Benefits of silicon-enhanced root nodulation in a model legume are contingent upon rhizobial efficacy

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Abstract

Our study determined the impacts of silicon (Si) supplementation on *Medicago truncatula* inoculated with *Ensifer meliloti* rhizobial strains that differed in their capacity for nitrogen fixation: Sm1021 ('low-efficiency') or Sm1022 ('high-efficiency'). We then examined how Si and rhizobial efficacy influence plant resistance to a polyphagous insect, *Helicoverpa armigera*. These combinations were supplied with Si or untreated in a glasshouse experiment, where we quantified nodule flavonoids and foliar chemistry (free amino acids, soluble protein, elemental C, N and Si). Si supply increased nodule number per plant, specific nodule flavonoids, contents of foliar nitrogenous compounds and foliar C, but not foliar Si. We also demonstrated that rhizobial efficacy altered the magnitude of Si effects on various traits. For example, Si significantly promoted concentrations of foliar N and soluble protein in the plants associated with the 'low-efficiency' strain only and this was not the case with the 'high-efficiency' one. Additionally, increases in foliar free amino acids in response to Si addition did not increase susceptibility to *H. armigera*. Collectively, our study indicates that Si enrichment generates positive effects on *M. truncatula*, particularly when the association with rhizobia is relatively inefficient, and may play a more prominent role in rhizobial functionality than previously thought.

KEYWORDS

Flavonoids, insect, legume, *Medicago*, nitrogen, rhizobia, root nodules, silicon, symbiosis

INTRODUCTION

Silicon (Si) uptake and accumulation in plants (silicification) confers a range of benefits, especially amelioration from biotic and abiotic stresses (Debona, Rodrigues and Datnoff, 2017). For example, silicification provides physical protection against herbivores (Massey and Hartley, 2009) and pathogens (Wang *et al.*, 2017). Silicification can also allow plants to tolerate nutrient deficiencies (Miao, Han and Zhang, 2010; Hernandez-Apaolaza, 2014) and increase yield (Detmann *et al.*, 2012). However, despite the manifold roles of Si in plant biology, most Si studies focus on Si-high accumulator plants, mainly grasses - Poaceae (Katz, 2014) and overlook other plant functional groups, such as legumes - Fabaceae (Putra *et al.*, 2020). Some legume species, such as pigeonpea (*Cajanus cajan*) and soybean (*Glycine max*) can accumulate a considerable amount of Si, but the other species, such as *Medicago* spp. are known to accumulate low amount of Si in the foliage (Hodson *et al.*, 2005). However, low silicification may not necessarily remove the beneficial role of Si in plant functions. For example, Si supply promoted resistance in *Arabidopsis thaliana* against a fungal pathogen although this model plant was a low Si accumulator (Fauteux *et al.*, 2006). More recently, Johnson *et al.* (2018) showed that Si could increase plant growth and root nodulation in lucerne (*M. sativa*), including under combined elevated CO_2 and temperature, which mimicked projected climate change scenarios.

The family of leguminous plants are comprised of more than 20,000 species (Lewis *et al.*, 2005), including some which are ecological and agricultural significant (Foyer *et al.*, 2016). Legumes have evolved distinct symbiotic associations with nitrogen-fixing bacteria (rhizobia) inside root nodules (Hirsch, 1992). Rhizobia convert atmospheric nitrogen (N_2) via nitrogenase into available ammonium (Vessey, 1994) in exchange for carbon-based photosynthates from the host plant (Checcucci *et al.*, 2017). However, along a symbiotic continuum, legumes are exposed to a plethora of rhizobia whose relationships with their host ranges from mutualistic to parasitic (Sachs, Quides and Wendlandt, 2018). In the latter case, which is believed to be more common in nature than previously understood, the result can be rhizobia with low efficiency that provide fewer benefits (e.g. fixed nitrogen) for their hosts (Gano-Cohen *et al.*, 2019). While host legumes can regulate or resist unfavourable rhizobia (Westhoek *et al.*, 2021), some rhizobia can persist

inside the root nodules by hijacking the host's ability to regulate the symbiosis (Sachs, Quides and Wendlandt, 2018). This may ultimately reduce plant fitness.

Cooke and Leishman (2016) hypothesised that the beneficial effects of Si on stress alleviation are greatest when plants are subjected to various environmental stresses, such as drought and salinity, or antagonistic biotic stresses, such as insect herbivory or pathogen attack, but it is possible that symbiotic microorganisms may impose similar stresses on their host when their interactions with the plant is disadvantageous. This may exist, for example, in the legume-rhizobia symbiosis where a rhizobial strain does not provision available N to the host plant efficiently (Terpolilli *et al.*, 2008). For example, the latter study found that a model legume barrel medic (*M. truncatula* genotype A17) possessed a lower symbiotic effectiveness when associated with a model rhizobial strain *Ensifer meliloti* Sm1021, but much higher of that when associated with a closely related strain *E. meliloti* Sm1022 (Terpolilli *et al.*, 2013). Furthermore, a low symbiotic effectiveness was indicated by the production of small and pale (inactive: lack of leghaemoglobin) nodules as opposed to that of relatively large and pink (active) nodules for a high symbiotic effectiveness (Terpolilli *et al.*, 2008). The former was often accompanied by decreases in nitrogen fixation, resulting in relatively low plant biomass and N content whereas the reverse was true for the latter (Terpolilli *et al.*, 2008).

A small, but growing, number of studies suggest that Si may have positive impacts on the legume-rhizobia symbiosis (reviewed by Putra *et al.*, 2020), potentially improving the efficacy of 'low-efficiency' rhizobia. Nelwamondo and Dakora (1999) demonstrated that Si supply promoted root nodulation and nitrogen fixation in symbiotic cowpea (*Vigna unguiculata*) with a *Bradyrhizobium* strain. Similarly, Johnson *et al.* (2017) also reported that Si supply benefitted root nodulation in symbiotic *M. sativa* with a commercial rhizobial strain and shoot biomass. However, the underlying mechanisms underpinning these benefits were unclear. It was recently reported that Si supply promoted nitrogenase activity in the model legume *M. truncatula*, which was positively associated with silicification in either the foliage or the root nodule, depending on host genotype (Putra *et al.*, 2021). Furthermore, some studies hypothesised that Si may promote root nodulation by increasing symbiotic chemical signals, such as flavonoids (Nelwamondo and Dakora, 1999; Johnson *et al.*, 2017). To date, no studies have investigated this hypothesis (reviewed by Putra *et al.*, 2020).

Flavonoids are a specialised class of plant metabolites, fulfilling a wide range of physiological and ecological functions, such as UV protection, defence against herbivores (Simmonds, 2003) and pathogens (phytoalexins) and microbial signalling (Dixon and Pasinetti, 2010). In the legume-rhizobia symbiosis, it is known that these specialised metabolites play a major role in attracting free-living rhizobia, regulating N-fixing rhizobia inside the root nodules and subsequent nodule development (Hassan and Mathesius, 2012). Some (iso-)flavonoids can act as Nod ('Nodulation') gene inducers or repressors for compatible rhizobia (Cooper, 2004). The effects of Si supply on flavonoid production in the legume-rhizobia symbiosis is largely unknown, but Fawe *et al.* (1998) reported that Si supply enhanced the synthesis of a flavonol aglycone rhamnetin in cucumber (*Cucumis sativus*), which was an effective antifungal to powdery mildew.

