



Leaf Ultrastructure and Metabolic Responses to Nickel Stress in *Calopogonium mucunoides* and *Canavalia ensiformis*

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Abstract

Nickel (Ni) is an essential micronutrient for plants, but its excess can disrupt physiological functions and impair growth. This study aimed to evaluate the morphophysiological responses of *Calopogonium mucunoides* and *Canavalia ensiformis* under increasing Ni concentrations (0, 120, 240, and 360 mg dm⁻³). Gas exchange, antioxidant enzyme activity, and leaf micromorphology were assessed using infrared gas analysis and scanning electron microscopy. *C. mucunoides* exhibited stable trichome and stomatal densities, increased chlorophyll concentration, and elevated peroxidase activity at 240 mg dm⁻³ Ni, but showed membrane instability and reduced water use efficiency. In contrast, *C. ensiformis* displayed higher morphological and physiological plasticity, evidenced by pronounced variation in stomatal conductance and superoxide dismutase activity (up to 54% RDPI), while maintaining photosynthetic rate and pigment stability across treatments. This enhanced plasticity under Ni stress suggests a greater acclimation capacity and highlights *C. ensiformis* as a promising candidate for phytoremediation of Ni-contaminated soils. These findings advance understanding of legume acclimation to nickel stress and provide valuable insights for sustainable agriculture in contaminated environments.

Keywords Ni toxicity · Fabaceae · Potentially toxic elements (PTEs) · Micronutrient

Introduction

Abiotic stressors—including heavy metals, pesticides, temperature fluctuations, irradiance variations, and water deficit—represent significant environmental challenges that disrupt plant metabolism and constrain ecosystem productivity (Mittler 2006; Hasanuzzaman et al. 2013). Among heavy metals, excess nickel (Ni) exerts pronounced phytotoxic effects, impairing nutrient uptake, metabolic pathways, root development, photosynthesis, and transpiration across higher plants. In wheat, for example, Ni toxicity has been shown to disrupt nutrient balance, oxidative status, and photosynthetic performance (Gajewska et al. 2006; Gajewska and Skłodowska 2008). Toxicity thresholds for Ni in wheat typically range from 10 to 100 mg kg⁻¹ of dry soil, above which visible symptoms such as chlorosis, stunted growth, and altered stomatal behavior emerge (Kabata-Pendias and Szteke 2015).

The complex interplay of metabolic, physiological, and anatomical responses under heavy-metal stress necessitates

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detailed investigations of organ- and tissue-level structural modifications (Yadav et al. 2021). Integrated morphophysiological approaches—combining gas-exchange measurements, antioxidant-enzyme assays, and high-resolution imaging—are essential for elucidating stress-induced mechanisms and for developing remediation technologies (Tadele 2019).

Canavalia ensiformis (L.) DC. and *Calopogonium mucunoides* Desv. are legume species with robust growth across diverse edaphic conditions, including low-fertility and contaminated soils (Costa et al. 2009; Formentini et al. 2008). *C. ensiformis* demonstrates adaptability to partial shading and the ability to remove sulfentrazone residues and heavy metals from soil (Araujo et al. 2024; Madalão et al. 2017; Mendes et al. 2024; Rangel et al. 2023; Santos et al. 2024). *C. mucunoides* is notable for its drought tolerance, osmotic adjustment capacity, and sustained nitrogen fixation even in the presence of mineral nitrogen (Camargos and Sodek 2010). Despite these agronomic advantages, their specific morphophysiological responses to Ni stress remain poorly described.

We hypothesize that (i) increasing Ni concentrations will induce species-specific alterations in leaf ultrastructure—modulating stomatal traits and trichome density—and concomitant changes in photosynthetic performance; (ii) antioxidant-enzyme activities will differ between *C. mucunoides* and *C. ensiformis*, reflecting distinct tolerance thresholds; and (iii) the degree of growth inhibition will correlate with the magnitude of these morpho-biochemical adjustments. To test these hypotheses, we will (1) characterize

leaf surface ultrastructure via scanning electron microscopy, (2) quantify gas-exchange parameters and photosynthetic pigment concentrations, (3) assay activities of catalase, superoxide dismutase, and peroxidase, and (4) assess biomass accumulation under graded Ni exposures. These integrative analyses aim to elucidate the adaptive strategies of these legumes to Ni stress and inform their application in phytoremediation and sustainable agriculture.

Materials and Methods

Experimental Site

The study was conducted in a naturally ventilated greenhouse equipped with automatic irrigation located at 20°25'58" S, 51°20'33" W, Brazil, from March to May 2023 (two months). The local daily average temperature was 24.5 ± 2.5 °C (max. 31.7 ± 2.8 °C; min. 18.7 ± 3.5 °C) and relative humidity averaged $81.5 \pm 8.4\%$. Soil (Typic Haplustox, Oxisol) was collected from 0 to 40 cm depth at 20°20'24.9" S, 51°24'19.7" W. Three composite samples (each comprising five subsamples) were analyzed for chemical fertility (Raij et al. 2001) and texture (Teixeira et al., 2017). Key properties are reported in Table 1.

The selected soil pH of 5.4 reflects acidic conditions typical of many tropical and subtropical soils where leguminous species like *Calopogonium mucunoides* and *Canavalia ensiformis* naturally grow. Acidic pH increases metal availability, including nickel, which was essential for our study to effectively evaluate the toxic impact of Ni on plant physiology and morphology. Using this pH ensured bioavailability of nickel ions, enabling realistic assessment of plant stress responses under environmentally relevant conditions.

Soil Contamination and Available Ni Quantification

Following the Guiding Values for Soil and Groundwater of the Environmental Company of the State of São Paulo (CETESB, 2021), the value of prevention is 30 mg kg^{-1} dry weight and the value of intervention nickel is 190 mg kg^{-1} dry weight of soil in agricultural areas. Based on these guidelines, the range and concentration intervals tested were defined.

The soil was artificially contaminated with sources of Ni sulfate hexahydrate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ - molecular weight: 262.84 g/mol) in the following doses 120(T120), 240 (T240), 360 (T360) mg dm^{-3} of soil, in addition to the control (T0) where no dose of Ni was added. The soil was homogenized manually and incubated in plastic bags for one month to stabilize the element.

