herbivory by placing one third instar *Helicoverpa armigera* larva inside the sheath, whereas the third would remain herbivore-free. Insects were supplied by CSIRO Agriculture & Food, Narrabri, Australia and reared at 20°C 15:9 h photoperiod (Light:Dark) according to Johnson et al. (2020). After 1 week of feeding, the three caged plants (two with herbivores, one herbivore-free) from each pot were removed from the soil and cleaned. *Epichloë*-status (Nil or AR584-infected) of each individual plant was confirmed immediately after harvest following Cibils-Stewart et al. (2021), which was derived from the method described by Simpson et al. (2012). Both immunoblotting results (nitrocellulose membrane) and further confirmation through histological staining (aniline blue) of an epidermal strip from the outermost leaf sheath from 20% of the plants was used to corroborate the presence or absence of characteristic *Epichloë* hyphae in intercellular spaces (Simpson et al., 2012). Immunoblotting results and histological tissue confirmed the attributed endophytic status of plants was accurate. Shoot tissue was oven dried, weighed and ground into a fine powder for elemental and alkaloid analysis (see below). Roots from herbivore-free plants were thoroughly cleaned with water, dried, and weighed to determine if CO<sub>2</sub>, Si supplementation or endophytic status affected root biomass. The remaining two plants in each pot were discarded.

## 2.4 | Leaf gas exchange measurements

Net photosynthetic rate (A), stomatal conductance (g<sub>s</sub>), and transpiration rate (E) were recorded on flag leaves using a portable infra-red gas analyser (LI-6400XT, Li-COR). One plant, selected at random, from each of the 40 pots was selected for gas exchange measurements using the first and second fully expanded leaves. Measurements were taken around midday (10:00 am to 2:00 pm). The RH of the reference air was fixed at 65%–70%. The CO<sub>2</sub> concentration of the reference air entering the leaf chamber was adjusted with a CO<sub>2</sub> mixer control unit keeping the CO<sub>2</sub> concentration of the reference air at approximately 400 ppm with a constant flow rate of 500 μmol s<sup>-1</sup> and a light-saturating photon flux density at 1500 μmol m<sup>-2</sup> s<sup>-1</sup> supplied by blue and red light-emitting diodes. Instantaneous leaf-level water use efficiency (WUE<sub>i</sub>) was calculated (A/E). Measurements were taken on four occasions (3–4, 10–11, 17–19 and 24–25 November 2020).

## 2.5 | Elemental analysis

Based on the method described by Reidinger et al. (2012), shoot Si and P concentrations were measured using c. 100 mg of dry ground tissue using X-ray fluorescence spectrometry (Epsilon 3x; Malvern Panalytical). Samples were calibrated against a certified control (NCS ZC73018, China National Institute for Iron and Steel; Hiltbold et al., 2017). Carbon and nitrogen concentrations

were measured on a sub-sample using c. 6–7 mg of ground tissue using an elemental analyser (FLASH EA 1112 Series CHN analyser, ThermoFinnigan).

## 2.6 | Alkaloid analysis

Alkaloid concentration of foliar tissue from both endophytic and non-endophytic plants was determined following Cibils-Stewart et al (2021) using a modification of the method reported in Bastías et al. (2019). Only the loline-derivatives (N-acetyllooline, NAL; N-acetylornoline, NANL, and N-formyllooline, NFL) and peramine were quantified since these are the only known endophyte alkaloids reported to be produced by the AR584 strain. For the analysis of the loline-derivatives, sub-samples (20 mg) of freeze-dried and ground herbage were extracted in the dark for 1 h using 40% methanol/5% ammonia (50  $\mu$ L) and 1,2-dichloroethane (500  $\mu$ L, containing 51.5 ng/mL 4-phenylmorpholine as an internal standard). After filtering through 10  $\mu$ m pore filters, extracts were analysed on a gas chromatography-flame ionisation detector (GC2010Plus, Shimadzu Corporation) equipped with a TG-5MS Amine capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m film; Thermo Fisher Scientific Inc.). The detection limit using this technique is 25  $\mu$ g/g dry mass (DM).

Sub-samples (20  $\mu$ g) of the same freeze-dried herbage were analysed for peramine after extraction with 80% methanol (500  $\mu$ L, containing 1.7  $\mu$ g/mL homoperamine as internal standard) for 1 h in the dark by over-over mixing at 30 rotations per minute. The filtered (0.45  $\mu$ m) supernatant was analysed on a LTQxl linear ion-trap mass spectrometer (Thermo Fisher Scientific Inc.) using the parameters described in Rasmussen et al. (2012). Limit of quantitation was 0.1  $\mu$ g/g DM.

