Silicon-mediated herbivore defence in a pasture grass under pre-industrial and Anthropocene levels of CO₂

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Abstract

The uptake and accumulation of silicon (Si) in grass plants plays a crucial role in alleviating both biotic and abiotic stresses. Si supplementation has been reported to increase production of defence related antioxidant enzymes which helps to reduce oxidative stress caused by reactive oxygen species (ROS) following herbivore attack. Atmospheric CO\textsubscript{2} levels are known to affect Si accumulation in grasses; pre-industrial CO\textsubscript{2} concentrations increase Si accumulation whereas elevated CO\textsubscript{2} concentrations often decrease Si accumulation. This can potentially affect antioxidant enzyme production and subsequently insect herbivory, but this remains untested. We examined the effects of Si supplementation and herbivory by *Helicoverpa armigera* on antioxidant enzyme (catalase, CAT; superoxide dismutase, SOD; and ascorbate peroxidase, APX) activity in tall fescue grass (*Festuca arundinacea*) grown under CO\textsubscript{2} concentrations of 200, 410, and 640 ppm representing pre-industrial, current and future CO\textsubscript{2} levels, respectively. We also quantified foliar Si, carbon (C) and nitrogen (N) concentrations and determined how changes in enzymes and elemental chemistry affected *H. armigera* relative growth rates and plant consumption. Rising CO\textsubscript{2} concentrations increased plant mass and foliar C but decreased foliar N and Si. Si supplementation enhanced production of APX and SOD activity under the ranging CO\textsubscript{2} regimes. Si accumulation and antioxidant enzyme production were at their highest level under pre-industrial CO\textsubscript{2} conditions and their lowest level under future levels of CO\textsubscript{2}. The latter corresponded with increased herbivore growth rates and plant consumption suggesting that tall fescue could become more susceptible to herbivory under future CO\textsubscript{2} conditions.

Introduction

Many grasses are hyper-accumulators of silicon (Si), which can account for up to 10% of their dry mass (Epstein 1994). Si uptake and accumulation is a functional trait with multiple implications for plant biology and ecology (Epstein 2009). Si is taken up as silicic acid (Si(OH)\textsubscript{4}) via the roots, and after being transported into plant tissues, is deposited within and between plant cells, the cell wall and silicified structures such as trichomes or other phytoliths (Perry et al. 1984, Kumar et al. 2017). Although the role of Si in protecting plants against multiple biotic (e.g. herbivores, pathogens) and abiotic (e.g. drought, salinity) stresses has been widely reported (Cooke and Leishman 2011, Debona et al. 2017), an understanding of the exact mechanisms underpinning such protection remains incomplete (Coskun et al. 2019). However, the consensus is that Si supplementation enhances plant physical defences and integrates with the regulation of secondary metabolite defences (Reynolds et al. 2016, Alhousari and Greger 2018, Hall et al. 2019, Waterman et al. 2020).

In terms of physical defences, it is well established that Si confers plant resistance and reduces plant damage caused by both vertebrate and invertebrate herbivores (Massey and Hartley 2009, Hartley et al. 2015, Alhousari and Greger 2018). Si deposition within and around plant cells makes plant tissues tougher and abrasive, causing wear on herbivore mouthparts, damages digestive organs, inhibits movement and reduces the feeding efficiency of insect herbivores (Massey et al. 2006, Reynolds et al. 2009). Moreover, Si is known to alter grass physical defences such as macrohairs, silica cells and prickle cells, which are linked to reduced feeding by insect herbivores (Hartley et al. 2015, Hall et al. 2020, Biru et al. 2021). Additionally, Si uptake and accumulation has been shown to be induced following herbivory (Massey et al. 2007, Islam et al. 2020, Biru et al. 2022).
In addition to direct physical defences, Si potentially protects plants against herbivores by influencing production of plant biochemical defences (Reynolds et al. 2016, Yang et al. 2017), although there is much uncertainty about this since Si has limited chemical reactivity within the plant. Herbivore attack is associated with the induction of oxidative stress in plants, resulting from overproduction of reactive oxygen species (ROS) (Bi and Felton 1995, Kerchev et al. 2012). For instance, insect herbivore attacks can induce various ROS such as hydrogen peroxide \( (\text{H}_2\text{O}_2) \), superoxide \( (\text{O}^-\text{2}) \), singlet oxygen \( (\text{^1}\text{O}_2) \), or hydroxyl radicals \( (\text{OH}) \) in cells (Sharma et al. 2012, Das and Roychoudhury 2014). Despite the fact that ROS plays an important signaling role in plants (Hasanuzzaman et al. 2020), particularly in response to biotic environmental stresses such as herbivory (Fichman and Mittler 2020), excessive levels of ROS can potentially exacerbate oxidative damage to the cell structures (Tripathy and Oelmüller 2012, Das and Roychoudhury 2014). In order to reduce excessive ROS production caused due to the imbalance between free radical formation and the capability of cells to detoxify them (Pizzino et al. 2017), plants have developed efficient antioxidant enzymatic machinery to scavenge ROS (Tripathy and Oelmüller 2012). The antioxidant defence system in the plant cell includes both enzymatic constituents such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and non-enzymatic constituents like cysteine (Cys), reduced glutathione (GSH), ascorbic acid (Asc) (Faroq et al. 2013, Kim et al. 2017). Plants possess either constitutive or induced antioxidants (Sudhakar et al. 2001), and the increased activities of these enzymes in plant cells appear to better control oxidative stress (Das and Roychoudhury 2014, Huang et al. 2019).

