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Targeted plant defence: silicon conserves hormonal defence signalling impacting chewing but not fluid-feeding herbivores

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Running Title: Silicon integrates into defence signalling

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Abstract

Plants deploy an arsenal of chemical and physical defences against arthropod herbivores, but it is most cost efficient to produce these only when they are attacked. Herbivory activates complex signalling pathways involving several phytohormones, including jasmonic acid (JA), which regulate production of defensive compounds. The Poaceae also have the capacity to take up large amounts of silicon (Si) which accumulates in plant tissues. Si accumulation has anti-herbivore properties, but it is poorly understood how Si defences relate to defence hormone signalling. Here we show that Si enrichment causes the model grass *Brachypodium distachyon* to show lower levels of JA induction when attacked by chewing herbivores. Triggering this hormone even at lower concentrations, however, prompts Si uptake and physical defences (leaf hairs) which negatively impact chewing herbivores. Removal of leaf hairs restored performance. Crucially, activation of such Si-based defence is herbivore-specific and occurred only in response to chewing and not fluid-feeding (aphid) herbivores. This aligns with our meta-analysis of 88 studies that showed Si defences were more effective against chewing herbivores than fluid-feeders. Our results suggest integration between herbivore defences in a model Si-accumulating plant, which potentially allows it to avoid unnecessary activation of other costly defences.

Key-words: Herbivory, insects, jasmonic acid, plant defence, physical defence, silica, silicon

Introduction

Grasses obtain more Si than any other inorganic constituent (Raven 1983, Cooke and Leishman 2011) and there is consensus that Si has an important functional role in plants for alleviating diverse biotic and abiotic environmental stresses (Coskun et al. 2019). Amongst these, Si has been widely reported to have anti-herbivore properties (McNaughton et al. 1985, Reynolds et al. 2016). Si deposition between and within cell walls most likely confers physical resistance to herbivory (Clissold 2008, Hunt et al. 2008). Moreover, Si may augment leaf trichomes / hairs and form discrete structures (e.g. opaline phytoliths) on the leaf surface (Hartley et al. 2015). These can interfere with feeding, wear down mouthparts and reduce nutrient acquisition by herbivores once ingested (Massey et al. 2006, Massey and Hartley 2009).

In addition to physical defences, plants have the capacity to synthesise a diverse range of defensive secondary metabolites with anti-herbivore properties. These defences may exist constitutively, but often they are produced following herbivore attack (Karban and Baldwin 1997). Herbivory activates complex signalling pathways involving several phytohormones, including jasmonic acid (JA), salicylic acid (SA) and ethylene, which regulate expression of defence genes and downstream production of defensive compounds (Howe and Jander 2008, Erb et al. 2012). The JA signalling pathway is regarded as the master regulator of plant resistance to arthropod herbivores and pathogens (Erb et al. 2012). In general, JA regulates defences against tissue-chewing herbivores, whereas defences against fluid-feeding (i.e. piercing/sucking) herbivores are regulated by both SA and JA pathways (Züst and Agrawal 2016).

Despite the emergence of numerous ecological theories to explain the evolution and maintenance of anti-herbivore plant defences (Stamp 2003), Si-based defences and secondary metabolite-based defences have largely been studied separately. In particular, attempts to characterise how Si interacts with the JA pathway when challenged by herbivory (real or simulated) have received limited attention (four studies to our knowledge; Ye et al. 2013, Kim et al. 2014, Hall et al. 2019, 2020). Moreover, these studies found conflicting patterns and only considered chewing-type damage despite plant defences operating very differently depending on how herbivores feed (i.e. chewing or fluid-feeding herbivory) (Schweiger et al. 2014).