Silicon-induced root nodulation could also indirectly affect host plant primary metabolites, such as amino acids, and hence ecological interactions aboveground (Johnson *et al.*, 2017). For example, Si-induced root nodulation was associated with increases in essential amino acids and thus increasing aphid abundance on *M. sativa* (Johnson *et al.*, 2017). However, the relationship between impacts of Si-induced root nodulation mediated by rhizobia on host amino acids and foliar-chewing insects remains poorly understood. Furthermore, there are well-characterised interactions between Si and other elemental components of plants, such as carbon (C) and nitrogen (N) (Cooke and Leishman, 2011; Schaller, Brackhage and Dudel, 2012; Klotzbücher *et al.*, 2018; Quigley *et al.*, 2020), although few studies have been done in legumes. In grasses, there is generally a negative relationship between Si vs C, reflecting a 'trade-off' between Si and C as cell structural components in which the incorporation of the former is postulated to be metabolically cheaper than the synthesis of the latter (Raven, 1983), but the relationship between Si vs N is less clear cut (Klotzbücher *et al.*, 2018). Understanding potential changes in host chemistry caused by Si supply is crucial to predict relative cost and benefit of accumulating Si and its potential consequences on plant-associated herbivores (Massey, Ennos and Hartley, 2007) and symbiotic microbes (Frew *et al.*, 2017; Putra *et al.*, 2020).

To understand how Si supply impacts legume-rhizobial interactions, we used the model legume *M. truncatula* genotype A17 in association with either symbiotic strain Sm1021 ('low-efficiency') or Sm1022 ('high-efficiency'), grown under N-limited conditions. Besides its significance in molecular studies of legumes (Young, Debellé and Oldroyd, 2011), *M. truncatula*

is also a model for understanding plant ecological interactions with a myriad of organisms above- and belowground, such as microbes and insects (Rose, 2008), and is one of the most important forage crops worldwide (Lewis *et al.*, 2005).

We measured plant growth, root nodulation and changes in metabolites within nodules (flavonoids) and plant shoots (free amino acids, soluble protein, and elemental C, N and Si) in response to Si supply and rhizobial association and how this affected plant resistance against the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) – a polyphagous chewing insect. Additionally, we determined if rhizobial efficacy affected the outcomes because the relationship between Si and rhizobia could potentially depend on the efficiency of the strain. For example, Si might have more beneficial effects in plants with low-efficiency strains because these are more likely to be under (symbiotic) stress. Moreover, Si-induced changes in plant chemistry, for example N and flavonoids, could affect susceptibility to herbivores, apart from Si deposition. Therefore, we hypothesised that Si supply (see Fig. S1):

(i) increases nodule number which may be linked to increases in flavonoid synthesis,
(ii) resulting in higher foliar amino acids, soluble protein and thus higher N content,
(iii) will have the biggest impacts on plants associated with the low-efficiency rhizobial strain due to those plants being more likely to experience N stress and this may corroborate with the 'stress hypothesis' suggested by Cooke and Leishman (2016), and finally,
(iv) will have no overall impact on a foliar-chewing insect because the benefits of higher host plant nutritional quality mediated by Si-enhanced root nodulation may be cancelled out by higher levels of specific foliar amino acids that act predominantly as precursors for downstream plant defensive metabolites.

MATERIALS AND METHODS

1. Plant material and rhizobial inoculation

The model legume species, barrel medic (*Medicago truncatula* Gaertn., Jemalong A17, hereafter 'JM') was chosen for this study. Barrel medic is an annual species and symbiotically associated with rhizobia (Terpolilli *et al.*, 2008). Seeds of this genotype were obtained from the Australian Pasture Genebank, Adelaide, Australia. Seeds were surface-sterilised in 70% ethanol (v/v), 40% sodium hypochlorite (v/v) and then washed in a sterile MQ-water following methods from Putra *et al.* (2021) and Terpolilli *et al.* (2008). Afterwards, seeds were singly inoculated either with

Ensifer meliloti (previously known as *Sinorhizobium meliloti*) Sm1021 or *E. meliloti* Sm1022 strains. It was reported that barrel medic associates more effectively with *E. meliloti* Sm1022 than Sm1021, e.g. better nodulation, higher plant biomass and shoot N content (Terpolilli *et al.*, 2013). For simplicity, hereafter we refer to 'low-efficiency strain' (LE) and 'high-efficiency strain' (HE) for *E. meliloti* Sm1021 and Sm1022, respectively.

Both strains were originally provided by the Rhizobium Stock Centre, Murdoch University, Australia and subsequently sub-cultured on yeast mannitol agar (YMA) according to Howieson and Dilworth (2016). Growing conditions and inoculations of rhizobia (OD_{595nm}= 0.1 or 10^8 CFUs ml^{-1}) were similarly adopted from Putra *et al.* (2021) prior to sowing seeds.

2. Soil media

An equal mixture of soil and sand (1:1 ratio by mass, hereafter 'soil') were obtained from Australian Native Landscapes Pty Ltd (NSW, Australia) and γ-sterilised (50 kGy; Steritech, NSW, Australia). Soil had low concentrations of bioavailable Si (11 mg kg^{-1}). See Table S1, for all soil chemical properties.

3. Design of experiment

We conducted a full factorial experiment using one-litre pots to grow 80 individuals of JM plants (see full details in Fig. S2). Each plant was singly inoculated either by LE or HE rhizobial strains. Half of the plants were supplemented either by potassium silicate (+Si) or potassium chloride (-Si) in the form of liquid solutions. The pH solutions were adjusted to ± 7.00. During seed germination and seedling growth, only tap water (± 30 ml) was given for 17 days once a day prior to Si supplementation. Afterwards, plants were supplemented either with +Si or -Si (± 65 ml) once every other day for 12 weeks, but not irrigated during a 5-day interval of larval infestation (see section 5 below). To avoid position bias, all plants were randomly assigned and rotated carefully on a weekly basis. Plant growth conditions were as described in Putra *et al.* (2021).

4. Insect infestation

To examine whether Si supply affects plant resistance against an insect herbivore, we sacrificed a subset of plants (6-7 plants) from each of the group treatment for *in situ* (intact) herbivory

assay (see n_h for numbers of replicates in Fig. S2), after 15 days of Si supplementation. We infested plants with larvae of *H. armigera*. Larvae of this insect were reared on an artificial diet until reaching the second late instar, starved for 12 hours and weighed for initial body mass prior to individual infestation on the foliage. The first instar larvae and artificial diets were originally obtained from CSIRO Agriculture & Food, NSW, Australia.

A single larva of *H. armigera* was transferred to the foliar surface of each plant and allowed to feed on plants for 5 days. During larval infestation, plants were not irrigated with -Si or +Si solutions. Cages were applied to pots (see Johnson *et al.*, 2019 for details). After 5 days, larvae were removed and weighed for final body mass to calculate the relative growth rate (RGR) (Johnson *et al.*, 2021) as a proxy for plant resistance.

5. Plant harvest

Shoots and roots were harvested by gently taking out the whole plant, cutting it at the soil surface, and washing it with running water. Fresh nodules were excised from the roots. All plant parts including nodules were separately and immediately stored in a 50 ml Falcon tube, snap-frozen in liquid nitrogen and directly freeze-dried at -60 °C for 72 hours. Freeze-dried plant and nodule samples were weighed for dry mass, and nodules per plant were counted by eye. Freeze-dried foliar samples were finely ground (ball-milled), stored inside a closed 1.5 ml tube at room temperature, whereas freeze-dried nodules inside a closed 1.5 ml tube were stored at -80 °C. Both samples were used for further chemical analyses.