Exogenous Si application has been linked to enhanced production of antioxidant enzyme defences such as CAT, APX, and SOD (Kim et al. 2016, Hasanuzzaman et al. 2018, Ahanger et al. 2020). However, the mode of action by which Si regulates antioxidant capacities is poorly understood. Previous work has shown that Si enhances SOD, CAT, APX and peroxidase activities in rice \( (\text{Oryza sativa}) \) (Han et al. 2016), wheat \( (\text{Triticum aestivum} \text{ L.}) \) (Gong et al. 2005) and maize \( (\text{Zea mays} \text{ L.}) \) (Moussa 2006). Si-mediated regulation of antioxidant defences, therefore, reduces the harmful effects of herbivore-induced oxidative stress (Ahanger et al. 2020, Acevedo et al. 2021). Atmospheric concentrations of carbon dioxide \( (\text{CO}_2) \) have emerged as an important environmental driver of Si accumulation (Johnson and Hartley 2018). In general, several studies report that elevated \( \text{CO}_2 \) concentrations \( (\text{eCO}_2) \) decrease Si accumulation (Ryalls et al. 2017, Johnson and Hartley 2018), but see Frew et al. (2017) and Fulweiler et al. (2014). In contrast, pre-industrial levels of atmospheric \( \text{CO}_2 \) can lead to increased Si accumulation (Biru et al. 2020, 2021). These effects are likely due to carbon \( (\text{C}) \) being either more available under \( \text{eCO}_2 \) (Johnson and Hartley 2018, Johnson et al. 2022) or less available under pre-industrial \( \text{CO}_2 \) conditions (Biru et al. 2020). Si accumulation is often negatively correlated with \( \text{C} \) potentially due to stoichiometric dilution or Si-substitution for C-based structural or defensive compounds (Raven 1983, Hodson et al. 2005).

Given \( \text{CO}_2 \) is such an important driver of Si accumulation, which has been shown to influence enzymatic responses (e.g. Kim et al. 2016, Ahanger et al. 2020, Acevedo et al. 2021), \( \text{CO}_2 \) may also affect production of plant biochemical defences (i.e. antioxidant enzymes), potentially via enhanced plant susceptibility to herbivore-induced oxidative stress. To our knowledge, no studies have yet investigated the effects of variable rates of Si accumulation on antioxidant enzyme production and regulation of herbivore-induced oxidative stress under different \( \text{CO}_2 \) concentrations. Using tall fescue \( (\text{Festuca arundinacea}) \) and the generalist insect herbivore, cotton bollworm \( (\text{Helicoverpa armigera}) \) (Hübner), we investigated the effect of Si treatments on
antioxidant enzyme activities and foliar chemistry of plants grown under three CO$_2$ concentrations (200, 410, and 640 ppm) and the consequences for insect herbivory. The objective of this study was to determine how Si treatments (+ Si or − Si) under different CO$_2$ concentrations affect antioxidant enzymes production and foliar chemistry (e.g. C, N) in tall fescue, and the subsequent impacts on performance and feeding efficiency of a chewing insect herbivore. We hypothesised that treatments of high Si together with reduced CO$_2$ are associated with increased antioxidant enzyme activity, whereas low Si together with high CO$_2$ are associated with reduced antioxidant enzyme activity. Here we correlate the rate of herbivore feeding on foliar tissues of tall fescue grown under different CO$_2$ concentrations providing a new perspective towards mitigating challenges of CO$_2$ enriched environment.