Accumulation of Si in higher concentrations (> 1% dry mass) relies on active uptake which has a metabolic cost to the plant (Ma and Yamaji 2015). Furthermore, Si accumulation is known to be an inducible defence across a range of plant species experiencing herbivory (Massey et al. 2007, Johnson et al. 2019). This induction is also an active process, so potentially costly to the plant (Ma and Yamaji 2015, McLarnon et al. 2017). If herbivores induce active Si uptake, plants may therefore downregulate production of secondary metabolite-based defences, which are potentially even more costly than Si-based defences (Hall et al. 2019). For example, Si accumulation imposed lower penalties on plant growth than synthesis of phenolic compounds in several grass species (Johnson and Hartley 2018). Such “trade-offs” between the allocation of plant resources to Si-based versus secondary metabolite defences is likely to be a tightly controlled process regulated by phytohormones.

We investigated the defensive role of Si in the model grass *Brachypodium distachyon* challenged by chewing herbivores (*Helicoverpa armigera*) and piercing/sucking (fluid-feeding) herbivores (*Rhopalosiphum padi*). We determined whether herbivory and stimulation of the JA pathway with methyl jasmonate (MeJA) induced Si uptake and the activity of the JA and SA pathways, whether Si modified these responses and how these factors affected herbivore performance. We also determined whether Si supply shaped physical defences (leaf macro-hairs). We predicted that the chewing herbivore would induce Si uptake and JA activity, but the increase in JA concentrations would be lower in +Si plants because these plants possessed constitutive physical Si defences. In contrast, aphids would not affect Si uptake or JA activity, regardless of feeding intensity (i.e. single or multiple attacks), and aphids would be unaffected by Si defences. Although there has been very little published work on the interactions between Si and phytohormones and the consequences for different herbivore feeding guilds, the subject of this study, there is now a substantive body of work on Si impacts on different types of herbivores. Hence we conducted a meta-analysis of published literature to determine whether Si adversely affected chewing and fluid-feeding arthropods to the same extent.

Methods

Plants and Herbivores

Brachypodium distachyon (Bd21-3), supplied by the French National Institute for Agricultural Research (INRA, Versailles, France), were grown hydroponically using the system and procedures outlined by Hall et al. (2020). The hydroponics system allows individual plants to be grown in opaque cups filled with 330 mL nutrient solutions. Silicon inclusion for +Si plants was achieved by adding liquid potassium silicate (K_2SiO_3) (Agsil32, PQ Australia, SA, Australia) at a concentration of 2 mM (SiO_2 equivalent) to the nutrient solution and adjusted to pH 5.5 using HCl to reduce the polymerisation of silicates. Silicon-free (–Si) plants had KCl added to balance additional K^+ and Cl^- in the Si+ treatments and adjusted to pH 5.5 using HCl (see Hall et al. 2020 for full details). Solutions were replaced weekly when cups were also rotated within the glasshouse chamber. Plant propagation and experiments were conducted in naturally lit glasshouse chambers maintained at 22/18ºC Light:Dark on a 14:10 hour cycle. Humidity was controlled at 50% (±6%). Conditions were monitored and regulated using the PlantVisorPRO (Carel Industries, Padova, Italy) system.

Cultures of the phloem-feeding herbivore (*Rhopalosiphum padi* L.) were established from a single parthenogenetic female obtained from laboratory cultures at Agriculture Victoria Research (Horsham, VIC, Australia). Cultures were reared on barley plants (*Hordeum vulgare* cv. 'Hindmarsh'). The chewing herbivore (*Helicoverpa armigera* Hübner), supplied by CSIRO Agriculture & Food, Narrabri Australia, were individually hatched from eggs and fed on growing media (see Johnson et al. 2019) at 20ºC 15:9 hour photoperiod (Light:Dark) until required.