## 2.7 | Statistical analysis

Plant biomass and elemental chemistry were analysed with four-way analysis of variance (ANOVA) that included CO<sub>2</sub>, Si supplementation, endophyte status and herbivore presence as fixed factors, with interactions between all factors. Root biomass and leaf gas exchange measurements were analysed with three-way ANOVAs with herbivore presence removed from the analysis as herbivore-treated plants were not included in these measurements. Similarly, alkaloid concentrations were analysed with three-way ANOVAs (CO<sub>2</sub>, Si supplementation and herbivore presence) as no alkaloids were detected in the endophyte-free plants. Chamber number was included as a “block term” in all analyses to avoid pseudo replication of CO<sub>2</sub> treatments within the analysis. For leaf gas exchange measurements, which included repeated measurements, week number was included as a ‘block term’ in interaction with chamber number. Pearson’s correlation tests were conducted to explore relationships between elemental chemistry. All responses produced residual diagnostic plots that fitted a normal distribution and showed low heteroscedasticity. Analyses were conducted in Genstat (version 18, VSN International).

## 3 | RESULTS

### 3.1 | Biomass and elemental chemistry

Averaged across treatments, shoot biomass increased by 52% when plants were grown under eCO<sub>2</sub> and by 31% with Si supplementation (Figure 2; Table 1). In contrast, herbivory reduced shoot biomass by 43% in –Si plants and 35% in +Si plants (Figure 2). The relative similarity in these decreases, and the lack of statistical significance for the Si  $\times$  Herbivory interaction (Table 1), indicates that Si supplementation did not substantively deter feeding. Endophytes had no impact on shoot mass (Table 1) and were therefore not distinguished in Figure 2 (or Figures 3 and 4). Root mass was similarly unaffected by CO<sub>2</sub>, Si supplementation or endophytes (Table 1).

When averaged across herbivore treatments, eCO<sub>2</sub> suppressed shoot Si concentrations in –Si plants by 17% and by 21% in +Si plants (Figure 3; Table 1). As might be anticipated, Si supplementation caused the biggest increase in Si concentrations (Table 1), but herbivory also caused increases (+24%) in Si concentrations (Figure 3; Table 1) indicative of herbivore-induced Si defence. There was weak interactive effect for CO<sub>2</sub>  $\times$  Si  $\times$  Herbivory ( $F_{1,94} = 4.11, p = 0.046$ ) whereby the negative effects of eCO<sub>2</sub> on Si concentrations were less pronounced in non-endophytic +Si plants (–4%), compared with endophytic +Si plants (–28%) or endophytic or non-endophytic –Si plants (–26% and 16%, respectively). Endophytic status had no discernible impact on Si concentrations (Table 1).

Contrary to our hypothesis, shoot C concentrations were not discernibly affected by eCO<sub>2</sub> (Figure 4a), although the negative impact of eCO<sub>2</sub> on shoot N concentrations (Figure 4b) and subsequent increase in shoot C:N ratios (Figure 4c) were consistent with our

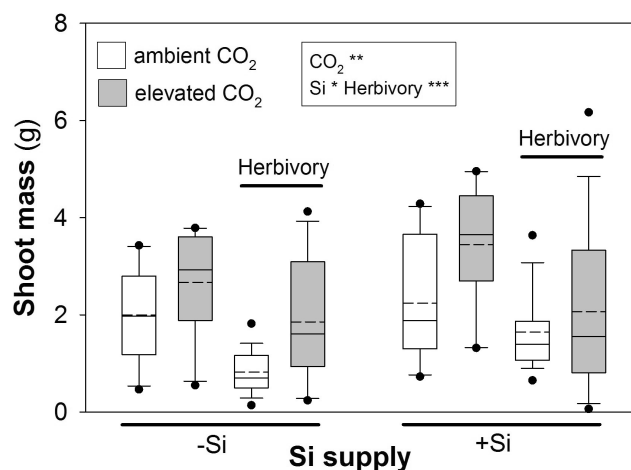
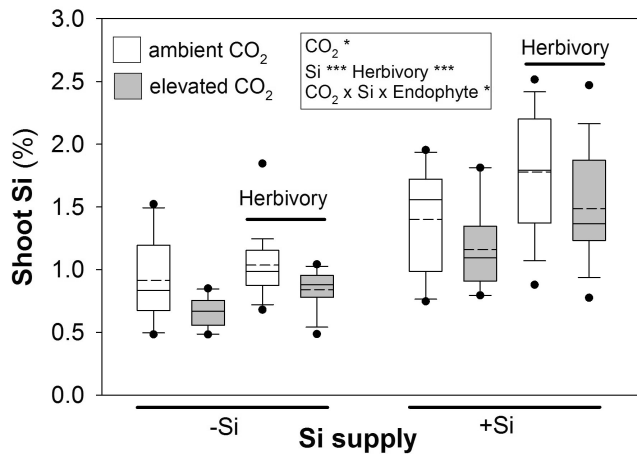