**Materials And Methods**

**Plant material and growth conditions**

Tall fescue (*Festuca arundinacea*) is a common pasture grass and a high Si accumulator (Hodson et al. 2005). Seeds of tall fescue (accession T 9627), obtained from Margot Forde Germplasm Centre (Palmerston North, New Zealand), were surface sterilized and inserted into perlite irrigated with water. Plants were grown in the glasshouse for two weeks to achieve uniform seedling growth. Two weeks after germination, individual plants were transferred to hydroponics cups. The hydroponics setup consisted of two nested disposable plastic cups as per Hall et al. (2019). Each cup was filled with approximately 350 ml of full strength standard hydroponic solutions following the protocol of Jung et al. (2015). Seedlings were grown in three plant growth chambers (TPG-1260TH, Thermoline Scientific, NSW, Australia), maintained at reduced CO$_2$ of 200 ppm, ambient CO$_2$ of 410 ppm and elevated CO$_2$ of 640 ppm CO$_2$ concentrations, the latter CO$_2$ concentration predicted for 2100 under the RCP6.0 scenario outlined by the IPCC. (2014). Chambers were illuminated with five 400 W Sunmaster Dual Spectrum High-Pressure Sodium globes at 350 µmol m$^{-2}$ s$^{-1}$ at the plant canopy level. Daytime air temperature was regulated at 26°C and fell to 18°C at night on a 15L:9D photoperiod cycle. Humidity was controlled at 50% (± 6%). Carbon dioxide within the chambers was monitored by a Li-Cor LI-820 CO$_2$ gas analyser, with CO$_2$ (food grade, Air Liquide, Australia) injected from pressurised cylinders through solenoid valves. For reduced CO$_2$ treatment in the chamber, the computer controller constantly monitors CO$_2$ and powers fans to direct chamber air through the scrubbers filled with Sodasorb® (W.R. Grace & Co, Chicago, USA).

**Experimental design**

The experimental design comprised 252 hydroponically grown tall fescue plants. The experiment consisted of a factorial combination of CO$_2$ concentrations (200, 410 or 640 ppm), Si (+ Si or − Si) and herbivore treatments. Si treatments (+ Si) used liquid potassium silicate (K$_2$SiO$_3$) (Agsil32, PQ Australia, SA, Australia) at a concentration equivalent to 2 mM SiO$_2$. Chemically, silicic acid polymerises to form silica gel when the concentration of silicic acid exceeds 2 mM (Ma and Yamaji 2006). Potassium chloride was added to the control (− Si) cups to balance additional K$^+$ ions in + Si treatments. The pH of both + Si and − Si solutions was adjusted to 5.6 using hydrochloric acid to reduce silicate polymerisation (Ma and Yamaji 2006). Solutions were replaced weekly for the first two weeks and then three times a week afterwards. Cups were rotated and
chambers were swapped weekly to minimize chamber effects and pseudo-replication as previously described by Johnson et al. (2018). Plants were grown hydroponically for a further six weeks before insect inoculation.

**Herbivore performance**

To assess the impacts of different CO$_2$ concentrations and Si supplementation on the growth of *Helicoverpa armigera* larvae, a feeding performance assay was conducted. *Helicoverpa armigera* third instar larvae supplied by CSIRO Agriculture & Food, Narrabri, Australia, reared on an artificial diet (Teakle 1985), were used for feeding assays. Initially, larvae were starved for 24 hours, weighed and a single larva was applied per each plant leaf. Plants were then caged with transparent Perspex sheaths and herbivores were allowed to feed for six days, after which they were removed and starved for a further 24 hours to allow the frass to pass, before being reweighed. All frass were collected, dried, and weighed. Frass production was used as a surrogate for plant consumption. Herbivore relative growth rate (RGR) was calculated according to Massey and Hartley (2009). RGR estimates the change in larval fresh mass relative to initial mass and was calculated as mass gained (mg)/initial mass (mg)/time (days).

**Antioxidant enzyme activity assays**

Immediately after herbivore removal, plants from all treatment groups were harvested into liquid nitrogen and stored at -80 °C until analysis. For the measurement of enzymatic activities, ca. 0.05 g of leaf tissue was ground in liquid N and homogenized in 3 ml ice-cold 100 mM K-phosphate buffer (pH 6.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 16000 g for 15 min and the supernatant was used as the source of crude extracts. The supernatant was utilized to measure the production of antioxidant enzymes such as catalase, ascorbate peroxidase, and superoxide dismutase. Enzyme activities were determined according to Hartmann and Asch (2019).