Experimental Design and Procedures

One hundred and eighty-six *B. distachyon* plants were grown hydroponically for six weeks, half in –Si solution and half in +Si solution and used for different components of the study (summarised in Fig. S1). For each Si treatment, 57 plants were assigned at random to five treatments: (1) no herbivory, N = 15; (2) caterpillar herbivory, N = 10, (3) aphid herbivory, N = 10, (4) three bouts of aphid herbivory, N = 10 and (5) application of methyl jasmonate (MeJA), N = 12. Caterpillar and aphid (single bout) herbivory treatments comprised applying a single third-fourth instar *H. armigera* or four adult *R. padi* to each plant for 7d, respectively, when plants were 5 weeks old. These herbivores were also used to measure the impacts of Si on performance. For *H. armigera*, relative growth rates (RGR) were calculated [mass gained (mg)/initial mass (mg)/time (days)] by measuring larval mass upon application and removal from the plant. For *R. padi*, the total number of individuals were counted after 7d. The repeated bouts of aphid herbivory treatment (4) comprised three periods of 3d of exposure to aphids, with 4d intervening aphid-free days, starting when plants were three weeks old. All plants, including those without herbivores, were caged using transparent cylinders with mesh apertures (similiar to those described in Johnson et al. 2019) fixed to the cups. The 12 plants from both Si treatments assigned for MeJA treatment were sprayed at close proximity with 1 mM MeJA solution prepared with 0.01% Tween 20. Twelve more plants from the two Si treatments were used as controls by spraying with 0.01% Tween 20 in water (treatment 6 in Fig. S1).

The adaxial leaf surfaces of *B. distachyon* are typically covered with small prickle cells and macro-hairs, with the latter potentially linked to Si supply (Glazowska et al. 2018). Three leaves from each herbivore-free plant were selected at random and a 2×2 mm ($4\ \mathrm{mm}^2$) section from the middle of the leaf photographed at ×40 magnification. Leaf macro-hairs were subsequently quantified. All plants (1–6 in Fig. S1) were harvested for analysis of Si, JA and SA when plants were six weeks old. Plant tissue was immediately snap-frozen in liquid nitrogen and stored at –80ºC prior to chemical analysis (details of Si and JA/SA analysis is given in supplemental material).

To determine whether leaf macro-hairs influenced the RGR of *H. armigera*, as previously hypothesised (Hall et al. 2019), we physically removed a proportion of macro-hairs from 12 +Si and 12 –Si plants, leaving a further 12 +Si and 12 –Si plants intact (7 and 8 in Fig. S1). Leaf macro-hairs were removed by gently scraping the adaxial and abaxial leaf surfaces (the latter possessed relatively few hairs) using a knife blade taking care to not damage the leaf. Forty-eight forth instar *H. armigera* were starved for 24 h and weighed before being placed individually in a container with a tiller of fresh leaf material from all 48 plants. The cut end of the tiller was inserted into a 1.5 mL tube with water to maintain turgor. Larvae were kept at 22 °C and allowed to feed for 48 h, after which time they were starved for a further 24 h to allow the frass to be evacuated, before being reweighed. Herbivore RGR was calculated and leaf macro-hairs were quantified (as described above) from all plants to determine the extent of macro-hair removal relative to intact leaves.

Statistical Analysis

One-way ANOVAs with Si as a fixed factor were used to analyse leaf Si, leaf macro-hair density, chewer RGR and aphid abundance. Two way ANOVAs were used to analyse impacts of Si and MeJA on JA concentrations and Si and Si and leaf hair removal on chewer RGR. Generalised linear models with Si and Herbivory/MeJA as fixed factors were used to analyse leaf JA and SA concentrations with Poisson and gamma distributions, respectively. A Pearson's correlation test was used to identify any relationship between JA and SA and between chewer RGR and leaf macro-hairs. All analysis was conducted in either Genstat v19 (VSN International, Hemel Hempstead, UK) or the R statistical platform (R Core Team 2018). Salient statistical results are reported in text and figures, will full details and complete results reported in Table S1.

Meta-analysis

Full details of data collection, screening, meta-analysis and publication bias testing are given in the supplemental material. In brief, we identified studies that quantified arthropod herbivore performance responses when feeding on both +Si plants (e.g. Si supplemented or plants containing naturally high Si concentrations) and –Si plants (e.g. with relatively lower levels of Si than +Si plants). Herbivores were classified according to feeding behaviour; chewing (mandibulate) or fluid-feeding. This constituted 95% of observations with the remaining 5% coming from other feeding guilds.