FIGURE 2 Shoot mass of *L. arundinaceum* grown under ambient and elevated CO<sub>2</sub>, with (+Si) and without (–Si) Si supplementation, and either subjected to insect herbivory or maintained without herbivores. Dashed lines represent mean values; solid lines depict the inclusive median.  $p$  values  $\leq 0.05$  summarised in the inset box (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ) with full test results given in Table 1. For clarity, and because endophyte status did not have a discernible impact, endophytic and non-endophytic plants are not distinguished.

**TABLE 1** Results of statistical analysis (Fisher values given with statistical significance indicated) for plant biomass, shoot chemistry and leaf gas exchange measurements as affected by CO<sub>2</sub>, Si supply, endophyte status and herbivore presence (when included as factorial treatment) and their interactive impacts. Factors with *p* values <0.05, \*\**p* <0.01 and \*\*\**p* <0.001 in bold.

Factors	F values										
	Shoot mass			Shoot chemistry			Leaf gas exchange measurements				
	Shoot mass	Root mass	Si	C	N	CN	P	A	g <sub>s</sub>	E	WUE <sub>t</sub>
Corresponding figure	2	—	3	4A	4B	4C	5	6A	6B	6C	—
<i>df</i>	1,96	1,24	1,94	1,93	1,94	1,93	1,95	1,114	1,114	1,114	1,114
CO <sub>2</sub> ( <i>df</i> =1,8)	14.06**	2.89	5.94*	0.52	10.65*	15.14**	0.01	1.18	0.370	0.01	0.05
Si	5.82*	0.63	106.40***	58.27***	2.05	3.60	0.56	0.27	0.735	0.01	0.01
Endophyte	0.01	1.12	0.11	1.05	1.93	2.90	18.72***	7.06**	6.01*	7.73**	1.48
Herbivory	19.28***	—	15.38***	7.36**	0.14	0.29	0.05	—	—	—	—
CO <sub>2</sub> × Si	0.28	0.87	0.38	0.09	0.84	2.33	0.27	0.69	0.36	0.01	0.36
CO <sub>2</sub> × Endophyte	0.19	0.01	1.16	0.65	1.70	1.38	6.62*	0.01	0.85	0.06	0.71
Si × Endophyte	1.94	0.02	0.03	0.04	0.39	0.05	2.07	0.35	1.48	2.70	2.59
CO <sub>2</sub> × Herbivory	0.23	—	0.01	1.29	0.19	0.04	0.30	—	—	—	—
Si × Herbivory	0.01	—	2.63	0.55	0.21	0.07	1.86	—	—	—	—
Endophyte × Herbivory	0.64	—	0.01	1.50	0.26	0.31	1.78	—	—	—	—
CO <sub>2</sub> × Si × Endophyte	1.05	0.17	4.11*	0.75	0.54	1.12	0.85	0.01	0.83	0.88	3.44
CO <sub>2</sub> × Si × Herbivory	1.63	—	0.15	1.61	0.09	0.44	0.33	—	—	—	—
CO <sub>2</sub> × Endophyte × Herbivory	0.04	—	0.32	0.72	0.31	0.01	2.56	—	—	—	—
Si × Endophyte × Herbivory	0.21	—	0.19	1.43	0.01	0.01	0.01	—	—	—	—
CO <sub>2</sub> × Si × Endophyte × Herbivory	0.25	—	0.16	0.01	1.14	1.14	1.20	—	—	—	—

hypothesis. Si supplementation resulted in consistent declines in C concentrations; herbivory also caused decreases in C concentrations (Figure 4a; Table 1). There was a strong negative correlation between



**FIGURE 3** Shoot Si concentration of *L. arundinaceum* grown under ambient and elevated CO<sub>2</sub>, with (+Si) and without (-Si) Si supplementation, and either subjected to insect herbivory or maintained without herbivores. Dashed lines represent mean values; solid lines depict the inclusive median. *p* values ≤0.05 summarised in the inset box (\**p* < 0.05 and \*\*\**p* < 0.001) with full test results given in Table 1. For clarity, and because endophyte status did not have a discernible impact, endophytic and non-endophytic plants are not distinguished.

shoot Si and shoot C concentrations (Figure 4d). Endophytes did not discernibly affect shoot concentrations of C or N (Table 1).