Catalase (CAT) activity was measured spectrophotometrically following the method of Fimognari et al. (2020) and Maksimović and Živanović (2012). Reaction mixture consists of 50 mM potassium phosphate buffer, pH 7.0, 20 mM hydrogen peroxide (H$_2$O$_2$) and crude extract. Absorbance at 240 nm was recorded for 130s using CLARIOstar® in 96 well plates (96 well Flat Bottom Plate, Greiner Bio-one, Australia). For control reactions, H$_2$O$_2$ was omitted. Enzyme activity was calculated using the molar extinction coefficient 36 × 10$^3$ mM$^{-1}$ m$^{-1}$ and expressed as µmol H$_2$O$_2$ oxidized g$^{-1}$ FW min$^{-1}$.

Ascorbate peroxidase (APX) activity was assayed according to Hartmann and Asch (2019). Reaction mixture consists of 50 mM potassium phosphate buffer, pH 7.0, 0.2 mM ascorbate, 0.2 hydrogen peroxide and crude extract (Hartmann and Asch 2019). Absorbance at 290 nm was recorded for 130s using CLARIOstar® in 96 well plates. APX activity was calculated according to Hartmann and Asch (2019); one unit of APX is defined as the amount of enzyme that can be oxidized 1µmol of ascorbic acid per minute.

Superoxide dismutase (SOD) activity was estimated following the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) by the enzyme according to Hartmann and Asch (2019). Reaction mixture containing 0.05 M sodium carbonate, 13.3 mM methionine, 1.3 µM Riboflavin, 21 µM NBT and plant extract (Hartmann and Asch 2019). The reaction took place in a chamber under illumination of a 30 W fluorescent lamp at 25°C. The reaction was started by turning the fluorescent lamp on and stopped 5 min later by turning
it off. The blue formazan produced by NBT photoreduction was measured as increase in absorbance at 560 nm. The blank solution had the same complete reaction mixture but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction in comparison with wells lacking the plant extract, and expressed as units of enzyme activity g\(^{-1}\) FW min\(^{-1}\) (Cavalcanti et al. 2004).

**Analyses of foliar chemistry**

Harvested sample leaves were oven-dried for three days at 60°C and ball milled for further analysis. For foliar Si analysis, roughly 80 mg of ground leaf material was analysed using X-ray fluorescence spectrometry (Epsilon 3\(^{x}\), PANalytical, EA Almelo, The Netherlands) as per Reidinger et al. (2012). Si measurements were calibrated against a certified plant reference material of known Si concentrations (Hiltpold et al. 2017). For foliar C and N concentration, approximately 7 mg of ground leaf material was analysed using Elementar Vario EL Cube, CHNOS elemental analyser (Analysensysteme GmbH, Hanau, Germany), at a combustion temperature of 950 °C.

**Statistical analysis**

All data were analyzed using SPSS (version 27) statistical software. Before analysis, all data were checked for normality. Foliar Si was analysed using two-way analysis of variance (ANOVA) type = II, comparing CO\(_2\), and herbivory (larval fed vs. undamaged controls) as treatments and their interaction. For foliar Si analyses, control (−Si) plants were omitted since −Si plants had Si concentrations lower than the machine detection limits (Reidinger et al. 2012). Plant dry mass, antioxidant enzyme activities (CAT, APX, SOD), foliar C, N and C to N ratio concentrations were all analysed using three-way ANOVA type = II, comparing CO\(_2\), Si (Si supplemented vs. non-Si supplemented plants) and herbivory as treatments and their interaction. Additionally, we tested the independent effects of CO\(_2\) on antioxidant enzymes using a one-way ANCOVA, with CO\(_2\) levels as a fixed factor and foliar Si concentration fitted as a covariate. Plant dry mass was analysed on square-root transformed data whereas CAT and C:N ratio were analysed on log10 transformed data, as they did not meet the assumptions of normality. For herbivore RGR and frass produced, three insects escaped, so data were analysed using type = III ANOVAs due to the unbalanced design. RGR was log10 transformed before analysis, as it did not meet the assumptions of normality. Differences between CO\(_2\) concentrations and Si treatments were determined using Bonferroni post hoc tests (Aslam and Albassam 2020), which were also applied for pairwise multiple comparisons when interaction terms were statistically significant. Potential relationships between foliar Si and herbivore RGR, frass produced, CAT, APX and SOD enzymes production were investigated using Spearman's rank correlation test.