Table 1 Analysis of soil fertility of the surface layer 0–40 cm deep of Typic Haplustox (Oxisol) used in the experiment

Variable	Result
pH	5.4
Base saturation	57
Sum of bases	$16 \text{ mmol}_c.\text{dm}^{-3}$
Calcium	$7 \text{ mmol}_c.\text{dm}^{-3}$
Potassium	$1 \text{ mmol}_c.\text{dm}^{-3}$
Magnesium	$8 \text{ mmol}_c.\text{dm}^{-3}$
Potential acidity - $\text{H}^+ + \text{Al}^{3+}$	$12 \text{ mmol}_c.\text{dm}^{-3}$
Aluminum	$0 \text{ mmol}_c.\text{dm}^{-3}$
Phosphorus	6 mg dm^{-3}
Organic matter	13 g dm^{-3}
Cation Exchange Capacity	$28 \text{ mmol}_c.\text{dm}^{-3}$
Manganese	5.5 mg dm^{-3}
Iron	8 mg dm^{-3}
Copper	0.3 mg dm^{-3}
Zinc	0.2 mg dm^{-3}
Boron	0.03 mg dm^{-3}
Clay	121 g kg^{-1}
Sand	876 g kg^{-1}
Silt	3 g kg^{-1}
Density	1.47 kg dm^{-3}

The extraction of micronutrients from the soil (5 g) was performed using the Mehlich 1 double-acid extracting solution (25 mL) (HCl 0.05 M + H₂SO₄ 0.0125 M) at a 1:5 soil-to-extractor ratio. The sample was placed on a circular shaker at 40 rpm for five minutes and inductively coupled plasma optical emission spectrometry was used to quantify Ni (Table 2). The minimum limit of quantification for Ni in the soil is estimated at 1.8 mg kg⁻¹ (Silva 2009).

Experimental Design and Growth Conditions

Commercially obtained seeds of *Calopogonium mucunoides* Desv. and *Canavalia ensiformis* (L.) DC. were used. The seeds were selected based on size and shape, with no treatments or inoculation with rhizobia applied. Four seeds were placed in each experimental unit (polypropylene pot with a capacity of 4 dm³ of soil) at a depth of 1 cm. After seedling emergence, two seedlings were thinned and the experiment was conducted with two plants per pot. The completely randomized design had four replications and four Ni concentrations over 55 days. Although conducted separately, experiments for both species were carried out simultaneously.

Therefore, each replicate consisted of two plants, resulting in a total of 8 plants per treatment (4 replicates × 2 plants each) for statistical analysis. Both species were evaluated simultaneously but in separate experimental units. This design ensures adequate replication and statistical power to detect treatment effects while maintaining manageable plant density per pot.

For measurements involving leaf-level variables, such as stomatal traits data were collected from both adaxial and abaxial surfaces of the leaves. Therefore, the reported sample size ($n=12$) corresponds to combination of species × treatment × surface.

Morphological Characterization - Scanning Electron Microscopy

Completely expanded leaves were fixed in 70% FAA (Johansen 1940) to avoid loss of material due to natural degradation and after 48 h the material was stored in 70% alcohol. Samples of leaves were selected in midrib region. The fixed samples were dehydrated, dried to the critical point Leica EM CPD300 (Horridge and Tamm 1969) fixed

on an aluminum support with double-sided carbon tape and metallized with a layer of 30–40 nm gold on the Quorum Q150T E device. The analyses were carried out using a Zeiss scanning electron microscope model EVO/LS15, operated at 20kv with the scales printed directly on the electron micrographs.

These images were analyzed using the Image J software 1.52r (Schneider et al. 2012), which made it possible to measure the following variables as described in Fig. 1: trichome density, stomatal density, percentage of open stomata, and length of the stomatal orifice (Poole and Kürschner 1999; Lima et al. 2010; Zhao et al. 2022) using the following equation:

$$\text{Trichome density} = \text{Number of Trichome} / \text{Leaf area} \quad (1)$$

$$\text{Stomatal density} = \text{Number of stomata} / \text{Leaf area} \quad (2)$$

$$\text{Percentage of open stomata} = (\text{Number of open stomata} / \text{Total number of stomata}) \times 100 \quad (3)$$

Furthermore, the characteristics and morphological changes in cells were described.

Sampling and Diurnal Aperture Note

SEM imaging and stomatal density quantification were conducted on samples collected on different days. Because stomatal aperture exhibits diurnal dynamics that affect pore opening and surface contrast, stomata can appear visually sparse or inconspicuous in SEM images taken at certain times of day. Importantly, stomatal density was quantified from standardized micrographs by counting stomata per unit area, independent of aperture state. This timing-related visual discrepancy explains why the qualitative appearance in Fig. 2 may not mirror the quantitative densities reported in Fig. 3.

Electrolyte Leakage

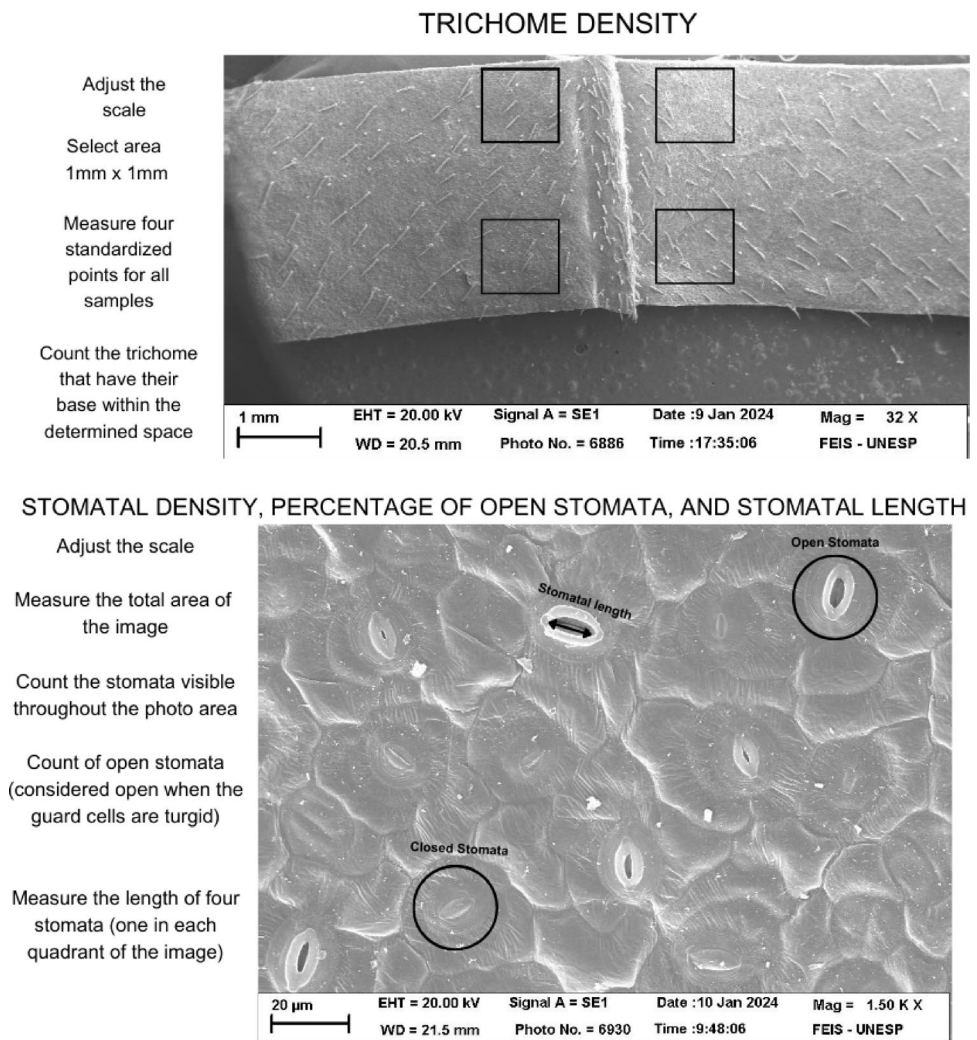
The leaves were washed and weighed, and approximately 0.1 g of leaves with an approximate diameter of 4.5 mm were obtained. The first step of the analysis is to place them in tubes with 20 mL of distilled water at 25 °C for 90 min in a water bath and after this period the initial conductivity (E1) was measured (Deshmukh et al. 1991; Onwueme 1979; Sairam et al. 2008). After the first reading, the tubes were heated to 90 °C for 90 min, cooled and the final conductivity (E2) was measured and calculated using the following equation:

Table 2 Amount of Ni available at the beginning of the experiment

Treatment	Ni (mg kg ⁻¹)
0 (T0 - Control)	1.8*
120 (T120)	67.3
240 (T240)	140.5
360 (T360)	244.7

*Limit of quantification for Ni

Fig. 1 *Canavalia ensiformis* scanning electron microscopy (SEM) image illustrating the standardized steps for measuring trichome density, stomatal density, percentage of open stomata, and stomatal orifice length across all treatments and species



$$EL = \left[1 - \left(E \frac{1}{E} 2 \right) \right] \times 100 \quad (4)$$

Determination of Peroxidase activity (EC 1.11.1.7)

The POX activity was determined in a reaction with 25 mM potassium phosphate buffer, pH 6.8, 20 mM pyrogallol and 20 mM H₂O₂ (Kar and Mishra 1976; Peixoto et al. 1999). The increase in production and absorbance of purpurugalin (420 nm) was measured for one minute. After reading, the activity was calculated using the molar extinction coefficient of 2.47 L mmol⁻¹ cm⁻¹ (Chance and Maehly 1955).

Determination of Superoxide dismutase activity (EC 1.15.1.1)

The SOD activity was determined in a reaction 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 µM p-nitro blue tetrazolium (NBT), 0.1 mM EDTA and 2 µM riboflavin (Longo et al. 1993; Peixoto et al. 1999). The

reaction was carried out in a reaction chamber, under lighting from a 15 W fluorescent lamp, kept inside a closed box. The illumination was stopped after 5 min of light exposure and the NBT was measured as 560 nm (Giannopolitis and Ries 1977). To inhibit NBT photoreduction by 50%, one unit of SOD is the amount of enzyme required (Beauchamp and Fridovich 1971).

Determination of Catalase Activity (EC 1.11.1.6)

The CAT activity was determined in a reaction of 50 mM potassium phosphate buffer, pH 7.0 and 12.5 mM H₂O₂ (Havir and McHale 1987; Peixoto et al. 1999). The decrease in absorbance at 240 nm was measured for one minute. The enzyme activity was calculated from the molar extinction coefficient of 36 L mol⁻¹ cm⁻¹ (Anderson et al. 1995).