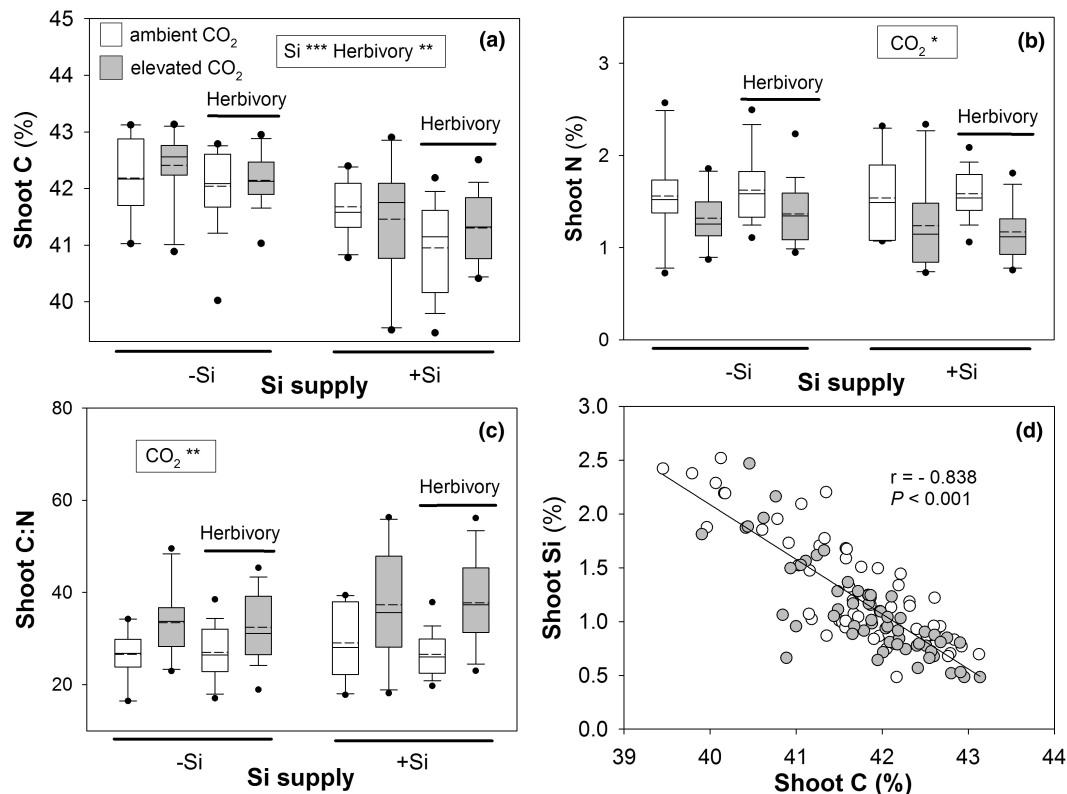
While endophytes had no discernible impact on plant biomass or concentrations of Si, C or N, they had a negative impact on shoot P concentrations, which decreased by 26% when averaged across all treatments (Figure 5; Table 1). There was interaction between CO<sub>2</sub> and endophyte status (Table 1) reflecting that this effect was seen to a greater extent when endophytic plants when grown under eCO<sub>2</sub> (Figure 5).

### 3.2 | Leaf gas exchange measurements

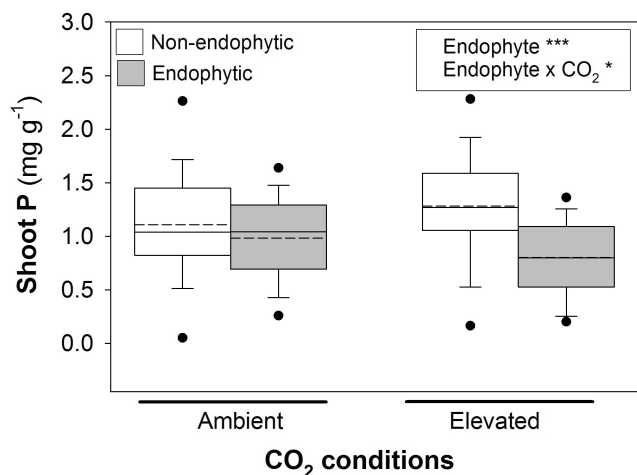
Photosynthesis, stomatal conductance, transpiration rates and WUE were unaffected by CO<sub>2</sub> or Si supplementation (Table 1), whereas endophytic plants had lower rates of photosynthesis (Figure 6a), stomatal conductance (Figure 6b) and transpiration (Figure 6c). These decreases become more pronounced in the last 2 weeks of the experiment, being up to 11%, 17% and 20%, respectively, lower in endophytic plants compared to non-endophytic plants. Endophytes did not have a clear impact on WUE (Table 1).