**Results**

**Plant dry mass and foliar chemistry**

Averaged across CO\(_2\) treatments, Si supply increased plant dry mass by 160% relative to those grown without Si supply, whereas elevated CO\(_2\) increased plant dry mass by 566% and 448% compared to reduced CO\(_2\) and ambient CO\(_2\), respectively (Fig. 1A, Table 1). Further, herbivory decreased plant dry mass in Si-free (control) plants by 134%, 234% and 210% under reduced, ambient and elevated CO\(_2\), respectively, compared to Si
supplemented plants (Fig. 1A). Si supplementation decreased foliar C concentrations under all CO₂ regimes. This effect was reversed when herbivores were present and foliar C concentrations increased to levels observed in Si-free plants (Fig. 1B, Table 1). Si supply decreased foliar C by 149%, 177% and 107% under reduced, ambient and elevated CO₂, respectively, regardless of herbivore treatments (Fig. 1B). Reduced CO₂ significantly decreased foliar C (Fig. 1B, Table 1). Si supply decreased foliar N under all CO₂ regimes. But there was also an effect of CO₂ whereby reduced CO₂ increased foliar N by 408% and 707% compared to ambient and elevated CO₂, respectively, irrespective of herbivore treatments (Fig. 1C, Table 1). In addition to variations in C and N concentrations, there was also effect on their ratio. Si supply increased foliar C:N ratio especially when plants were damaged by herbivores or in herbivore free plants only under elevated CO₂ (Fig. 1D, Table 1). While herbivory increased foliar C:N ratio regardless of CO₂ levels, reduced CO₂ decreased foliar C:N ratio relative to elevated CO₂ (Fig. 1D, Table 1). Foliar Si accumulation (% dry weight (DW)) was significantly higher under reduced CO₂ relative to ambient and elevated CO₂ (Fig. 2, Table 1).
<table>
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<th>Response variables</th>
<th>Figure reference</th>
<th>Factors</th>
<th>CO₂</th>
<th>Si</th>
<th>Herbivory</th>
<th>CO₂ × Si</th>
<th>CO₂ × Herbivory</th>
<th>Si × Herbivory</th>
<th>CO₂ × Si × Herbivory</th>
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<tr>
<td>Biomass</td>
<td>1A</td>
<td>(F_{2,60} = 153.1)</td>
<td>(F_{1,60} = 44.61)</td>
<td>(F_{1,60} = 14.23)</td>
<td>(F_{2,60} = 5.761)</td>
<td>(F_{2,54} = 0.545)</td>
<td>(F_{1,60} = 0.489)</td>
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<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>(p = 0.005)</td>
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<tr>
<td>C</td>
<td>1B</td>
<td>(F_{2,84} = 41.34)</td>
<td>(F_{1,84} = 10.54)</td>
<td>(F_{1,84} = 145.5)</td>
<td>(F_{2,84} = 0.211)</td>
<td>(F_{2,84} = 0.515)</td>
<td>(F_{1,84} = 0.062)</td>
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<td>(p &lt; 0.001)</td>
<td>(p = 0.002)</td>
<td>(p &lt; 0.001)</td>
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<td>N</td>
<td>1C</td>
<td>(F_{2,84} = 54.73)</td>
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<td>(F_{1,84} = 123.1)</td>
<td>(F_{2,84} = 0.072)</td>
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<td>(p &lt; 0.001)</td>
<td>(p = 0.006)</td>
<td>(p &lt; 0.001)</td>
<td>(p = 0.930)</td>
<td>(p = 0.019)</td>
<td>(p = 0.424)</td>
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<td>C:N</td>
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<td>(F_{2,84} = 0.102)</td>
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<td>2</td>
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<td>(p &lt; 0.001)</td>
<td>(p = 0.044)</td>
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<td>(p = 0.993)</td>
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<td>CAT</td>
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<td>(F_{2,45} = 19.19)</td>
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<td>(p = 0.989)</td>
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**a**Analysed using a two-way ANOVA.