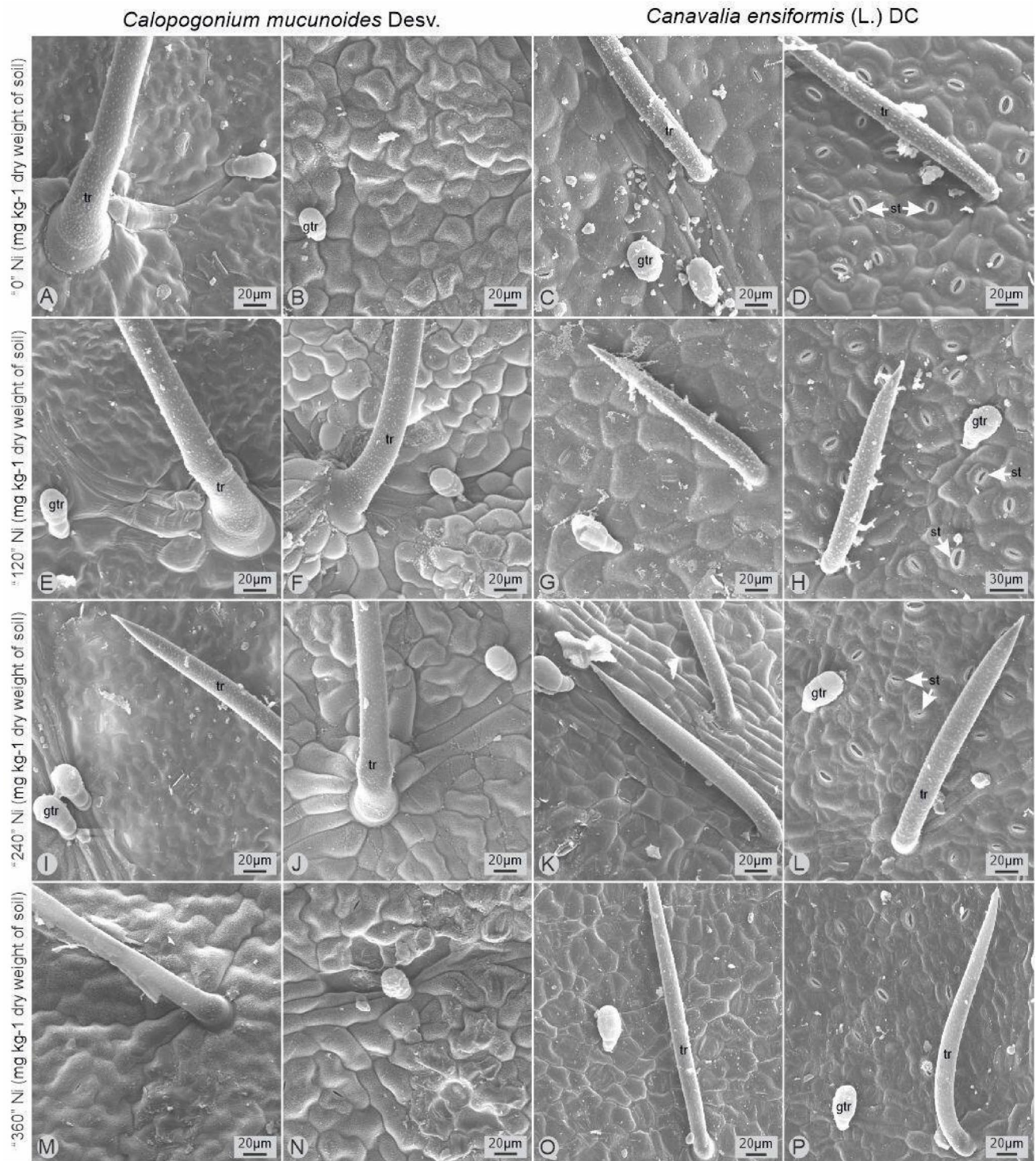


Fig. 2 Electron micrographs of leaf surfaces of: (A, B, E, F, I, J, M, N) *Calopogonium mucunoides*; (C, D, G, H, K, L, O, P) *Canavalia ensiformis*. Panels A, C, E, G, I, K, M, O show adaxial surfaces; B, D, F, H, J, L, N, P show abaxial surfaces. Abbreviations: gtr=glandular

trichome; st=stomata; tr=tector trichome. Note: SEM images and stomatal counts were done on samples collected on different days. Diurnal variation in stomatal aperture may affect visual appearance in SEM images, but density was quantified independently of aperture

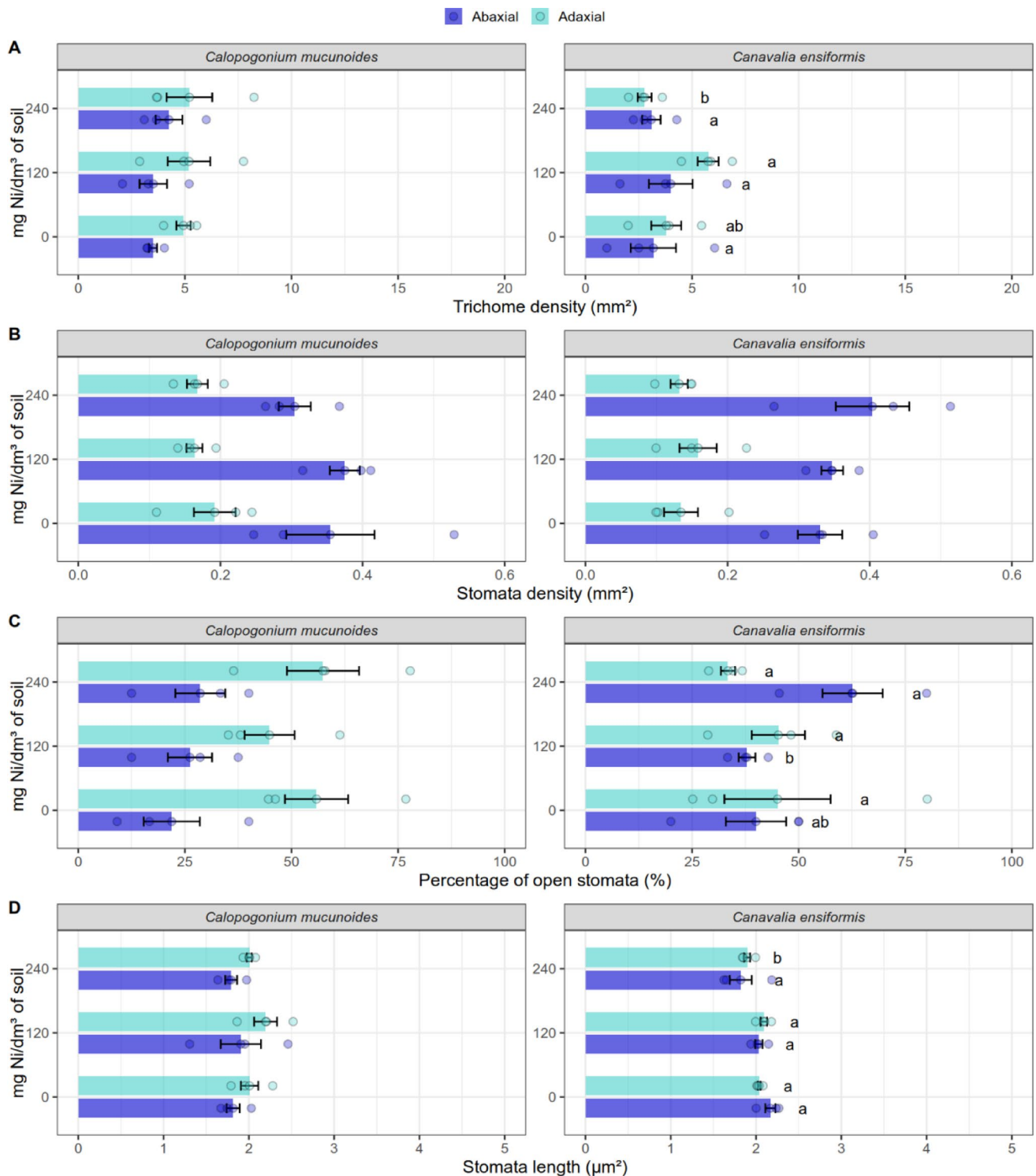


Fig. 3 Morphological measurements from SEM images of *C. mucunoides* and *C. ensiformis* under varying soil nickel (Ni) concentrations (mg dm⁻³): (A) Trichome density (number/mm²); (B) Stomatal density (number/mm²); (C) Percentage of open stomata (%); (D) Length of stomatal orifice (μm). Number of observations: 12. Different letters indicate significant differences among treatments within the same leaf sur-

face, according to Tukey's test ($p < 0.05$). Treatment codes: T0=0 mg dm⁻³ Ni, T120=120 mg dm⁻³ Ni, T240=240 mg dm⁻³ Ni. Note: SEM images and stomatal counts were done on samples collected on different days. Diurnal variation in stomatal aperture may affect visual appearance in SEM images, but density was quantified independently of aperture

Proteins

Total proteins were measured using 2 mL of Bradford solution. After resting for 5 min, total protein content was determined in a spectrophotometer at $\lambda=595$ nm (Bradford 1976).