### 3.3 | Alkaloids

No alkaloids were found in non-endophytic plants; endophyte status was therefore not included in the statistical analysis. Total



**FIGURE 4** Shoot (a) C, (b) N concentrations and (c) shoot C:N ratio (details as for Figures 2 and 3). (d) The negative correlation between shoot Si and C concentrations for plants grown under ambient (white circles) and elevated (grey circles) CO<sub>2</sub> concentrations. *p* values ≤0.05 summarised in the inset box (\**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001).



**FIGURE 5** Shoot P concentration of endophytic (grey bars) and non-endophytic (white bars) of *L. arundinaceum* grown under ambient and elevated  $\text{CO}_2$ . Dashed lines represent mean values; solid lines depict the inclusive median.  $p$  values  $\leq 0.05$  summarised in the inset box (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ) with full test results given in Table 1. Si supply and herbivore presence not distinguished for clarity and not having a discernible impact.

lolines, peramine, NFL and NANL were detected in all endophytic plants but were not discernibly impacted by  $\text{CO}_2$ , Si supplementation or herbivory (Table S2). NAL was not detected in any of the plants.

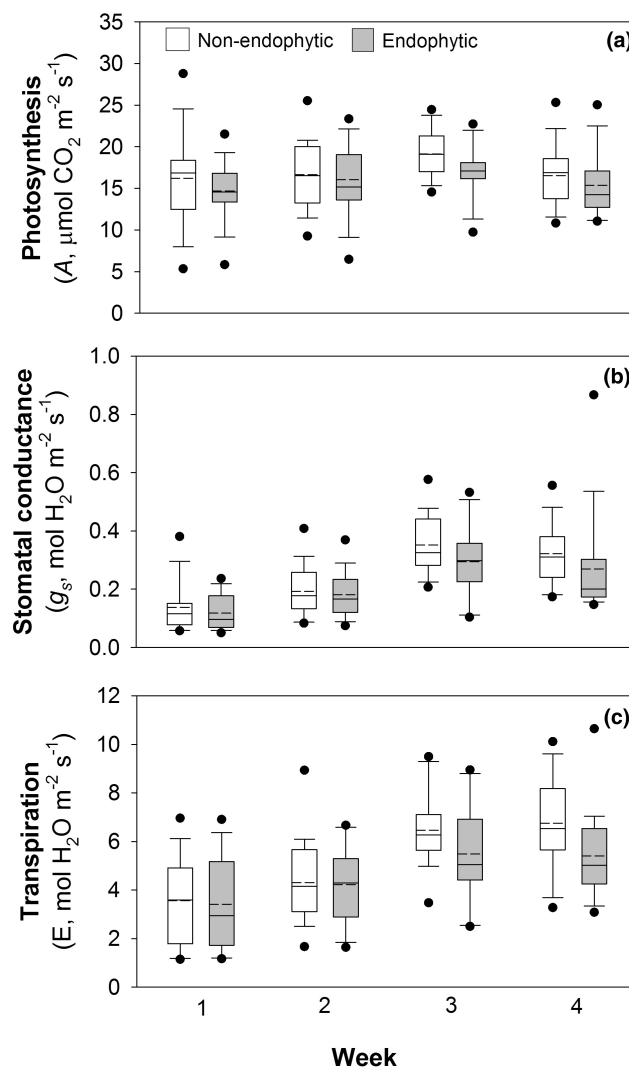
### 3.4 | Summary

The key findings from the study are summarised in Figure 7.  $e\text{CO}_2$  and Si supplementation increased shoot biomass, whereas herbivory reduced shoot biomass. Herbivory induced Si accumulation, which was also increased with Si supplementation; shoot Si concentrations, in contrast decreased with  $e\text{CO}_2$ . Si supplementation and herbivory reduced shoot C concentrations; shoot N concentrations were reduced under  $e\text{CO}_2$  which increased shoot C:N ratios. Shoot P concentrations were lower in endophytic plants, compared to non-endophytic plants, which was further exacerbated under  $e\text{CO}_2$ . Endophytes also reduced net photosynthesis rates, stomatal conductance and transpiration.