**b**Analysed using a three-way ANOVA.

**z**Analysed using a three-way ANOVA on log10 transformed data.

**y**Analysed using a three-way ANOVA on square-root transformed data.
<table>
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<td>APX³</td>
<td>3B</td>
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<td>F₂,₃₄ = 13.69</td>
<td>F₁,₃₄ = 33.36</td>
<td>F₁,₃₄ = 20.57</td>
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<td>3C</td>
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<td>F₁,₄₅ = 8.781</td>
<td>F₁,₄₅ = 2.047</td>
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<td>p = 0.002</td>
<td>p = 0.005</td>
<td>p = 0.159</td>
<td>p = 0.941</td>
<td>p = 0.983</td>
<td>p = 0.743</td>
<td>p = 0.910</td>
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**Herbivore performance**

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<tr>
<td>RGR⁴</td>
<td>5A</td>
<td></td>
<td>F₂,₃₉ = 6.067</td>
<td>F₁,₃₉ = 10.03</td>
<td>F₂,₃₉ = 0.224</td>
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<td>p = 0.005</td>
<td>p = 0.003</td>
<td>p = 0.801</td>
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<tr>
<td>Frass⁴</td>
<td>5B</td>
<td></td>
<td>F₂,₃₉ = 2.510</td>
<td>F₁,₃₉ = 4.825</td>
<td>F₂,₃₉ = 0.635</td>
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<td>p = 0.094</td>
<td>p = 0.034</td>
<td>p = 0.536</td>
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| a^Analysed using a two-way ANOVA. |
| x^Analysed using a three-way ANOVA. |
| z^Analysed using a three-way ANOVA on log10 transformed data. |
| y^Analysed using a three-way ANOVA on square-root transformed data. |

**Antioxidant enzyme production was enhanced by Si uptake and reduced CO₂**

CAT production increased in response to Si supply and herbivore damage overall, although this was only apparent under reduced CO₂ concentrations (Fig. 3A, Table 1). The significant interaction between Si and CO₂ reflects that Si impacts were only most apparent under reduced CO₂ concentrations (Table 1). In contrast, Si supply increased APX enzymes production under all CO₂ regimes and SOD enzymes production under reduced and ambient CO₂ (Fig. 3B, C, Table 1). Herbivory caused higher APX production specifically in Si supplemented plants (Fig. 3B, Table 1), however, it had no significant effect on SOD enzyme production (Fig. 3C, Table 1). Overall, antioxidant enzyme production (CAT, APX, SOD) declined with increasing CO₂ concentrations (Fig. 3A – C, Table 1). Including foliar Si as a covariate in ANCOVA indicated that the changes in antioxidant enzymes...
production were linked to CO\(_2\) levels, which fully explained the observed changes in CAT (\(F_{1,53} = 11.67, p = 0.001\)) and APX (\(F_{1,42} = 5.79, p = 0.021\)) but not in SOD (\(F_{1,53} = 1.130, p = 0.258\)). There was a positive correlation between foliar Si concentrations and concentration of CAT under reduced CO\(_2\), and concentration of APX under elevated CO\(_2\) (Fig. 4A, B). Interestingly, frass produced was negatively correlated with SOD (\(r = -0.310, p = 0.038\)), and marginally insignificant negatively correlated with APX (\(r = -0.286, p = 0.057\)). However, there was no such relationship observed between CAT and frass produced (\(r = -0.191, p = 0.208\)).

**Si supply and low CO\(_2\) environment suppressed herbivore RGR and feeding activity**

Si supplementation decreased herbivore relative growth rate (RGR) under all CO\(_2\) regimes; RGR was significantly lower under reduced CO\(_2\) compared to elevated CO\(_2\) (Fig. 5A, Table 1). Si supply decreased the amount of frass produced by caterpillars (indicative of feeding activity) under all CO\(_2\) levels; CO\(_2\) has no significant effect on frass production, although there was a big increase in production in Si-free plants grown under elevated CO\(_2\) (Fig. 5B, Table 1). While herbivore RGR and frass produced were negatively correlated with foliar Si under ambient CO\(_2\), there was no such relationship observed under the other two CO\(_2\) regimes (Fig. 5C, D).

**Discussion**

We demonstrated that pre-industrial levels of atmospheric CO\(_2\) caused plants to accumulate more Si and produce higher levels of antioxidant enzymes relative to future levels of atmospheric CO\(_2\). These increased levels of Si and antioxidant enzymes concentrations under pre-industrial levels of CO\(_2\) were associated with reduced insect herbivore performance. In contrast, herbivore performance and plant consumption (frass production as proxy) were highest under elevated atmospheric CO\(_2\) conditions which typically had the lowest levels of Si and antioxidant enzymes. To our knowledge, this is the first study to address the relationship between Si defences and antioxidant enzyme production in the context of variable atmospheric CO\(_2\) conditions, which we summarise in Fig. 6.