Membrane Integrity - Evans blue

The location of loss of plasma membrane integrity was detected by staining four leaves in 10 mL of 0.025% Evans blue solution for 10 to 20 min. The stained leaves were washed thrice with 200 mL of CaCl_2 solution (pH 5.6) (Lv et al. 2017; Vijayaraghavareddy et al. 2017; Yamamoto et al. 2001). The four replicates were observed from 4 mm leaf discs made with hollow pliers and a cut in the median region of the leaf from the tip (0 mm) towards the basal region (approximately 4 mm), using a stereomicroscope.

Photosynthetic pigments

Leaf pigments were extracted using the dimethyl sulfoxide (DMSO) method described by Hiscox and Israelstam (1979). Fresh leaf tissue (0.05 g) was cut into ~1 mm fragments and incubated in 7 mL of DMSO at 65 °C for 30 min in the dark. After extraction, the absorbance of the supernatant was measured at 665, 649, and 480 nm using a spectrophotometer. The concentrations of chlorophylls and carotenoids were calculated according to the equations of Wellburn (1994), which are appropriate for DMSO-based extraction: (Eqs. 5–8).

$$\begin{aligned} \text{Chl } a (\mu\text{g mL}^{-1}) \\ = 12.47 \times A_{665} - 3.62 \times A_{649} \end{aligned} \quad (5)$$

$$\begin{aligned} \text{Chl } b (\mu\text{g mL}^{-1}) \\ = 25.06 \times A_{649} - 6.60 \times A_{665} \end{aligned} \quad (6)$$

$$\text{TotalChl } (\mu\text{g mL}^{-1}) = \text{Chl } a + \text{Chl } b \quad (7)$$

$$\begin{aligned} \text{CAR} (\mu\text{g mL}^{-1}) \\ = \frac{(1000 A_{480} - 29 \text{Chl } a - 53.78 \text{Chl } b)}{220} \end{aligned} \quad (8)$$

$$\frac{\text{Cl } a}{b} = \frac{\text{Cl } a}{\text{Cl } b} \quad (9)$$

$$\frac{\text{Cl } t}{\text{CAR}} = \frac{\text{Cl } t}{\text{CAR}} \quad (10)$$

Pigment concentrations were expressed on a fresh-weight basis (mg g^{-1} FW) using the extraction volume (7 mL) and tissue mass (0.05 g), according to:

$$\text{Pigment } (\text{mg g}^{-1} \text{FW}) = \frac{(C \times 7)}{(0.05 \times 1000)} \quad (11)$$

Gas Exchange Measurement

Gas exchange measurements were performed using a portable CIRAS-3 analyzer (PP Systems, 2024) on fully expanded leaves of the second trifoliate from the apex. The measured variables included photosynthetic rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air), and water use efficiency (WUE , $\text{mmol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$). Measurements were taken in the morning between 09:00 and 12:00 under ambient light, with photosynthetically active radiation (PAR) exceeding $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and reference CO_2 set to $390 \mu\text{mol mol}^{-1}$.

Leaf chamber setpoints were as follows: leaf temperature was monitored using the infrared (IR) sensor, vapor pressure deficit (VPD) was controlled by setting the reference humidity to 60%, cuvette flow rate was set to 300 cc min^{-1} , and analyzer flow to 100 cc min^{-1} . Two leaves were enclosed in the PLC3 universal cuvette (18×25 mm window), leaf area was set to 4.50 cm^2 , boundary layer conductance was $0.40 \text{ m}^2 \text{ s mol}^{-1}$, and stomatal ratio was 50%, with auto-zero calibration applied. These settings were maintained consistently across all treatments to ensure standardized measurements.

Minor fluctuations in gas exchange values may occur due to variability in ambient PAR during the 09:00–12:00 measurement window; however, all measurements were conducted under similar conditions across treatments to ensure consistency.

Length, Fresh Mass and Leaf Area

Growth measurements (Benincasa 1988; Hunt 1990) were carried out to obtain the total length and total fresh mass.

For the leaf area, after collecting the plants, the leaves were removed and identified and a millimeter ruler was placed next to the leaves and used to calibrate the software scale. All leaves were then photographed and the images were processed to calculate the area using ImageJ software (Schneider et al. 2012) — using instructions for evaluating leaf area (Martin et al. 2021) with adaptations.

Relative distance plasticity index (RDPI)

This variable was to be obtained for each species and variable through the equation below (Valladares et al. 2006):

$$RDPI = \sum \frac{(d_{ij} \rightarrow i'j' \frac{1}{(x_{i'j'} + x_{ij})})}{n} \quad (12)$$

where i referred to a given level of the environment treatment and i' another given environment different from i . j referred to a given species. Thus X_{ij} is the phenotypic value for a given trait for the species j in the environment i . n is defined as the number of distances calculated.

Statistical Analysis

For descriptive morphological analyses (e.g., SEM imaging and qualitative assessments), all Ni treatments (including T360) were considered to capture the full spectrum of leaf surface responses. However, treatment T360 was excluded from quantitative analyses (e.g., gas exchange, enzymatic activity, pigment concentration, and biomass) due to severely reduced plant development, which led to inconsistent or missing data for several replicates. To maintain statistical validity and balanced replication across treatments, only T0, T120, and T240 were included in the final quantitative dataset.

Analysis of variance (ANOVA) was performed with a significance level of $p < 0.05$, and data showing significant differences were further analyzed using Tukey's test ($p < 0.05$). Statistical tests (e.g., Tukey's test) were based on $n = 4$ replicates per treatment. The packages used for statistical analysis and data organization were ExpDes.pt (Ferreira et al. 2021), plyr (Wickham 2008), dplyr (Wickham et al. 2014) For graphical representation, ggplot2 (Wickham et al. 2023) e ggpubr (Kassambara 2016) were employed. The Pearson correlation analysis was conducted using the corplot package (Wei and Simko 2010), and the Plasticity package (Ameztegui 2017) was used to calculate RDPI.

Statistical analysis was conducted using protocols developed in the R software version 4.4.1 (2024-06-14) - "Race for Your Life" (Core Team 2022) and with Integrated Development Environment (IDE) RStudio (Posit team 2023).