6. Foliar elemental chemistry (Si, C and N)

A fine powder of freeze-dried foliar samples was processed for determining Si concentrations with an X-ray fluorescence spectrometer (Epsilon-3x; PANalytical-Almelo, The Netherlands). We followed procedures described in Reidinger *et al.* (2012) and Hiltpold *et al.* (2017) using a standard Si calibration from a certified plant reference material (i.e. citrus leaves SRM 1572). An automated dry combustion method (Dumas) using Elementar-Vario EL Cube Analyser (Elementar Analysensysteme GmbH, Hanau, Germany) was used to determine foliar concentrations of C and N from the same samples (burnt at 950 °C). Concentrations in % of dry mass were used to express foliar concentrations of Si, C and N.

7. Total soluble protein

Soluble protein concentrations were measured as Johnson *et al.* (2020).

8. Free amino acids

Approximately 35 mg of freeze-dried foliar tissue was extracted in 245 µL 80% MeOH. Samples were then centrifuged at 25 °C for 15 min (15,000 RMP) and 100 µL of supernatant was removed and added to a glass vial insert placed within a 1.5 mL microtube. Samples were combined with 20 µL of 10 µg mL^{-1} DL-norvaline (internal standard) and placed in a vacuum concentrator for 60 min at 30 °C until all liquid was evaporated. In order to derivatise AAs, 50 uL of N-tert-butyldimethylsilyl-N methyltrifluoroacetamide standard mixed with acetonitrile in a 1:1 ratio (v:v) was added to the glass inserts and sealed immediately. Samples were vortexed for 30 s and then mixed at 100 °C for 120 min at 300 RPM. Samples were then cooled to room temperature and analysed using an Agilent 7890A series gas chromatography (GC) system and a 5975C mass spectrometer (MS) detector operating in selected ion monitoring (SIM) mode. The samples were analysed with a J&W Scientific HP-5 column (30 m x 25 mm x 0.25 um) and a temperature program set to 70 °C for 2 min and then increased by 20 °C min^{-1} until reaching 230 °C. The flow rate was set to 1.2 mL min^{-1} with H2 as the carrier gas. Injection port and transfer line temperatures were set at 250 °C and 280 °C, respectively. The MS detector was run in electron ionisation (EI) mode with a collision energy of 70 eV and an ion source temperature of 230 °C. Analysis of clean AA standards was performed to determine high quality mass spectra of each compound. Due to their instability in their silylated (derivatised) form, arginine and glutamine were converted to and quantified as ornithine and pyroglutamic acid, respectively (Leimer, Rice and Gehrke, 1977). The most dominant ion for each amino acid was selected as the quantifying ion, however in some instances the strongest ion was identical to a highly abundant background ion. In those cases (methionine, asparagine, arginine/ornithine, glutamic acid, glutamine/pyroglutamic acid, serine, threonine and phenylalanine), the second most dominant ion was selected for quantification. Retention times of AAs ranged from 5.832 – 20.203 min. We detected 20 foliar free AAs which were grouped into 11 non-essential AAs and nine essential AAs. Non-essential AAs included alanine, glycine, proline, tyrosine, aspartic acid, glutamic acid, arginine, serine, cysteine, asparagine and glutamine whereas essential AAs included isoleucine, leucine, valine, phenylalanine, tryptophan, histidine, lysine, threonine and

methionine. Additionally, we also grouped phenylalanine, tryptophan and tyrosine into aromatic AAs as precursors for plant defence. These detected AAs were used for further statistical analyses. All chemical reagents and standards used for this assay were purchased from Sigma-Aldrich, NSW, Australia.

9. Flavonoids

Flavonoid extraction was performed based on Ng *et al.* (2015) with some modifications. In summary, pre-weighed and frozen nodule samples (25 mg per sample) were powderised in a Qiagen TissueLyser LT with a pre-cooled holder. Twenty ng of umbelliferone (internal standard; Sigma-Aldrich) was added into each sample tube, followed by 1 mL of 80% (v/v) LC-MS grade methanol (Merck). Samples were vortexed, sonicated at 4 °C for 30 min, followed by 15 min centrifugation at 16,000x g. The supernatant was concentrated to dryness in a speedvac centrifuge. Samples were resuspended in 200 µL of 80% (v/v) LC-MS grade methanol, vortexed for 10 s and filtered through a 0.2 µm regenerated cellulose micro-spin filter (CIRO, USA) and resuspended in 50 µL 80% (v/v) LC-MS grade methanol.

Samples were subjected to targeted analysis in a Thermo QE Plus UPLC-Orbitrap at the Joint Mass Spectrometry Facility of the Australian National University following the procedure by Ng *et al.* (2015) with some modifications. Samples and standards were separated in an Agilent Zorbax Eclipse 1.8 µm XDB-C18 2.1 x 50 mm column that was maintained at 40 °C, and separated on a linear gradient from 5-90 % of 0.1 % aqueous formic acid to 99.9% methanol containing 0.1% formic acid at a flow rate of 200 µL min^{-1}. Data were collected in the positive ion mode and collision energies optimised for each flavonoid. The heated electrospray ionisation (HESI-II) probe was operated with the following settings: Ultra-high purity nitrogen gas was used as the sheath gas (45 L min^{-1}), auxiliary gas (10 L min^{-1}) and sweep gas (2 L min^{-1}); the spray voltage was 3.5 kV and capillary temperature 250 °C; the S-lens RF level was 50 V; the auxiliary gas heater temperature was 300 °C. Tandem mass spectrometry was performed using the parallel reaction monitoring mode with a mass resolution of 17,500 at 1.0 microscan. The Automatic Gain Control target value was set at 1.0 E+05 counts, maximum accumulation time was 50 ms and the isolation window was set at m/z 4.0. Data were acquired and analysed using the Thermo Scientific Xcalibur 4.0 software.

Flavonoid standards were dissolved in 80% methanol at 1 ppm and analysed in the same analysis run. Flavonoids were sourced as follows: 2'-hydroxyflavone, 3'-hydroxyflavone, 6,7,4'-trihydroxyisoflavone, 7,3',4'-trihydroxyflavone, 7,4'-dihydroxyflavone, Afromosin, 5,7-dihydroxyflavone (Chrysin), Daidzein -7-O-glucoside (Daidzin), Eriodyctiol, Esculetin, Genistin, Glycitein, Isoliquiritigenin, Luteolin, Madecassoside, Naringenin-7-O-glucoside (Prunin), Formononetin-7-O-glucoside (Ononin), Prunetin, Puerarin, Resveratrol, Rutin, Taxifolin (Indofine Chemical Company, Hillsborough NJ, USA); Genistein, Hesperitin, Kaempferol-7-O-glucoside, Kaempferol-3-O-glucoside (Astragalin); Liquiritigenin, Morin (Extrasynthese, Genay Cedex, France); Coumestrol, Daidzein, Kaempferol (Cayman Chemical Company); Medicarpin, 2'-O-methylliquiritigenin (Carbosynth, Compton, UK), Apigenin, Apigenin-7-neohesperidoside, Naringenin-7-O-rhamnoglucoside (Naringin), 3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside, Biochanin A, Formononetin, Naringenin, Quercetin, Quercetin-3-glucoside (Isoquercetin) (Sigma-Aldrich, Castle Hill Australia). From this source, we detected 17 flavonoid compounds out of 43 compounds, which were grouped into eight isoflavones and nine non-isoflavones based on their known chemical structures. We detected 8 isoflavones, such as Daidzein, Formononetin, 7,3',4'- trihydroxyisoflavone, 6,7,4- trihydroxyisoflavone, Glycitein, Afromosin, Daidzin and Ononin, and nine non-isoflavones, such as Resveratrol, 2'-hydroxyflavone, 3'-hydroxyflavone, 7,4-dihydroxyflavone, Chrysin, Liquiritigenin, 2'-O-methylliquiritigenin, Medicarpin and Naringenin. These detected compounds were used for further statistical analyses.