A meta-analysis was conducted using the package *metaphor* (Viechtbauer 2010) in the R statistical platform. The effect size (Hedges' d), the extent to which herbivores were affected, was calculated for each pair of performance responses on +Si and –Si plant, respectively. This measure of effect size compares two means using a pooled standard deviation and bias correction and reflects the number of standard deviations by which the means differ (Hedges and Olkin 1985). The 95% confidence intervals were used to interpret whether Si had significant impacts on herbivores (i.e. no overlap with zero) and differences between the two groups. Plots to the left of zero indicate increasingly negative impacts of Si, whereas any plots to the right of zero would reflect beneficial impacts of plant Si.

Results

Si uptake, Phytohormones and Leaf Macro-hairs

Si concentrations in –Si plants were below the levels of detection so statistical analysis was conducted on +Si plants only. Leaf Si concentrations were significantly elevated when plants had been subjected to chewing herbivory (Fig. 1A). Aphid herbivory had negligible impacts on Si concentrations. Application of the chemical stimulant (MeJA), corresponding to simulated chewing herbivory, also caused similar increases in leaf Si (Fig. 1A).

Chewers induced higher concentrations of JA (Fig. 1B), but this response was substantially higher in –Si plants than +Si plants. Aphids had no impact on JA concentrations, regardless of Si treatment. As might be expected with chemical stimulation of the JA pathway, MeJA caused a very large increase in JA concentrations ($F_{1,34} = 211.73$, $P < 0.001$) but this was much lower in MeJA treated +Si plants which was reflected in the significant interaction between Si and MeJA treatments (Fig. 1B; $F_{1,34} = 14.70$, $P < 0.001$).

Si had no impacts on SA concentrations, though we observed that repeated bouts of aphid herbivory stimulated SA synthesis (Fig. S2; Table S1). Moreover, chewers resulted in lower concentrations of SA than herbivore-free plants. Compatible with antagonistic cross-talk between SA and JA, we also observed a negative correlation between concentrations of the two hormones ($r = -0.285$, $P = 0.024$). MeJA and Si had no significant impact on SA concentrations (Table S1). Leaf surfaces, particularly the adaxial surface, possessed numerous non-glandular macro-hairs (Fig. 3). Si supply increased the density of these macro-hairs by c. 18% to 3.14 per mm^2 on +Si plants, compared to 2.61 per mm^2 on –Si plants ($F_{1,28} = 13.08$, $P < 0.001$).

Herbivore Performance

The RGR of chewing herbivores fell by over 70% when feeding on +Si plants (72.81 ± 14.08 mg mg^{-1} day^{-1}) compared to those feeding on –Si plants (317.29 ± 43.72 mg mg^{-1} day^{-1}) ($F_{1,18} = 29.98$, $P < 0.001$). In contrast, aphid abundance was similar when feeding on either –Si plants (15.40 ± 1.93) or +Si plants (12.90 ± 1.38) ($F_{1,18} = 1.11$, $P = 0.306$). Mean value ± standard error ($N = 10$) shown in all cases.

Partial removal of leaf macro-hairs reduced hair density (Fig. 4B and 4D) relative to intact leaves (Fig. 4A and 4C). After macro-hair removal, Si continued to have negative impacts on chewer RGR ($F_{1,44} = 239.86$, $P < 0.001$), with herbivores typically losing mass over 24 hours (Fig. 4E). RGR was, however, significantly improved with removal of leaf macro-hairs ($F_{1,44} = 53.95$, $P < 0.001$) to a similar extent on both –Si and +Si plants ($F_{1,44} = 0.86$, $P = 0.360$). Furthermore, there was a significant negative correlation between chewer RGR and the macro-hair density ($r = -0.293$, $P = 0.043$).

Meta-analysis

There were 478 herbivore responses from 88 studies that were suitable for inclusion in the meta-analysis (see Supplemental Material and Fig. S3). Overall, Si had substantially more defensive impacts on mandibulate (i.e. chewing) herbivores than on fluid-feeding herbivores

(Fig. 5). Si-based plant defences were around 2.5× times more harmful for the performance of chewing herbivores compared to that of fluid-feeders. Fluid-feeders were negatively impacted by plant Si but the effect size was relatively small (–0.37) and in line with the non-significant effect size generated in the present study (–0.45).