The results are presented as means with standard errors. Details on the analysis of variance, *post-hoc* tests, correlations, and RDPI categories are provided in Supplementary Material 1.

Results

SEM analysis of *C. mucunoides* leaves revealed two morphologically distinct trichome types on both adaxial and abaxial surfaces: long, non-glandular trichomes and shorter, glandular trichomes. Stomatal complexes were present on both surfaces. The adaxial epidermis exhibited subtle undulations of the anticlinal cell walls, whereas the abaxial epidermis showed pronounced grooves at cell junctions. Moreover, the adaxial cuticle was relatively smooth with scattered epicuticular wax granules, in contrast to the abaxial cuticle, which bore abundant, plate-like wax scales (Fig. 2A, B, E, F, I, J, M, N).

Scanning electron microscopy of *C. ensiformis* (Fig. 2C, D, G, H, K, L, O, P) similarly revealed two trichome morphotypes: elongated, non-glandular hairs—though notably shorter than those in *C. mucunoides*—and smaller glandular trichomes. Stomatal complexes occurred on both adaxial and abaxial surfaces, with density markedly greater on the abaxial side. The cuticular layer on both surfaces appeared smooth, punctuated by epicuticular wax granules (Fig. 2D, H, L, P).

Quantitative image analysis generated four metrics—non-glandular trichome density, stomatal density, percentage of open stomata, and stomatal pore length—across treatments and surfaces (Fig. 3). In *C. mucunoides*, non-glandular trichome density did not differ significantly among Ni treatments on either surface. In contrast, *C. ensiformis* exhibited a treatment-dependent response: adaxial non-glandular trichome density was significantly greater under T120 (5.75 ± 0.48) than under T240 (2.77 ± 0.32 ; $p < 0.05$) (Fig. 3A). Other morphological variables for *C. ensiformis* likewise displayed dose-specific shifts, as detailed in Fig. 3B–D.

Although the SEM micrographs (Fig. 2) of *C. mucunoides* appear to show few visible stomata, these images were acquired on a different day than the samples used for density counts. Because stomatal aperture varies diurnally and influences pore visibility in SEM, this qualitative impression does not contradict the quantitative stomatal densities reported in Fig. 3, which were calculated per unit area irrespective of aperture.

Stomatal density showed no statistically significant differences across species, leaf surfaces, or Ni treatments (Fig. 3B), although *C. ensiformis* exhibited slightly higher mean values on the abaxial surface. Likewise, *C. mucunoides* showed no treatment- or surface-dependent variation in the proportion of open stomata. In *C. ensiformis*, however, the abaxial surface under T240 exhibited a significantly higher percentage of open stomata (62.65 ± 7.05) than under T120 (37.89 ± 1.94 ; $p < 0.05$) (Fig. 3C).

Stomatal pore length in *C. mucunoides* also did not differ significantly among treatments or surfaces. In contrast, the adaxial pore length of *C. ensiformis* was significantly greater under T0 ($2.04 \pm 0.05 \mu\text{m}$) and T120 ($2.09 \pm 0.06 \mu\text{m}$) than under T240 ($1.90 \pm 0.04 \mu\text{m}$; $p < 0.01$). On the abaxial surface, pore length showed a similar but non-significant decreasing trend, with mean values of $2.17 \pm 0.08 \mu\text{m}$ (T0), $2.03 \pm 0.06 \mu\text{m}$ (T120), and $1.82 \pm 0.05 \mu\text{m}$ (T240) (Fig. 3D).

Electrolyte leakage and antioxidant enzyme activities revealed distinct species-specific responses (Fig. 4). In *C. mucunoides*, membrane permeability remained stable up to 120 mg kg^{-1} Ni, but increased significantly at 240 mg kg^{-1} (30.73 ± 5.00 vs. 17.85 ± 1.80 ; $p < 0.05$), indicating loss of membrane integrity only under severe Ni stress. By

contrast, *C. ensiformis* maintained membrane stability, with no significant differences in electrolyte leakage across all Ni treatments (Fig. 4A).

Superoxide dismutase (SOD) activity in *C. mucunoides* did not vary significantly among Ni treatments (Fig. 4B). In contrast, *C. ensiformis* exhibited a marked induction of SOD under T240 (8.01 ± 2.03) relative to T0 (1.82 ± 0.61) and T120 (1.37 ± 0.48 ; $p < 0.05$) (Fig. 4B).

Peroxidase (POX) activity in *C. mucunoides* increased significantly at $240 \text{ mg Ni kg}^{-1}$ soil (205.13 ± 8.56) compared to the T0 (154.52 ± 13.02). By contrast, *C. ensiformis* POX activity remained statistically unchanged across all treatments (Fig. 4C).

Catalase (CAT) activity in *C. mucunoides* followed a similar trend, rising to 4.66 ± 0.85 under T240, significantly higher than both T0 (1.81 ± 0.32) and T120 (1.97 ± 0.07 ; $p < 0.05$). *C. ensiformis* CAT activity did not differ among Ni levels (Fig. 4D).

Membrane permeability, assessed by Evans blue uptake, remained low in *C. mucunoides* across treatments, although visible chlorotic symptoms were noted under T120 and T240 (Fig. 5A), suggesting sublethal Ni stress.

Chlorophyll *a* concentration in *C. mucunoides* was significantly higher at T240 (1.26 ± 0.07) compared to T0 (0.74 ± 0.06) and T120 (0.90 ± 0.02 ; $p < 0.05$). No statistically significant changes in chlorophyll *a* were observed in *C. ensiformis* (Fig. 5B).

Chlorophyll *b* concentration in *C. mucunoides* increased significantly under T240 (0.33 ± 0.01) compared to T0 (0.23 ± 0.02) and T120 (0.29 ± 0.0 ; $p < 0.05$), whereas *C. ensiformis* showed no treatment-dependent variation (Fig. 5C). Consequently, total chlorophyll concentration in *C. mucunoides* was highest at T240 (1.59 ± 0.09), significantly exceeding T0 (0.98 ± 0.08) and T120 (1.20 ± 0.01 ; $p < 0.05$), with no corresponding differences in *C. ensiformis* (Fig. 5D).

Carotenoid levels mirrored this pattern: in *C. mucunoides*, T240 yielded 0.36 ± 0.03 , significantly above both T0 (0.24 ± 0.01) and T120 (0.26 ± 0.01 ; $p < 0.05$). Again, *C. ensiformis* carotenoids remained unchanged across Ni doses (Fig. 5E). Both *Chl a/b* and total chlorophyll/carotenoid ratios were statistically invariant among treatments in both species (Figs. 5G, H), indicating proportional pigment adjustments under Ni stress.