10. Statistical analyses

All statistical analyses were computed in R version 4.0.3 (R Core Team, 2021). To examine whether Si supply affected multiple plant traits in general (except for insect RGR), we employed multivariate analysis of variance (MANOVA) using 'Manova' function (type = 'II') from the 'car' package (Fox and Weisberg, 2019) with Rhizobia [low-efficiency strain or high-efficiency strain] and Si [-Si or +Si] as main and interacting factors. To accommodate the large number of response variables, relative to available degrees of freedom (the number samples and terms in the model), traits as response variables were divided into categories based on plant phenotypic and chemical groups. The MANOVA revealed that traits differed in their responses to the treatments (see Table S2); therefore, we focus in the main text on interpreting the outcomes of univariate

two-way ANOVAs calculated from each multivariate model to assess the individual traits. This was done using the 'Anova' function (type = 'II') from the 'car' package (Fox and Weisberg, 2019). Moreover, when *p*-values were corrected using an 'fdr' interference, no apparent quantitative changes between unadjusted and adjusted *p*-values were observed, suggesting that the interference could be ignored. Normality ('qqPlot') and homogeneity of variance ('residualPlot') plots were visually assessed and if the assumptions were not met then data were either square-root or $\log_e$ transformed. When the interactive effect between Rhizobia and Si on dependent variables was significant ($p < 0.05$), the Tukey's post hoc multiple comparison test was further conducted using the 'pairs' and 'cld' function from the 'multcomp' package (Hothorn *et al.*, 2021) based on the estimated marginal means in a fitted model using the 'emmeans' function from the 'emmeans' package (Russell *et al.*, 2021). Additionally, to understand how flavonoids and amino acids covaried and were clustered in response to the combination of group treatments, we analysed them separately with the principal component analysis (PCA) using 'prcomp' ('devtools' Wickham *et al.* (2021) and 'ggbiplot' in Vu Q *et al.* (2011) packages). To understand whether certain dependent variables (e.g. Si-induced nodule number vs flavonoids and/or foliar C) were associated with each other between -Si and +Si plants, Pearson's correlation tests ('cor') from the 'stats' package (R Core Team, 2021) were conducted. Finally, the 'ggboxplot' function from the 'ggpubr' package (Kassambara, 2018) was used for data visualisations.

RESULTS

1. Silicon increased nodule number per plant and nodule flavonoids

Total nodule numbers were significantly higher in HE compared to LE-inoculated plants (Fig. 1a). We found that there was a significant effect of Si on nodule number per plant ($F_{1,53}$ = 18.616; $p < 0.001$; Table 1). Nodule number was significantly increased in +Si relative to -Si plants, but relatively more so when the plant associated with LE rather than HE by +86% and +59%, respectively (Fig. 1a).

Si supply significantly increased nine nodule flavonoids (out of 17 detected compounds): Liquiritigenin ($F_{1,22}$ = 4.532; p = 0.046; Table 2), 2'-O-methylliquiritigenin ($F_{1,22}$ = 7.510; p = 0.013; Table 2), Formononetin ($F_{1,22}$ = 14.684; p = 0.001; Table 2), Glycitein ($F_{1,22}$ = 9.561; p = 0.006; Table 2) as well as total flavonoids ($F_{1,22}$ = 12.284.; p = 0.002; Table 2). Specifically,

Liquiritigenin was augmented in the +Si LE and +Si HE plants by +33% and +167%, respectively (Fig. 1b). 2'-O-methylliquiritigenin also increased, particularly in the +Si HE plants (+150%) (Fig. 1c). Moreover, Formononetin was augmented in the +Si LE and +Si HE plants by +200% and +53%, respectively (Fig. 1d). Glycitein also increased, particularly in the +Si LE plants (+157%) (Fig. 1e). Supply of Si increased total flavonoids, to a greater extent when the plant associated with LE rather than HE (+131% and +47%, respectively) (Fig. 1f). The percentage increases are summarised in Table S3. Additionally, how individual flavonoids covaried and were clustered are shown in Figure S4a.

2. Silicon altered free amino acids (AAs) and total soluble protein in the foliage

Non-essential AAs were strongly affected by Si supply ($F_{1,23}$ = 19.106; p < 0.001; Table 3), increasing in the +Si LE and +Si HE relative to the -Si LE and -Si HE plants by 90% and 141%, respectively (Fig. 2a). Essential AAs were also significantly affected by Si supply ($F_{1,23}$ = 8.192; p = 0.009; Table 3), increasing by 23% and 161% (Fig. 2b). Consequently, total AAs were affected by Si ($F_{1,23}$ = 19.499; p < 0.001; Table 3) where they were augmented by 73% and 144%. In addition, aromatic AAs were significantly affected by Si supply ($F_{1,23}$ = 5.983; p = 0.024; Table 3) and increased by 29% and 132% (Fig. 2c).

In terms of individual AAs, only five (i.e., tyrosine, cysteine, isoleucine, leucine and methionine) out of 20 AAs were not significantly affected either by Si or its interaction with rhizobia (Table 3). The percentage increases/decreases of how Si significantly altered individual AAs are summarised in Table S4. Additionally, how individual amino acids covaried and were clustered are explained in Figure S4b.

Si had a significant impact on total soluble protein ($F_{1,23}$ = 32.023; p < 0.001; Table 1). Its effect on that, however, depended on rhizobia ($F_{1,23}$ = 9.343; p = 0.006; Table 1). Total soluble protein was increased by Si in the LE plants by 84% (Fig. 2d). In contrast, total soluble protein was not significantly affected by Si in the HE plants (Fig. 2d).

3. Silicon affected concentrations of elemental C and N

Si significantly affected foliar concentrations of C ($F_{1,11}$ = 23.536; p = 0.001; Table 1), N ($F_{1,11}$ = 28.833; p < 0.001; Table 1) and C/N ($F_{1,11}$ = 29.881; p < 0.001; Table 1) and there was a significant interactive effect between rhizobia and Si on foliar concentrations of N ($F_{1,11}$ =

30.017; $p < 0.001$; Table 1) and of C/N ($F_{1,11} = 66.454$; $p < 0.001$; Table 1). Foliar concentrations of C were increased by Si by 5% in both LE and HE-inoculated plants (Fig. S3a). Si increased foliar concentrations of N in LE plants by 36%, whereas no significant effect of Si was found in HE plants (Fig. S3b). Consequently, Si decreased foliar C/N under in LE plants by 22%, whereas no significant effect of Si on that was found in HE plants (Fig. S3c).