Discussion

This study has established that Si causes a reduction in JA concentrations in *B. distachyon* in response to chewing herbivory. The fact that MeJA induced Si uptake demonstrates that induced Si defences are regulated by the JA pathway. Crucially, the burst in JA activity following MeJA application was lower in +Si plants than –Si plants which establishes that lower JA in +Si plants was not simply due to reduced feeding / damage by herbivores on these plants. Our results suggest that +Si plants are investing in physical defences and while chewing herbivores continued to trigger the JA pathway, associated with further Si uptake, it does not appear to be necessary to trigger this pathway to the same extent as in –Si plants to maintain effective defences. In contrast, fluid-feeding herbivores neither induced Si uptake or triggered the JA pathway and their performance was unaffected by Si. Meta-analysis indicated that this may be a general pattern: fluid-feeders show some responses to the presence of Si but are not affected by Si defence to the same extent as chewing herbivores.

Production of glandular (i.e. chemical secreting) trichomes is regulated by JA, whereas non-glandular trichomes or macro-hairs can be formed independently of the JA pathway (Li et al. 2004). In addition to forming discrete opaline phytoliths, Si can also augment trichomes and macro-hairs on the leaf surface (Hartley et al. 2015) so while JA activity is required for Si uptake it may not necessarily be directly involved in macro-hair formation. Moreover, once plant tissues become silicified this is irreversible so plants possibly do not need sustained JA activity (and continuous Si uptake) once physical defences are in place. Andama et al. (2020) also demonstrated the importance of silicified non-glandular trichomes for herbivore defence. In common with our study, physical removal of these trichomes improved herbivore performance.

Fluid-feeding aphids did not impact Si induction, whether feeding in single or multiple bouts, and Si had no impacts on aphid performance suggesting that Si is not an effective defence against aphids in this system. Many of the physical defences that Si confers probably have less impact on fluid-feeders than chewing insects (Massey et al. 2006). Fluid-feeders do not need to crush or macerate plant tissues and they can insert feeding stylets between physical structures (e.g. phyoliths and macro-hairs). Moreover, the majority of fluid-feeding herbivores feed on the phloem and therefore do not encounter Si which is transported in the xylem (Raven 1983, Ma and Yamaji 2015). Nonetheless, there are some examples of aphids being negatively impacted by Si (Keeping and Kvedaras 2008) and the current meta-analysis indicated that Si-enriched plants were at least sub-optimal for this group.

The relationships between different physical and chemical plants defences have occupied ecologists for decades (Koricheva et al. 2004). On the basis that plant defences are costly for the plant to produce, some theories predict that defences should trade-off against one another whereas others argue that plants deploy coordinated defence syndromes (Moles et al. 2013). In an extensive global study comprising 261 species from 80 families, Moles et al. (2013) found there was little consistent evidence for either proposition. They did, however, report that four of the five significant defensive pairwise relationships were between ash content (a proxy for Si and calcium oxalate defences) and other chemical defences. This could be compatible with a defensive trade-off between Si and other chemical defences, and in support of this, Cooke and

Leishman (2012) provide evidence from a broad range of plant taxa showing negative trade-offs between Si and carbon-based defences (phenolics and tannins).

Our observations that stimulation of the JA pathway leads to Si accumulation in *B. distachyon*, whilst Si addition regulates lower levels of JA, is compatible with a potential trade-off between defences. It would be metabolically costly for plants to simultaneously have (demonstrably effective) physical defences in place *and* produce defensive secondary metabolites. Our observation of how Si is integrated into plant defence signalling leads us to hypothesise that Si could be a mechanism for delivering a coordinated defence syndrome. We propose that Si may act as a form of cross-talk, similar to that between JA and SA (Thaler et al. 2012), that allows plants to regulate defensive responses in a coordinated manner, and to target them against particular types of herbivore. In addition to this, we provide the first evidence, to our knowledge, that the Si–JA interaction is specific to chewing herbivores and is not influenced by fluid-feeding herbivores.