Evans blue staining of *C. ensiformis* leaves was used as a qualitative measure of membrane integrity. No dye uptake was observed in T0 and T120, indicating intact membranes, whereas T240 induced localized staining along secondary veins and veinlets, indicative of Ni-induced membrane rupture (Fig. 5F).

Gas-exchange measurements revealed no significant effect of Ni on net photosynthetic rate (Fig. 6A) in *C.*

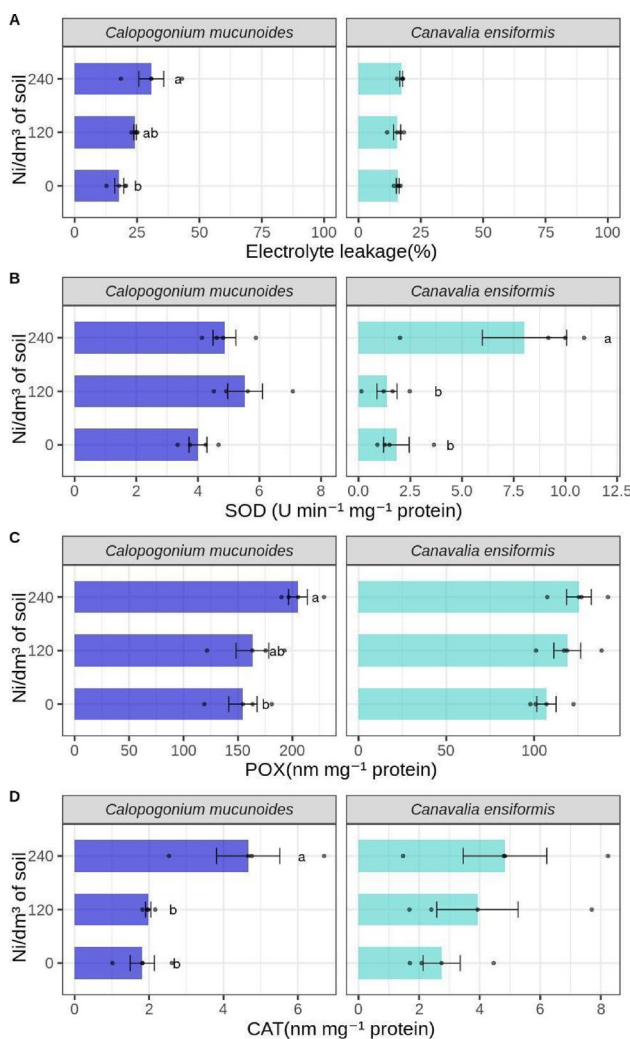


Fig. 4 Membrane stability and antioxidant enzyme activities of *C. mucunoides* and *C. ensiformis* at different Ni soil concentrations (mg dm^{-3}): (A) Electrolyte leakage (EXT, %); (B) Superoxide dismutase (SOD) activity (U mg^{-1} protein); (C) Peroxidase (POX) activity (nm mg^{-1} protein); (D) Catalase (CAT) activity (nm mg^{-1} protein). Number of observations: 12. Different letters indicate significant differences by Tukey's test ($p < 0.05$)