4. The effects of Si on plant biomass and insect growth

In terms of root biomass, there was a significant interactive effect between rhizobia and Si ($F_{1,53} = 4.718$; $p = 0.035$; Table 1). However, the multi-comparison test based on Tukey's HSD showed that Si tended to increase root biomass in the plant associated with LE by 14%, marginally non-significant with a 95% confidence interval ($p = 0.076$), whereas no significant difference on root biomass was found in the plants associated with HE (Table 1).

Si supply did not significantly affect shoot biomass ($F_{1,53} = 3.255$; $p = 0.077$; Table 1), total plant biomass ($F_{1,53} = 3.395$; $p = 0.071$; Table 1) or nodule biomass ($F_{1,52} = 3.049$; $p = 0.087$; Table 1). Foliar concentrations of Si were not significantly affected by Si regardless of plant association with rhizobia ($F_{1,29} = 0.828$; $p = 0.371$; Table 1). Finally, the insect RGR was not significantly affected by Si supply (Table 1; Fig. S3d), although there was a slight reduction of RGR for insects feeding on +Si LE (-9%) and +Si HE (-17%) plants.

5. Silicon-enhanced nodule number was linked to increased nodule flavonoids and foliar concentrations of elemental C

There was a positive correlation in Si+ (HE and LE) plants between nodule number and total flavonoids in Si+ plants ($r = 0.790$; $p = 0.002$; Fig. 3a). The increase in nodule number was positively correlated with increased foliar C in Si+ plants only ($r = 0.930$; $p = 0.008$; Fig. 3b). However, no significant correlation was found between nodule number and foliar N either in -Si ($r = -0.610$; $p = 0.200$) or +Si plants ($r = 0.190$; $p = 0.710$). Finally, mean and standard error (SE) values of all quantified parameters are provided in Table S5a – Table S5.

DISCUSSION

This study provides novel evidence that Si supply substantially improves the functioning of the root nodulation in the model legume *Medicago truncatula*. One potential mechanism includes increasing synthesis of specific flavonoids that could act as Nod gene regulators. Furthermore, we demonstrate that Si can improve root nodulation of a low-efficiency (LE) rhizobial strain. Besides these positive impacts of Si belowground, Si also strongly affects aboveground foliar primary metabolites, increasing free amino acids, total soluble protein and total N, possibly is facilitated by Si-enhanced root nodulation. However, this does not compromise plant resistance against a foliar-chewing insect pest.

Consequences of Si supply on nodule number and nodule flavonoids

Previous studies demonstrated that Si enhanced nodule number in several legume species, for example cowpea *Vigna unguiculata* (Nelwamondo and Dakora, 1999), lucerne *M. sativa* (Johnson *et al.*, 2017) and soybean *Glycine max* (Steiner *et al.*, 2018). A recent study also found that Si enhanced nitrogenase enzyme activity in *M. truncatula* (Putra *et al.*, 2021). Despite these consistent findings, however, the mechanistic explanation for these impacts has not been identified. Our current findings suggest that increased production in nodule flavonoids resulting from Si supply may underpin increased root nodulation.

Si increased specific nodule flavonoids differently depending on plant association with rhizobial strains. In plants inoculated with the HE strain, Si supply induced the concentrations of liquiritigenin and 2'-O-methylliquiritigenin by 167% and 150%, respectively. These flavonones are known to act as Nod-gene inducers in *E. meliloti* (Peck, Fisher and Long, 2006). In plants inoculated with the LE strain, formononetin was strongly induced by Si (up to 200%). Local induction of this isoflavone was reported to accelerate auxin breakdown, regulating nodule organogenesis in white clover *Trifolium repens* cv. Haifa (Mathesius, 2001). Formononetin is also active as an auxin transport inhibitor (Laffont *et al.*, 2010) and could thus play a role in nodule initiation (Wasson, Pellerone and Mathesius, 2006). We also found that Si significantly increased another isoflavone, glycitein (up to 157%) in LE plants. However, this compound is an inactive precursor which has to be activated as a Nod-gene inducer for *Bradyrhizobium* infecting soybean (Pueppke *et al.*, 1998) and its function as a Nod-gene inducer in *E. meliloti* is unknown.

Consequences of Si supply on foliar primary metabolites

Johnson *et al.* (2017) found that Si supplementation in lucerne (*M. sativa*) enhanced the production of essential, but not non-essential or total free amino acids (AAs), in the foliage, possibly was mediated *via* increases in nodule number. In support of their findings, we found that not only essential but also non-essential, aromatic and total foliar free AAs in its closely related species *M. truncatula* were augmented by Si. Changes in certain AAs may potentially alter host quality for herbivores, through changes in nutritional chemistry (Johnson, Hawes and Karley, 2009; Ryalls *et al.*, 2015) and specialised metabolites, such as flavonoids which are synthesised through the phenylpropanoid pathway (Simmonds, 2003). We found that phenylalanine, the key precursor of that pathway (Dixon and Pasinetti, 2010), was enhanced by Si. Moreover, the other aromatic AAs such as tryptophan and tyrosine are also main precursors for downstream defensive compounds, such as indole and alkaloids (Zeier, 2013). Although Si promoted total free AAs to a much greater extent in plants with the HE strain than those with the LE strain, we found that the impacts of Si on individual AAs were compound specific depending on rhizobial strains. For example, Si enhanced proline, histidine and valine more highly in plants inoculated with the HE strain and asparagine, serine and arginine in plants with the LE strain. Variation of Si impacts on these individual AAs might influence different metabolic routes and signalling processes (Hildebrandt *et al.*, 2015), and therefore plant functions. For example, the prominent increase in proline could help plants to better cope with environmental stresses (Hayat *et al.*, 2012) as well as physiological activities, such as flowering and seed development (Mattioli, Costantino and Trovato, 2009). A higher accumulation of asparagine could contribute to increased plant nitrogen and protein contents (Lea *et al.*, 2007).

We found total soluble protein, which is often used as a proxy for nutritional quality (Chapin, 1980; Schwab and Broderick, 2017; Johnson, Waterman and Hall, 2020), was augmented by Si supply. Increased total soluble protein might be, in part, associated with Si-increased total AAs.

Interestingly, we found that total AAs were higher and soluble protein was lower in -Si LE plants, suggesting that LE plants might utilise AAs as precursors for other (defensive) metabolites as opposed to protein synthesis when there was a potential symbiotic stress from the LE strain. However, more crucially, these nitrogenous metabolites were drastically increased when LE plants were supplemented with Si, indicating that Si might alleviate the stress by improving the host plant quality when having symbioses with LE rhizobia.

Consequences of Si supply on foliar elemental chemistry

Si supply slightly increased foliar concentrations of C but greatly increased concentrations of N, resulting in a significantly lower C:N in plants associated with the LE strain relative to those with the HE strain. Moreover, we found the opposite trend in the current legume system relative to that in a grass systems; increasing foliar C was positively linked with Si-increased nodule number. This suggests that this positive relationship might be related to allocation of more organic compounds as a feedback of Si-enhanced nodulation in the foliage. Increased foliar N in +Si LE plants might be related to the fact that Si enrichment could promote nitrogenase activity (Putra *et al.*, 2021). As a consequence, Si-enhanced nodule functionality could then contribute to higher foliar concentrations of foliar amino acids and soluble protein, resulting in higher concentrations of foliar N.