**Direct and Si-mediated impacts of CO\(_2\) on production of antioxidant enzymes**

The effects of CO\(_2\) on Si concentrations are broadly similar to the few studies exploring this, whereby elevated CO\(_2\) leads to decreased Si accumulation (Ryalls et al. 2017, Johnson and Hartley 2018, Johnson et al. 2022), whereas reduced CO\(_2\) leads to increased Si accumulation. To the best of our knowledge, the current study is only the third study to investigate the latter (Biru et al. 2020, 2021). Si has been hypothesized to act as a structural substitute for C at a lower metabolic cost, particularly when CO\(_2\) concentrations were lower in the Miocene (Craine 2009).

Exogenous application of Si has been shown to increase antioxidant enzymes including CAT, APX and SOD (Kim et al. 2016, Hasanuzzaman et al. 2018, Ahanger et al. 2020). It seems likely that changes in Si in
response to CO₂ growing conditions influenced production of antioxidant enzymes in the current study. Additionally, the ANCOVA results indicated that CO₂ affects antioxidant enzyme production (e.g. CAT and APX) which were mostly explained by the direct impacts of CO₂ levels on antioxidant enzymes. However, the observed positive correlation of leaf Si with CAT and APX under reduced CO₂ and elevated CO₂, respectively, suggests that CAT defence response may be linked to higher levels of Si under pre-industrial CO₂. Whereas APX defence response may be associated with increased induction of defence responses following herbivore attack under elevated CO₂. Overall, our results demonstrated significant augmentation of antioxidant enzymes responses via increased Si uptake under reduced CO₂ as well as by the direct effect of CO₂ levels (see Fig. 6).

In contrast, previous studies have reported that elevated CO₂ increases production of antioxidant enzymes in different plants (Wang et al. 2003, Moghimifam et al. 2020). For example, Moghimifam et al. (2020) found that elevated CO₂ enhances CAT, polyphenol oxidase (PPO), SOD, and proline activity in algae (Dunaliella sp.). Our result may reflect that reduced CO₂ often increases photorespiration (Moroney et al. 2013, Voss et al. 2013) and since photorespiration is the key source for ROS production (Voss et al. 2013), reduced CO₂ may cause increased antioxidant enzyme production in order to scavenge excessive ROS produced. However, these studies did not address whether Si played a role in these changes, while our findings suggest Si as an important influencer of the production of these enzymes.

**Herbivory and Si enhanced antioxidant enzyme production**

In addition to CO₂ having direct and Si-mediated impacts on antioxidant enzyme production, it is also possible that the amount of herbivore damage played a role in antioxidant enzyme production. Herbivores induced higher production of CAT and APX, so production is at least associated with the levels of damage done to the plant, which has been similarly reported in previous studies (Leitner et al. 2005, Yang et al. 2017). Increased levels of antioxidant enzymes under reduced CO₂ may reflect the fact that insects were doing less damage to the plant under these conditions and, therefore, there was less ROS oxidative stress that may have persisted in plant tissues to react with higher levels of antioxidant enzymes. Results from the ANCOVA and correlation tests also revealed that CO₂ directly altered antioxidant enzyme production and influenced feeding efficiency (leaf consumption) which eventually reduce growth rate of herbivores.

**Diminishing Si defence with rising CO₂**

Recent finding by Biru et al. (2021) has shown that H. armigera RGR was lowest when fed on the model grass Brachypodium distachyon grown under reduced CO₂ due to these plants having higher levels of Si defences compared to plants grown under ambient and elevated CO₂ concentrations. In the current study, we observed that tall fescue had the highest concentrations of foliar N when grown under reduced CO₂, which in theory could have promoted herbivore RGR because N is frequently the limiting factor in insect herbivore diets (Mattson 1980, Huberty and Denno 2006). The lower production of frass under reduced CO₂, which we used as a proxy for plant consumption, suggests that herbivores were deterred from feeding altogether so would have not been able to access these N resources.

Understanding whether diminishing levels of Si-based plant defences against herbivores under future elevated atmospheric CO₂ concentrations has received limited attention. Previous studies have shown that elevated
CO₂ concentrations decrease Si accumulation in different Poaceae genera (e.g. grasses species and wheat) (Johnson and Hartley 2018, Biru et al. 2022, Johnson et al. 2022), and this was associated with reduced Si defences while increasing herbivore performance; however, this effect is not always reproducible (Frew et al. 2017). The impact of elevated CO₂ on Si defences reflect that plants switch from Si defences to C-based defences due to higher C availability (Johnson and Hartley 2018, Johnson et al. 2022). Although not examined in the context of Si defences, previous studies have demonstrated that elevated atmospheric CO₂ increased consumption and growth rate of the generalist (Pseudaletia unipuncta) and specialist (Spodoptera frugiperda) insect herbivores when fed on C₃ grass relative to lower atmospheric CO₂ conditions (Barbehenn et al. 2004). Johnson et al. (2020) also reported that H. armigera RGR increased under elevated CO₂ as a result of lower plant defence signalling and minimal reductions in the nutritional quality of lucerne (Medicago sativa).