## 4 | DISCUSSION

### 4.1 | $\text{CO}_2$ suppression of shoot Si in a field setting

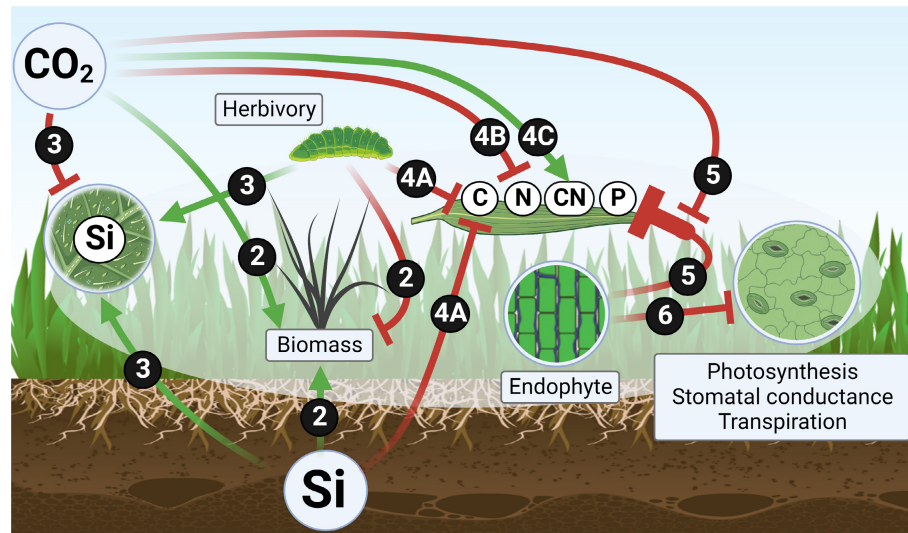
To our knowledge this is the first study to demonstrate that  $e\text{CO}_2$  suppresses Si accumulation in plants under semi-field conditions. Previous studies, using different plant species, have shown this in hydroponic (e.g. Biru et al., 2020) and soil pot (e.g. Ryalls et al., 2017) experiments conducted in growth chambers or glasshouses, respectively. These approaches have proved important for developing



**FIGURE 6** Leaf gas exchange measurements of endophytic and non-endophytic *L. arundinaceum* plants showing (a) rates of photosynthesis,  $A$ , (b) stomatal conductance,  $g_s$ , and (c) transpiration rates,  $E$  over a four-week growth period.  $\text{CO}_2$  growing conditions. Dashed lines represent mean values; solid lines depict the inclusive median. Si supply and herbivore presence not distinguished for clarity and not having a discernible impact. See Table 1 for results of statistical analysis.

conceptual models, but the current findings suggest that  $e\text{CO}_2$  is a powerful driver of Si accumulation in grasses even when temporal heterogeneity, stochastic variation in climatic variables and exposure to natural microbial and invertebrate communities is involved. As discussed by Lindroth and Raffa (2016), outdoor (field) studies help confirm findings from indoor studies because they introduce a level of ecological realism. We agree with Lindroth and Raffa (2016), however, that OTCs can still impose some constraints such as limiting plant growth, increasing temperature and incidence or pest outbreaks. Our specific design for the OTCs seems to have limited these constraints to some extent, for example we observed minimal increases in temperature ( $1.63^\circ\text{C}$ ) inside the OTCs compared to the external environment.





**FIGURE 7** Schematic summarising the key findings. Green arrows represent positive impacts on plant traits (i.e. increases); red attenuating lines indicate negative impacts on plant traits (i.e. reductions) with the relevant Figure numbers given in black circles, which are also used in the following text. In summary,  $\text{CO}_2$  and Si supply increased shoot biomass, whereas herbivory reduced shoot biomass (2). Herbivory induced Si accumulation, which was also increased with Si supplementation; shoot Si concentrations, in contrast decreased with  $\text{eCO}_2$  (3). Si supplementation and herbivory reduced shoot C concentrations (4A),  $\text{eCO}_2$  reduced shoot N concentrations (4B) which resulted in higher shoot C:N ratios (4C). Endophytes reduced shoot P concentrations particularly strongly under  $\text{eCO}_2$  (5). Endophytes also reduced net photosynthesis rates, stomatal conductance and transpiration (6).