Unlike plant C and N, Si addition had no significant impact on foliar concentrations of Si in *M. truncatula* regardless of plant association with rhizobial strains. This might be explained by the fact that most *Medicago*-legumes are considered as low-Si accumulators relative to high-Si accumulators, such as grasses (Poaceae) in shoot by % dry mass (Hodson *et al.*, 2005; Putra *et al.*, 2020). However, a recent study found that Si addition in *M. truncatula* significantly increased Si accumulation in root nodules but not in foliar tissues (Putra *et al.*, 2021), suggesting that silicification might occur in other plant organs besides leaves (Lux *et al.*, 2020) more frequently than expected, especially in non-grasses (Katz, 2014). A previous study by Fauteux *et al.* (2006) concluded that low Si uptake in *Arabidopsis thaliana* was sufficient to confer plant resistance against a fungal pathogen, suggesting that low silicification may not necessarily preclude Si functions, especially in non-grass taxa.

Negligible impacts of Si supply on nodule biomass, plant biomass and plant resistance against an insect herbivore

Silicon supply only had minor impacts on nodule biomass and plant biomass, though Si increased root and shoot biomass more in plants with the LE strain and the HE strain, respectively. Previous studies have shown that Si supply increased root growth in *V. unguiculata* and this might be related to increased concentrations of endogenous phytohormone abscisic acid (Dakora and Nelwamondo, 2003; Mali and Aery, 2009). Moreover, previous studies also found that Si supply increased shoot

biomass in a symbiotic lucerne, a *Medicago* species closely related to barrel medic (Johnson *et al.*, 2017, 2018; Putra *et al*., 2021).

Despite the positive impacts of Si on potential nutritional quality, we found that this did not compromise plant resistance against *H. armigera*, possibly due to Si-enhanced nodulation increasing the concentrations of specific aromatic amino acids (i.e. phenylalanine and tryptophan), which play a crucial role in the synthesis of defensive metabolites (Zeier, 2013). Therefore, this potentially negated the benefits of improved host plant nutrition. Nevertheless, how Si affects other anti-herbivore metabolites in legumes is still understudied and thus, worthy of further investigation.

Conclusion

In summary, our findings point to the underlying biochemical mechanism whereby Si supply profoundly increases nodule number, which is positively correlated with increased concentrations of nodule flavonoids, leading to higher content of foliar nitrogenous chemistry in the model legume *M. truncatula* associated with two distinct rhizobial strains varying in their symbiotic efficacy. Intriguingly, Si may potentially improve host plant symbiosis with the low-efficiency rhizobial strain. In essence, a stimulating effect of Si in the production of (iso-)flavonoids could enhance the symbiosis. Some of these benefits, however, did not reduce plant resistance against a global polyphagous pest *H. armigera*. Further investigation should explore and consider Si impacts on nodule properties and host plant quality (including defensive metabolites) in a broad range of legume species whose associations with their rhizobial symbionts are relatively poor to better understand whether Si is a key driver for a beneficial legume-rhizobia symbiosis.

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CONFLICT OF INTEREST STATEMENT

We declare that all authors have no conflict of interest.

AUTHORS' CONTRIBUTIONS

R.P., J.R.P., S.E.H., and S.N.J. constructed the idea and design of the experiment; R.P. conducted the experiment; U.M. assisted with flavonoid analysis; J.M.W. and D.W. ran the amino acids and soluble protein analyses; R.P. analysed the data with some statistical advice from J.R.P. and led the writing of the manuscript. J.M.W., U.M., D.W., J.R.P., S.E.H. and S.N.J. critically contributed to manuscript drafts. Lastly, all authors gave final approval for publication.

DATA AVAILIBILITY STATEMENT

Data can be accessed through Dryad Digital Repository when a DOI is available for the data.

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725

Table 1 Effects of rhizobial strain (Rhizo), Si and their interactions on multiple parameters of *M. truncatula* and insect RGR based on a two-way ANOVA test. *p*-values highlighted in bold indicate statistical significance at $p < 0.05$.

Parameters	*df*	Rhizo		Si		Rhizo x Si	
		F	*p*	F	*p*	F	*p*
Nodule number per plant	1,53	4.597	**0.037**	18.616	**< 0.001**	< 0.001	0.983
Foliar soluble protein	1,23	4.657	**0.043**	32.023	**< 0.001**	9.343	**0.006**
Foliar C	1,11	10.218	**0.013**	23.536	**0.001**	0.024	0.880
Foliar N	1,11	30.017	**< 0.001**	28.833	**< 0.001**	30.017	**< 0.001**
Foliar C/N	1,11	42.938	**< 0.001**	29.881	**< 0.001**	66.454	**< 0.001**
Foliar Si	1,29	0.273	0.606	0.828	0.371	< 0.001	0.990
Shoot biomass	1,53	8.148	**0.006**	3.255	0.077	2.743	0.104
Root biomass	1,53	3.998	0.051	1.764	0.190	4.718	**0.035**
Total plant biomass	1,53	8.303	**0.006**	3.395	0.071	0.327	0.570
Nodule biomass	1,52	12.249	**0.001**	3.049	0.087	0.208	0.650
Insect RGR	1,25	0.950	0.340	1.145	0.296	0.179	0.676

729 **Table 2** Effects of rhizobial strain (Rhizo), Si and their interactions on detected individual and total flavonoids in root nodules of *M.*
730 *truncatula* based on a two-way ANOVA test. *p*-values highlighted in bold indicate statistical significance at $p < 0.05$.

Flavonoids	*df*	Rhizo		Si		Rhizo x Si	
		F	*p*	F	*p*	F	*p*
Afromosin	1,22	5.069	**0.036**	3.465	0.078	4.078	0.058
Daidzein	1,22	0.041	0.841	1.044	0.320	2.182	0.156
Daidzein-7-O-glucoside (Daidzin)	1,22	0.042	0.840	6.501	**0.019**	0.026	0.873
Formononetin	1,22	13.864	**0.001**	14.684	**0.001**	0.228	0.638
Formononetin-7-O-glucoside (Ononin)	1,22	3.992	0.060	8.531	**0.009**	0.255	0.619
Glycitein	1,22	10.142	**0.005**	9.561	**0.006**	2.560	0.126
6,7,4-trihydroxyisoflavone	1,22	3.579	0.074	8.964	**0.007**	0.041	0.842
7,3',4'-trihydroxyisoflavone	1,22	8.993	**0.007**	5.625	**0.028**	3.051	0.097
5,7-dihydroxyflavone (Chrysin)	1,22	5.518	**0.030**	0.408	0.530	0.633	0.436
7,4-dihydroxyflavone	1,22	0.537	0.472	0.835	0.372	0.578	0.456
2'-hydroxyflavone	1,22	0.952	0.341	0.563	0.462	2.046	0.169
3'-hydroxyflavone	1,22	1.773	0.199	1.912	0.183	0.388	0.541
Liquiritigenin	1,22	0.061	0.808	4.532	**0.046**	1.097	0.308
Medicarpin	1,22	23.171	< **0.001**	0.001	0.972	0.613	0.443
Naringenin	1,22	0.085	0.773	1.317	0.265	0.480	0.497
2'-O-methylliquiritigenin	1,22	0.556	0.465	7.510	**0.013**	2.447	0.134
Resveratrol	1,22	0.723	0.406	15.907	< **0.001**	8.909	**0.008**

Total	1,22	10.360	**0.004**	12.284	**0.002**	0.160	0.694

731

732 **Table 3** Effects of rhizobial strain (Rhizo), Si and their interactions on individual, non-essential, essential, aromatic and total free
733 amino acids in the foliage of *M. truncatula* based on a two-way ANOVA test. *p*-values highlighted in bold indicate statistical
734 significance at $p < 0.05$.