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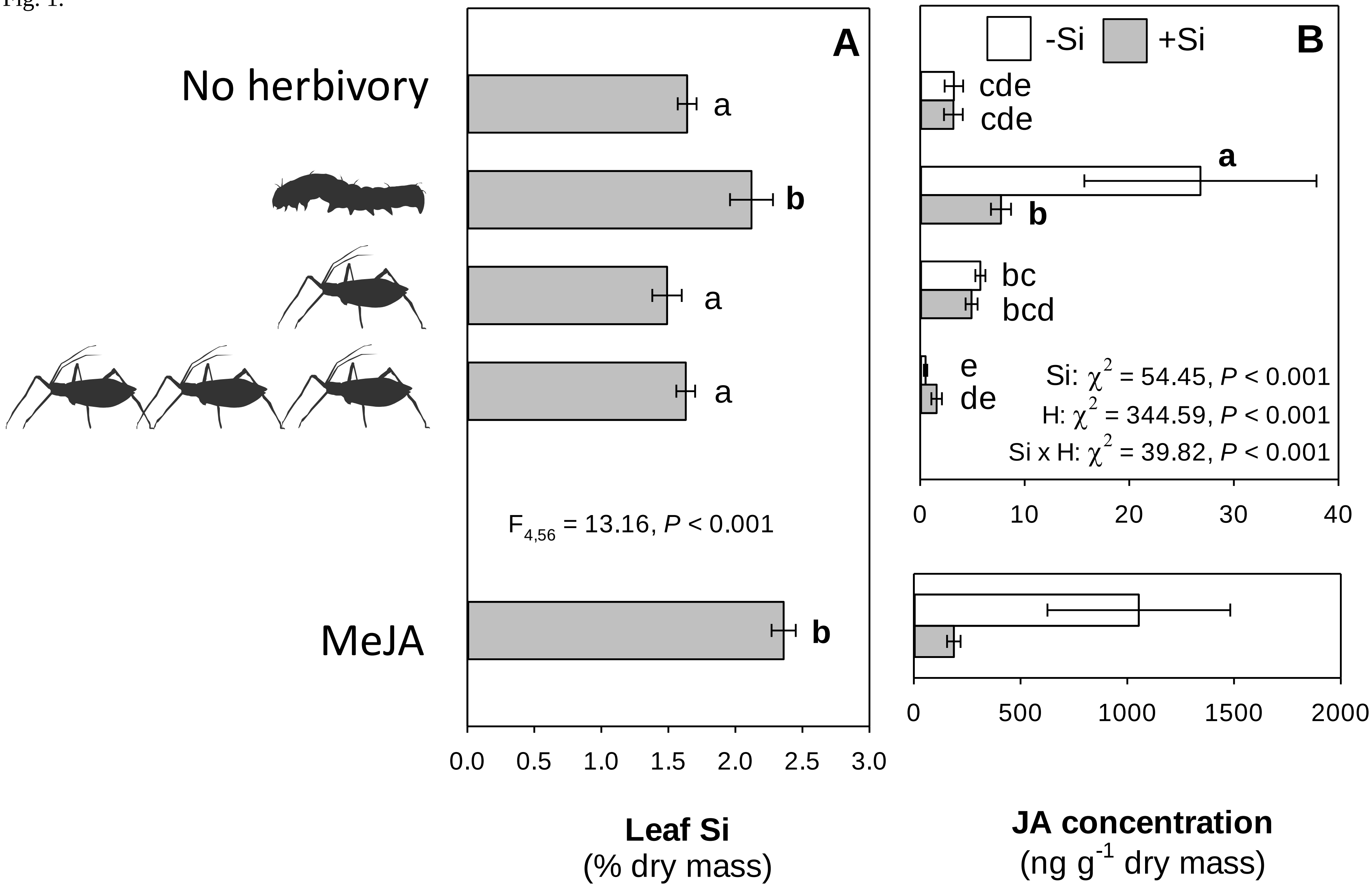
Figure Legends

Fig. 1. Leaf (A) Si and (B) JA concentrations in response to no herbivory, caterpillar herbivory, aphid (single or multiple bouts) herbivory or application of MeJA. Lowercase letters indicate significant differences between treatments arising from Fisher's LSD tests. Bold letters indicate significant differences with plants with no herbivory (controls). Mean ± standard error shown. (A) N = 15, 10 and 12 for no herbivory, herbivore combinations and MeJA application, respectively. (B) N = 8 for herbivores and N = 10 for MeJA.