The negative impact of  $\text{eCO}_2$  on Si accumulation most likely arises because of the increased availability of C under  $\text{CO}_2$ , although we did not observe consistent increases in shoot C concentrations under  $\text{eCO}_2$ . This may be due, in part, to the strong negative impact of Si supplementation on C concentrations which introduced experimental “noise”. The increase in shoot C:N ratio under  $\text{eCO}_2$  was more consistent with predicted impacts of  $\text{eCO}_2$ . The negative relationship between plant Si and C concentrations is widely reported (e.g. Klotzbücher et al., 2018; Rowe et al., 2020), although not fully understood (Hodson & Guppy, 2022). It is interesting to consider that Si supplementation may be useful for limiting or reversing increased levels of C-based structural constituents (e.g. cellulose and lignin) under  $\text{eCO}_2$ , where this is an undesirable outcome (e.g. paper pulping and forage quality), although this should be balanced against potential disadvantages of increased silicification (Johnson et al., 2021; Raven, 1983).

#### 4.2 | Insect herbivore induction of shoot Si in a field setting

To our knowledge, this is also the first demonstration of insect herbivore-induced Si accumulation under semi-field conditions. Previous field studies have sought to link grass silicification with small (e.g. Huitu et al., 2014; Ruffino et al., 2018) and large (e.g. McNaughton & Tarrants, 1983) grazing mammals, but here we demonstrate that invertebrate herbivores may also play a role in grass silicification. Moreover, the 24% increase in shoot Si concentration in plants experiencing insect herbivory in the current study was higher than plants experiencing vole herbivory (+13%) (Huitu et al., 2014).

Glasshouse experiments previously reported that *H. armigera* can induce Si defences in *L. arundinaceum* (Cibils-Stewart et al., 2022), although to a greater extent (+94%) than in the current study (+24%). This is consistent with a recent meta-analysis of 647 experiments which demonstrated that plant defences, in general, are induced to a greater extent when measured in glasshouse settings compared to field situations (Ojha et al., 2022).

#### 4.3 | Endophyte impacts: Patterns and variations

*Epichloë* endophytes reduced P concentrations in *L. arundinaceum*, which we did not specifically predict. Surprisingly few studies characterise the effects of *Epichloë* endophytes on plant nutrients and many provide conflicting results. Nutrient uptake is primarily mediated by three mechanisms: mass flow, diffusion, and root interception (Gregory, 2006). These can be affected by *Epichloë* endophyte infection, for instance, via endophyte induced changes to leaf area, transpiration, root architecture and manipulation of soil nutrient pools via root exudation (Cibils-Stewart et al., 2020). We observed that endophytic plants had reduced rates of photosynthesis, transpiration and stomatal conductance which may explain why these plants had lower shoot P concentrations (i.e. reduced mass flow). In resource poor environments, *Epichloë* endophytes can act as a sink, consuming the plant's photosynthetic products and nutrients (Lehtonen et al., 2005). *Epichloë* endophytes are also known to reduce transpiration and stomatal conductance to minimise water losses, particularly when experiencing drought stress (Decunta et al., 2021; Elmi & West, 1995; Malinowski & Belesky, 2000). Our plants were not visibly water-stressed, but we did maintain them

at 80% field capacity, so this may have been sufficient to induce greater regulation of water loss in endophytic plants.

In contrast to the current findings, Malinowski, Belesky, et al. (1998) found that endophytic *L. arundinaceum* increased P uptake when growing in low P soils, which they attributed to increased root exudation of phenolics that increased P solubilising activity (Malinowski, Alloush, et al., 1998; Malinowski & Belesky, 1999). In that study, endophytic impacts on diffusion and root interception seem to facilitate P uptake. Our findings are more consistent with Soto-Barajas et al. (2016) who reported that *Epichloë* endophytes reduced concentrations of shoot P in *Lolium perenne*, suggesting that various mechanisms including altered evapotranspiration rates and negative impacts on arbuscular mycorrhizal fungi might underpin these decreases.