Free amino acids	*df*	Rhizo		Si		Rhizo x Si	
		F	*p*	F	*p*	F	*p*
Alanine	1,23	1.841	0.190	8.515	**0.008**	0.073	0.789
Arginine	1,23	0.635	0.435	8.267	**0.009**	0.462	0.504
Asparagine	1,23	0.011	0.917	12.784	**0.002**	0.223	0.640
Aspartic acid	1,23	12.485	**0.002**	3.635	0.071	8.814	**0.007**
Cysteine	1,23	0.316	0.580	0.538	0.472	0.100	0.755
Glutamic acid	1,23	7.090	**0.015**	4.585	**0.045**	6.661	**0.018**
Glutamine	1,23	0.075	0.786	5.165	**0.034**	< 0.001	0.982
Glycine	1,23	1.061	0.315	6.850	**0.016**	0.284	0.600
Proline	1,23	23.291	**< 0.001**	20.295	**< 0.001**	0.033	0.858
Serine	1,23	< 0.001	0.981	12.536	**0.002**	0.744	0.399
Tyrosine	1,23	3.176	0.090	2.532	0.127	0.053	0.820
Histidine	1,23	6.651	**0.018**	22.320	**< 0.001**	1.158	0.295
Isoleucine	1,23	15.416	**< 0.001**	0.918	0.349	0.689	0.416
Leucine	1,23	12.244	**0.002**	2.883	0.105	1.102	0.306

Lysine	1,23	0.273	0.607	5.841	**0.025**	0.010	0.922
Methionine	1,23	3.580	0.073	2.093	0.163	1.979	0.175
Phenylalanine	1,23	25.452	**< 0.001**	6.005	**0.023**	0.417	0.526
Threonine	1,23	0.134	0.718	9.598	**0.006**	0.412	0.528
Tryptophan	1,23	9.251	**0.007**	8.148	**0.010**	0.695	0.415
Valine	1,23	31.973	**< 0.001**	17.011	**< 0.001**	1.565	0.225
Non-essential	1,23	12.074	**0.002**	19.106	**< 0.001**	0.363	0.553
Essential	1,23	19.428	**< 0.001**	8.192	**0.009**	1.092	0.308
Aromatic	1,23	14.913	**< 0.001**	5.983	**0.024**	0.246	0.625
Total	1,23	15.542	**< 0.001**	19.499	**< 0.001**	1.005	0.754

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SUPPORTING INFORMATION

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

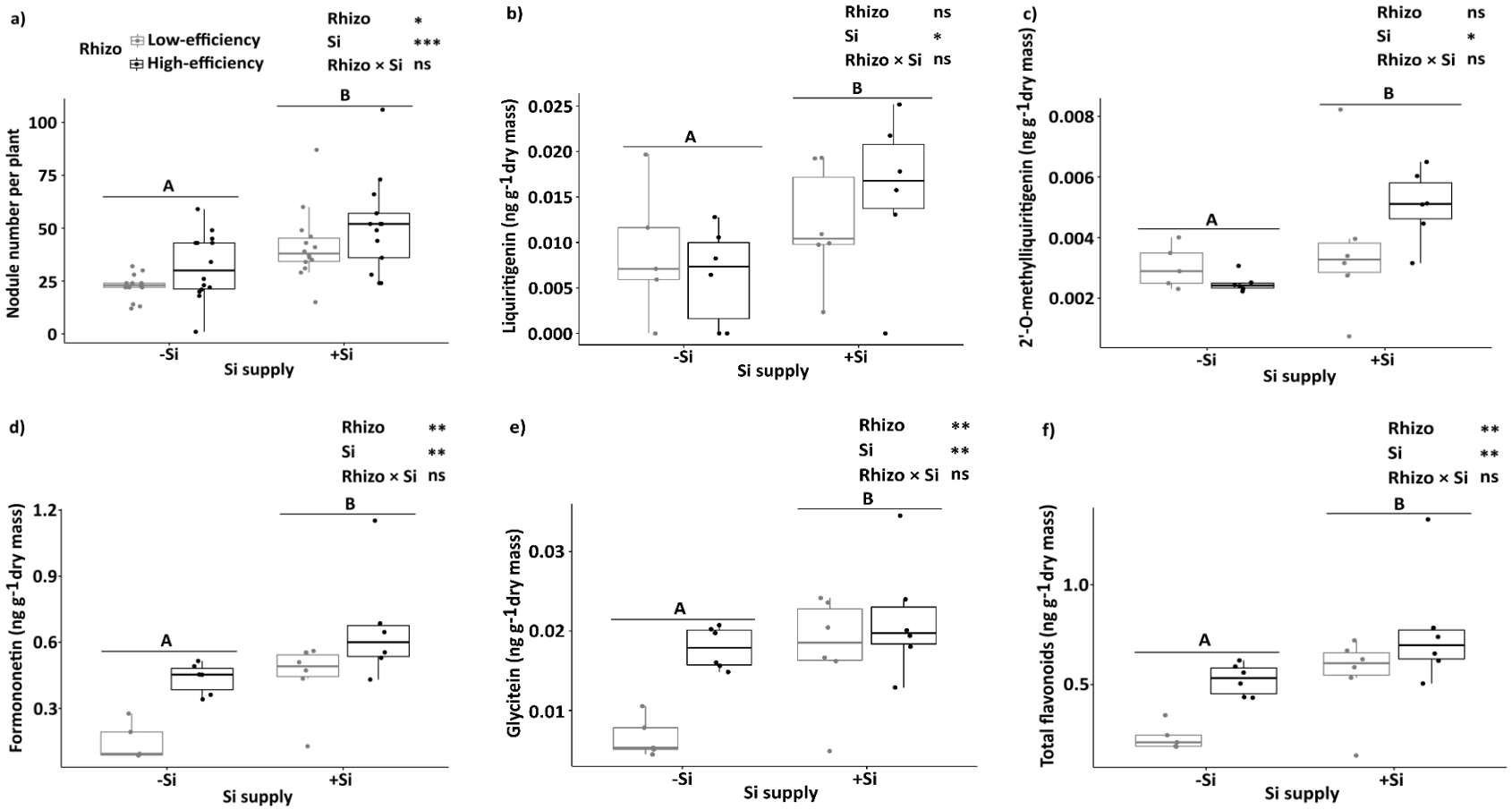

Fig. 1 The impacts of Si supply on: a) nodule number per plant and nodule concentrations (ng g^{-1} dry mass) of b) Liquiritigenin, c) 2'-O-methylliquiritigenin, d) Formononetin, e) Glycitein and f) total flavonoids in the plants associated with low-efficiency and high-efficiency rhizobial strains. Dots represent individual measurement per plant (*n*). Statistically significant factors, namely rhizobial strain (Rhizo), Si, and their interactions are indicated as: ns (not significant), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Different capital letters and the horizontal lines indicate significance at $p < 0.05$.