Fig. 2. Confocal image of *B. distachyon* leaf surface showing macro-hairs. White bar = 250 µm. Images of –Si leaves that were (A) intact, (B) subject to hair removal and equivalent +Si leaves (C and D, respectively). White bar = 1 mm. (E) Relative growth rates (RGR) of *H. armigera* when feeding on such leaves over a 24hr period. Mean ± standard error shown; N = 12.

Fig. 3. Effect size (±95% confidence interval) of plant Si defence against arthropod herbivores classified as mandibulate (chewing) or fluid-feeding.

Fig. 1.


A
No herbivory
MeJA
a
b
a
a
b
F$_{4,56}$ = 13.16, P < 0.001
0.0
0.5
1.0
1.5
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2.5
3.0
Leaf Si
(% dry mass)
B
-Si
+Si
cde
cde
a
b
bc
bcd
e
de
Si: χ^2 = 54.45, P < 0.001
H: χ^2 = 344.59, P < 0.001
Si x H: χ^2 = 39.82, P < 0.001
0
10
20
30
40
0
500
1000
1500
2000
JA concentration
(ng g^{-1} dry mass)

Fig. 2

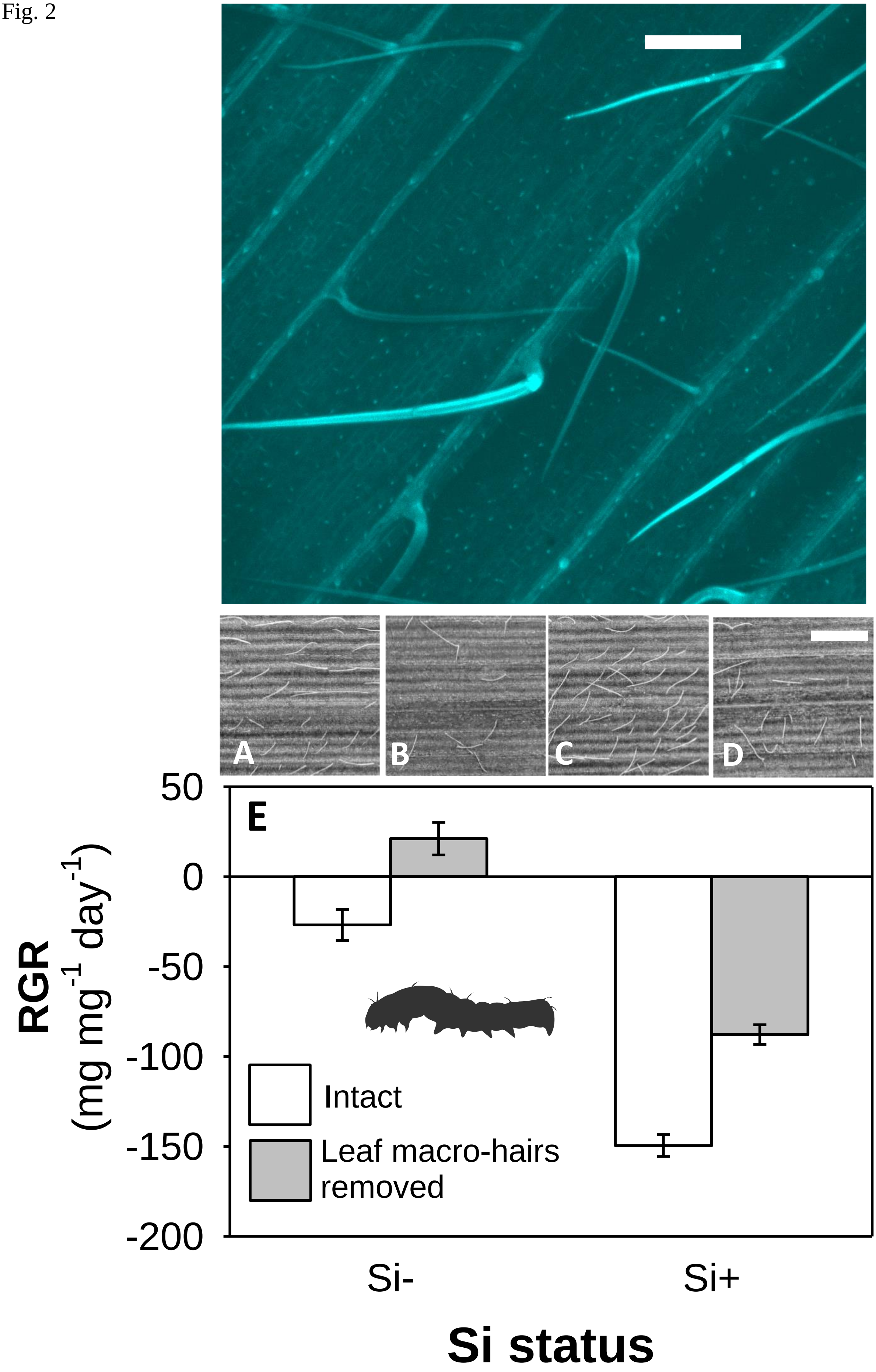
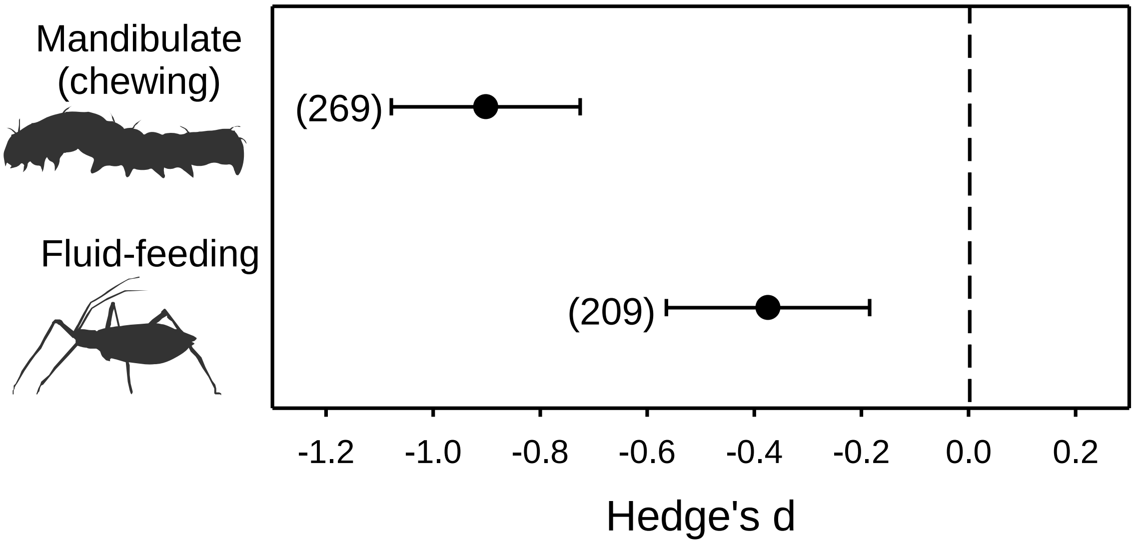

A
B
C
D
E
50
0
-50
-100
-150
-200
RGR
(mg mg^{-1} day^{-1})
Intact
Leaf macro-hairs removed
Si-
Si+
Si status

Fig. 3.


Mandibulate
(chewing)
(269)
Fluid-feeding
(209)
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
Hedge's d