The negative impacts of endophytes on P concentrations in the shoots was worsened under eCO<sub>2</sub>, possibly because of increased plant growth which could have had a dilution effect since the plants were grown in soil with relatively low P. We found that endophytes had no further impacts on other plant traits and did not interact with CO<sub>2</sub>. Newman et al. (2003) similarly found that endophytes had no impact on biomass but, as in the current study, eCO<sub>2</sub> promoted biomass in both endophytic and non-endophytic tall fescue plants. Chen et al. (2017) reported that endophytes increased growth in tall fescue, but only in plants grown under aCO<sub>2</sub>. These differences between studies may be due to different experimental platforms and levels of eCO<sub>2</sub> used; Newman et al. (2003) used OTCs and 700 ppm whereas Chen et al. (2017) used indoor growth chambers and 800 ppm. We did not detect any impacts of eCO<sub>2</sub> on endophyte-derived alkaloids, unlike Brosi et al. (2011), who reported that ergovaline and loline concentrations decreased by c. 30% under eCO<sub>2</sub>. Ryan et al. (2014) also reported that total lolines decreased under eCO<sub>2</sub>, but ergovaline concentrations were less impacted. We adopted a less extreme climate change scenario (eCO<sub>2</sub>; +186 ± 77 ppm) than Brosi et al. (2011) (+300 ppm) and Ryan et al. (2014) (+400 and +1000 ppm), which may explain why alkaloid production was unaffected by CO<sub>2</sub> at this lower level.

The AR548 *Epichloë* endophyte strain, used in the current study, has been reported to enhance Si accumulation in tall fescue in both hydroponic (Cibils-Stewart et al., 2020) and soil-based glasshouse studies (Cibils-Stewart et al., 2022). We did not observe this in the current study for two possible reasons. Firstly, transpiration was negatively impacted by endophyte infection and since Si uptake and accumulation is partly driven by mass flow (Ma & Yamaji, 2015) this may have negated any beneficial impacts of endophytic infection. Secondly, the current field study introduced more ecological realism which may have masked effects seen in highly controlled conditions (Cibils-Stewart et al., 2020; Cibils-Stewart et al., 2022).

## 5 | CONCLUSIONS

The current study has shown for the first time that eCO<sub>2</sub> and insect herbivory have contrasting impacts on Si accumulation under

semi-field conditions. Moreover, we report that *Epichloë* endophytes may reduce plant uptake of P via reduced mass flow, a situation which may be aggravated by eCO<sub>2</sub>, particularly if increased demand of P due to increased plant growth cannot be met (Jin et al., 2015). Reductions in Si accumulation in a globally important pasture grass could increase susceptibility to various environmental stresses with negative implications for plant productivity, particularly if eCO<sub>2</sub> had concurrent negative impacts on grass-endophyte interactions as reported here.

Cooke et al. (2016) highlight how so much of our understanding of the functional role of Si in plant biology comes from small-scale laboratory and glasshouse experiments and there is an imperative to scale-up and introduce ecological context. In reporting these findings, which diverge to some extent from previous findings, including our own work, we hope to illustrate the importance of investigating these functional responses of plant in more realistic community and field conditions. This can either confirm or moderate important predictions derived from smaller-scale controlled experiments.

## AUTHOR CONTRIBUTIONS

Scott N. Johnson and Ximena Cibils-Stewart conceived the ideas and designed methodology; Craig V.M. Barton constructed the experimental platform and collected climatic data; Rhiannon C. Rowe and Ximena Cibils-Stewart conducted the experimental procedures not listed below. Fikadu N. Biru and Tarikul Islam conducted leaf gas exchange measurements and analysed the data. Wade J. Mace analysed all endophyte components of the research. Rhiannon C. Rowe collected all of the remaining data; Scott N. Johnson analysed the data; Scott N. Johnson led the writing of the manuscript, with specific contributions from all authors. All authors contributed critically to the drafts and gave final approval for publication.

## ACKNOWLEDGEMENTS

This research was funded by the Australian Research Council in the form of Future Fellowship (FT170100342) awarded to Scott N. Johnson. Pushpinder (Simmy) Matta is thanked for analysis of plant C and N. Burhan Amiji is thanked for assisting with site maintenance and field assistance. Open access publishing facilitated by Western Sydney University, as part of the Wiley - Western Sydney University agreement via the Council of Australian University Librarians.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository: <https://doi.org/10.5061/dryad.6m905qg4p> (Johnson 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.

**Table S1.** Soil chemistry.

**Table S2.** Shoot concentrations of alkaloids in *L. arundinaceum*.

**How to cite this article:** Johnson, S. N., Barton, C. V. M., Biru, F. N., Islam, T., Mace, W. J., Rowe, R. C., & Cibils-Stewart, X. (2023). Elevated atmospheric CO<sub>2</sub> suppresses silicon accumulation and exacerbates endophyte reductions in plant phosphorus. *Functional Ecology*, 37, 1567–1579. <https://doi.org/10.1111/1365-2435.14342>