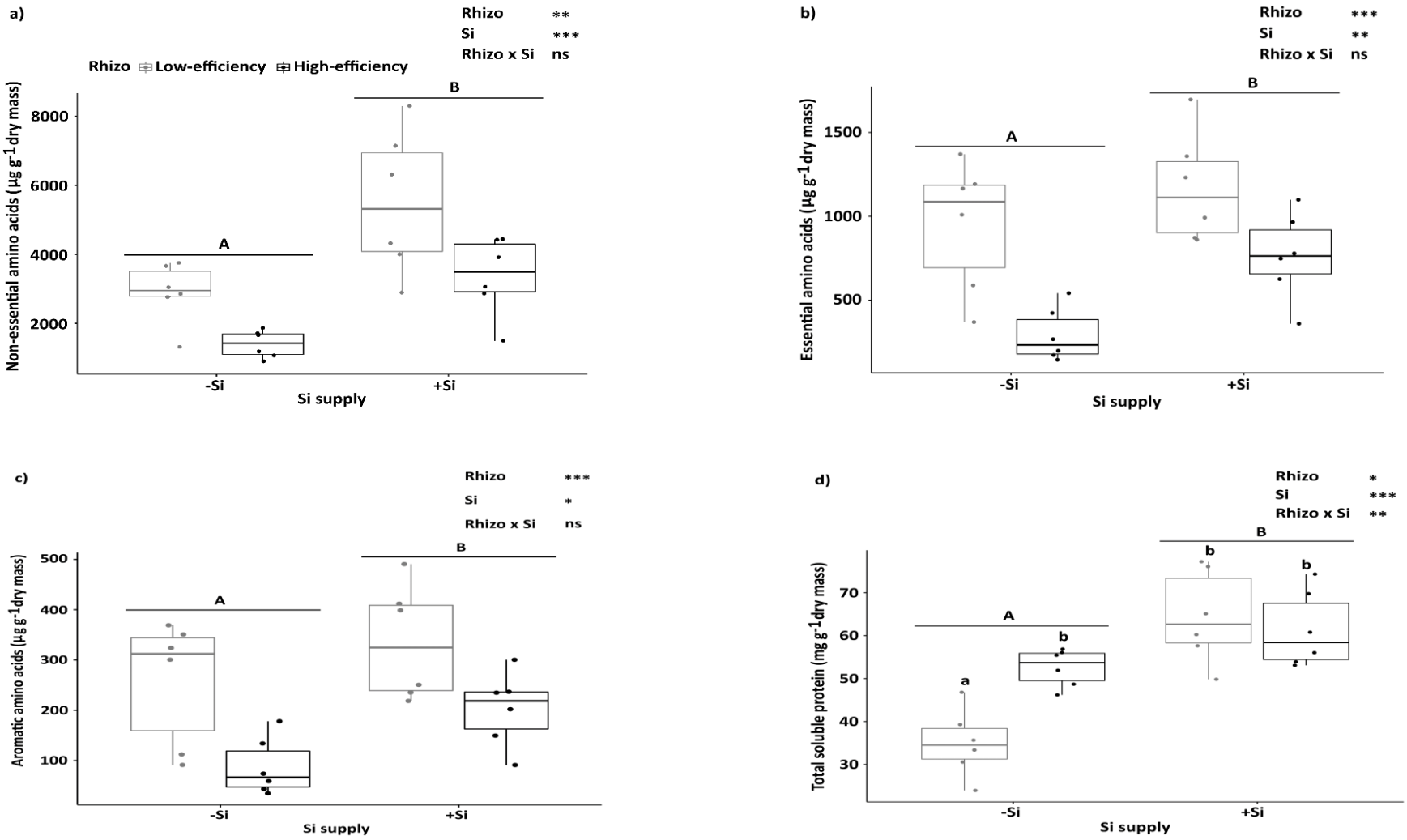

Fig. 2 The impacts of Si supply on foliar concentrations of free amino acids (µg g^{-1} dry mass): a) non-essential, b) essential and c) aromatic, and of d) total soluble protein (mg g^{-1} dry mass) in the plants associated with low-efficiency and high-efficiency rhizobial strains. Dots represent individual measurement per plant (*n*). Statistically significant factors, namely rhizobial strain (Rhizo), Si, and

their interactions are indicated as: ns (not significant), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Different capital letters and the horizontal lines indicate significance at $p < 0.05$.

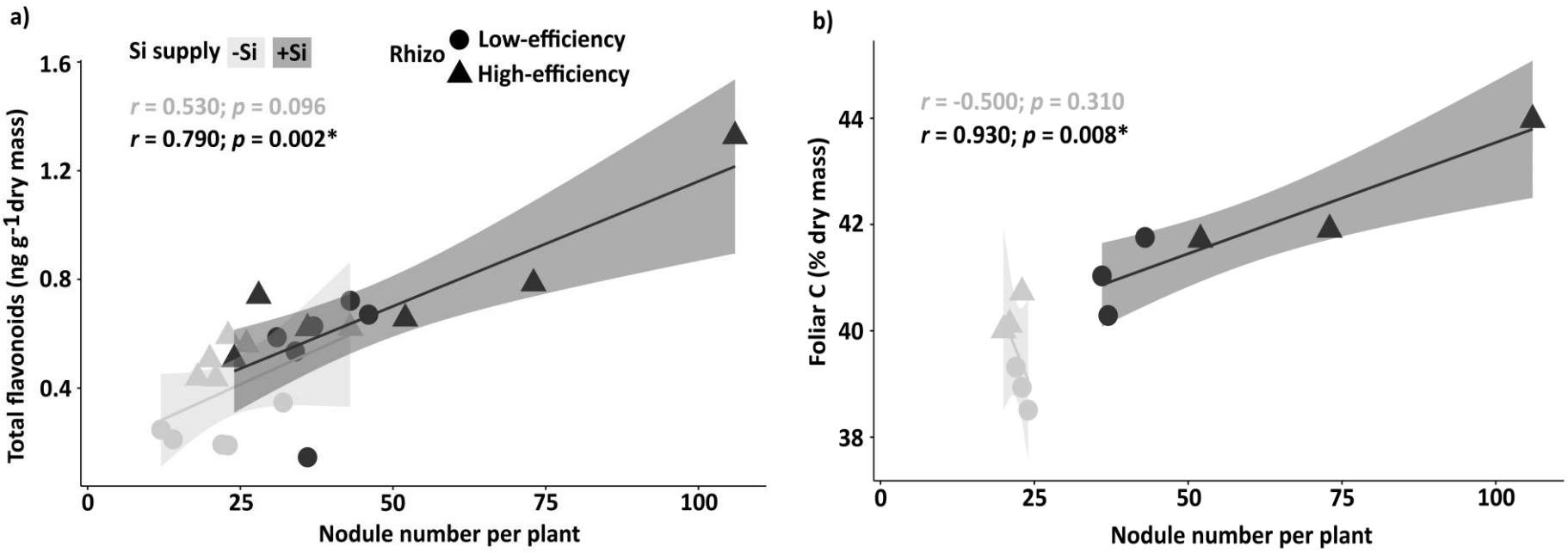

Fig. 3 Pearson's correlation tests between nodule number per plant and: a) total flavonoids (ng g^{-1} dry mass) and b) foliar C (% dry mass). Light grey indicates -Si plants and dark grey indicates +Si plants, associated with low-efficiency (closed circle) or high-efficiency (closed triangle) rhizobial strains. Circles and triangles represent measurements from individual plants (*n*), regression lines represent a slope of the model and ribbons shows 95% CI. Statistical significance was set at $p < 0.05$ and indicated by an asterisk (*).