In conclusions, our study provides further evidence that CO₂ concentrations are strong drivers of Si accumulation in an important plant species, not previously reported on. We found strong evidence that reduced CO₂ increased foliar Si concentration and antioxidant enzyme levels, which potentially linked to suppressed insect herbivore performance. This suggests that the negative effects of silicification, whether via physical or biochemical defence mechanisms are stronger under reduced CO₂. Further, we showed a strong linkage between Si supplementation and production of antioxidant enzymes which may help in alleviating the harmful effects of herbivore-induced oxidative stress on plant defence responses. Although Si defences are minimal under elevated atmospheric CO₂ conditions, many agricultural soils can become deficient in bioavailable Si (Haynes 2017), which points to the importance of maintaining Si levels in soils under future projected atmospheric CO₂ conditions.

Declarations

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Conflict of interests We declare that we have no competing interests.

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Data availability The data that support the findings of this study are available on request from the corresponding author, Biru.

Authors contributions: FNB and SNJ conceived the experimental design. FNB acquired and analysed the data. SNJ, CIC and RE supervised FNB. FNB wrote the initial draft of the paper, with CIC, RE and SNJ contributing critical edits to the manuscript. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

References


**Figures**
Effects of Si supply (+Si or −Si) and herbivory (+H or −H) on (A) plant dry mass, (B) foliar C, (C) foliar N, and (D) foliar C:N of tall fescue grass grown under reduced, ambient, and elevated CO$_2$ concentration. Means ± SE shown. N = 6–8. Uppercase letters represent differences between CO$_2$ concentrations whereas lowercase letters indicate differences between Si treatments (Panels 1A, 1B and 1D) or herbivore treatments (Panels 1C).
Figure 2

Effects of Si supply and herbivory (+H or –H) on foliar Si concentration of tall fescue grass grown under reduced, ambient, and elevated CO$_2$ concentration. Means ± SE shown. N = 10. Uppercase letters represent differences between CO$_2$ concentrations whereas lowercase letters indicate differences between herbivore treatments.

Figure 3

Effects of Si Si supply (+Si or –Si) and herbivory (+H or –H) on (A) CAT, (B) APX, and (C) SOD enzymes production of tall fescue grass grown under reduced, ambient, and elevated CO$_2$ concentration. Means ± SE shown. N = 3–5. Uppercase letters represent differences between CO$_2$ concentrations whereas lowercase letters indicate differences between Si treatments (Panels 1A, 1C).
Figure 4

The relationship between (A) CAT and foliar Si, and (B) APX and foliar Si. The solid line represents linear regression through all data points and dashed lines indicate that no significant relationship was observed.

Figure 5

Effects of Si supply (+Si or –Si) and CO₂ level on (A) relative growth rate (RGR) of Helicoverpa armigera fed on tall fescue grass, (B) frass produced, and the relationship between foliar Si concentrations and (C) RGR, (D) frass produced. Means ± SE shown. N = 7–8. Uppercase letters represent differences between CO₂ concentrations whereas lowercase letters indicate differences between Si treatments (Panels 1A, and 1B). For
(C) and (D), the solid line represents linear regression through all data points and dashed lines indicate that no significant relationship was observed.

Figure 6

Summary of the effect of Si (Si) and variable atmospheric CO$_2$ concentrations on the Si and antioxidant enzyme defences against herbivory. (A) current knowledge of the interactions effect between silicon (Si), and antioxidant enzyme (AOE) production on insect herbivory. Our key results from this study are indicated in panels B and C. (B) Reduced CO$_2$ enhances AOE defences by (1) directly enhancing AOE production and (2) indirectly increasing Si uptake, which leads to reduced herbivore performance. (C) Elevated CO$_2$ reduces AOE defences by (3) directly reducing AOE production and (4) indirectly decreasing Si uptake, which leads to increased herbivore performance. Positive and negative effects for both plants and insects are indicated by blue arrows and red lines, respectively.