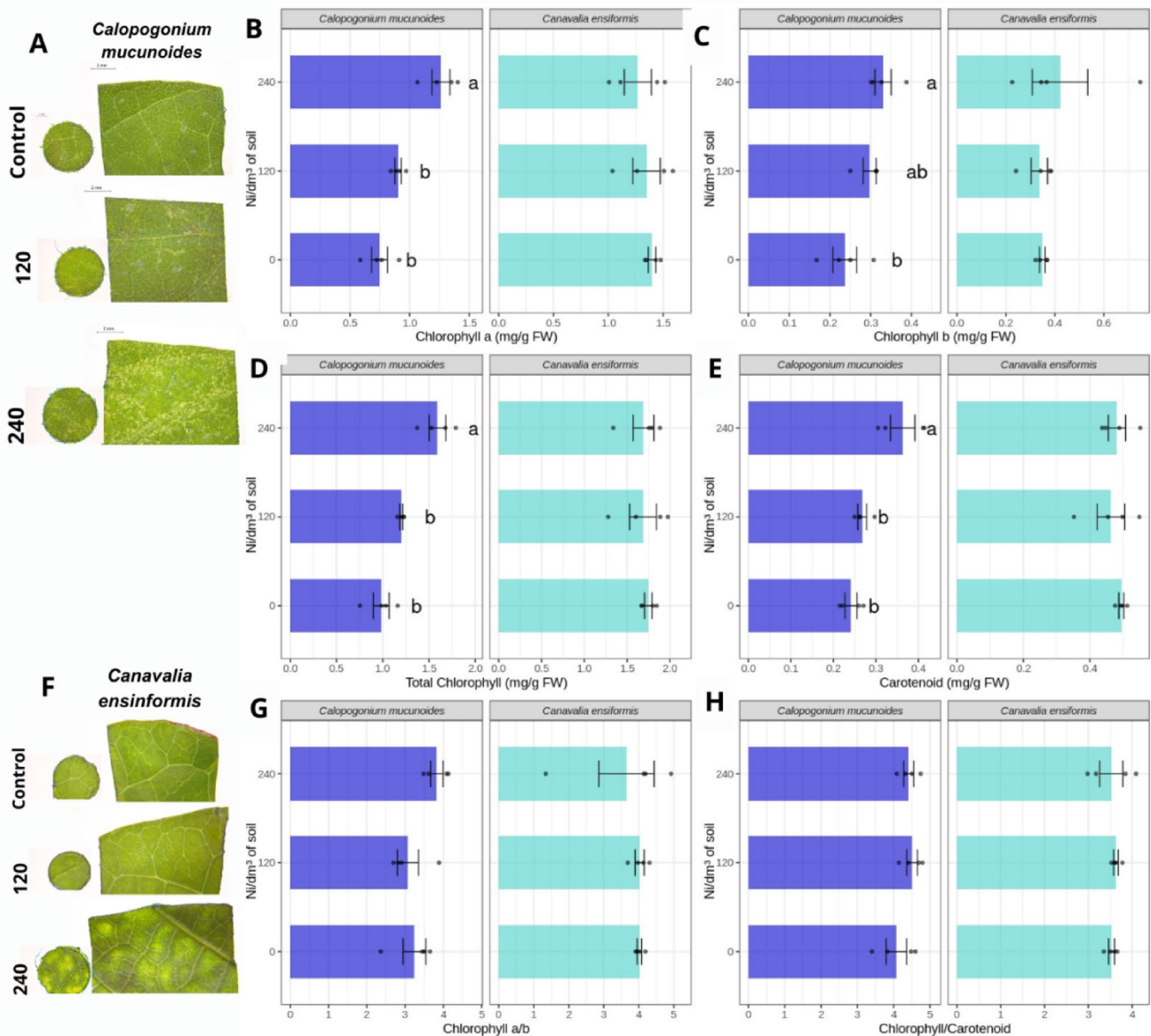


Fig. 5 Leaf pigment quantification and membrane permeability staining with Evans Blue for *C. mucunoides* and *C. ensiformis* under different Ni soil concentrations (mg dm^{-3}): (A, F) Representative leaves stained with Evans Blue; (B) Chlorophyll a (mg g^{-1} fresh weight); (C) Chlorophyll b (mg g^{-1} fresh weight); (D) Total chlorophyll (mg g^{-1}

fresh weight); (E) Carotenoids (mg g^{-1} fresh weight); (G) Chlorophyll a/b ratio; (H) Total chlorophyll/carotenoid ratio. Number of observations: 12. Different letters indicate significant differences by Tukey's test ($p < 0.05$)

mucunoides. In contrast, *C. ensiformis* exhibited a pronounced increase in A under T240 (10.67 ± 1.14) relative to T0 (4.82 ± 0.50) and T120 (3.45 ± 0.80 ; $p < 0.05$) (Fig. 6A). This suggests that moderate Ni exposure may stimulate photosynthetic capacity in *C. ensiformis*, whereas *C. mucunoides* maintains stable photosynthetic performance across the tested Ni gradient.

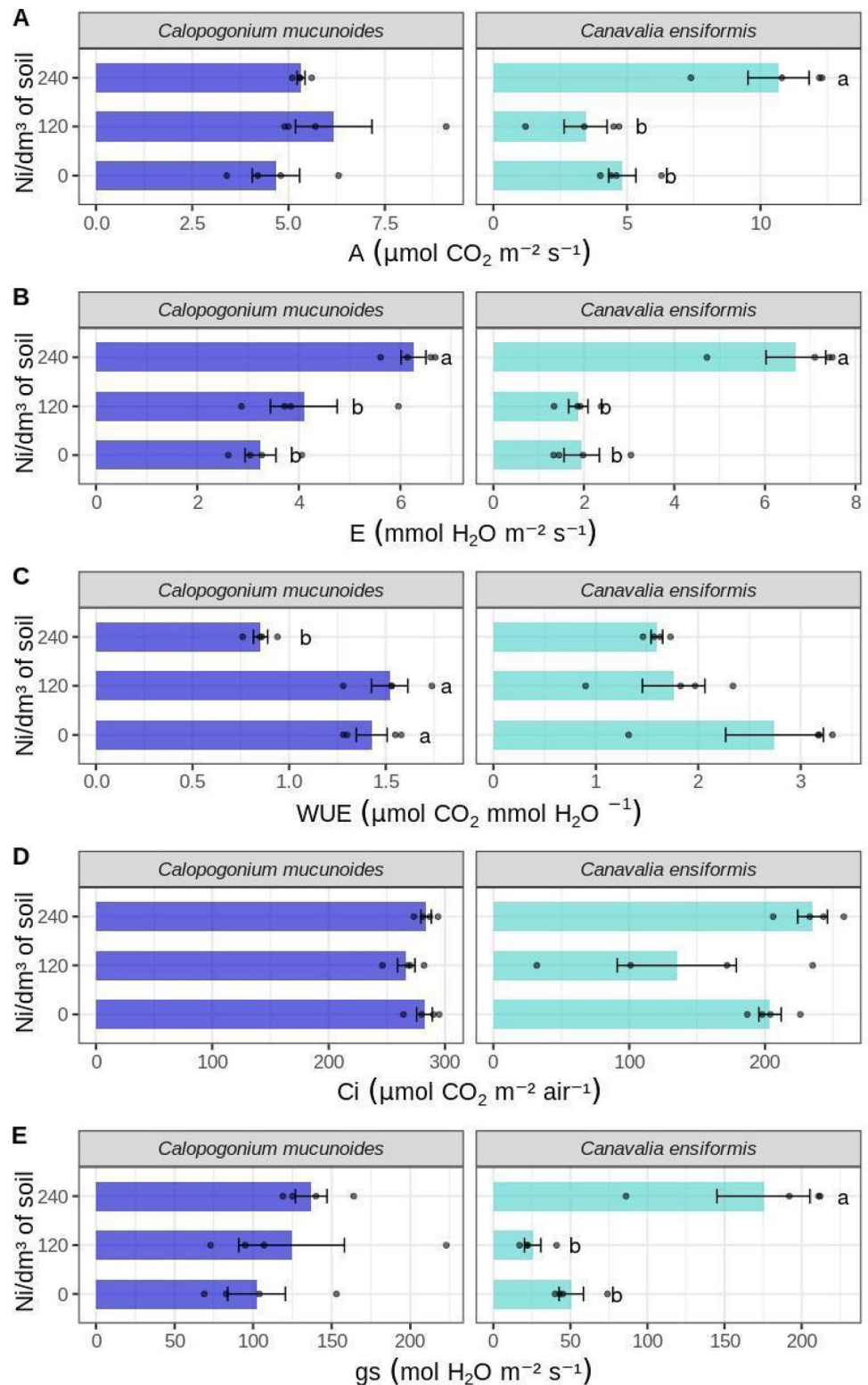
Transpiration rate (E) increased significantly under high Ni exposure in both species (Fig. 6B). In *C. mucunoides*, E rose from 3.24 ± 0.30 at T0 and 4.09 ± 0.65 at T120 to 6.26 ± 0.24 at T240 ($p < 0.05$). *C. ensiformis* exhibited a

similar pattern, with E increasing from 1.95 ± 0.38 (T0) and 1.87 ± 0.21 (T120) to 6.68 ± 0.65 at T240 ($p < 0.05$).

Water-use efficiency (WUE; A/E) in *C. mucunoides* declined significantly under T240 (0.85 ± 0.03) compared to T0 (1.42 ± 0.08) and T120 (1.52 ± 0.09 ; $p < 0.05$). In *C. ensiformis*, WUE remained statistically unchanged across Ni treatments (Fig. 6C).

Interacellular CO_2 concentration (C_i) did not differ significantly among treatments in either species (Fig. 6D), indicating that CO_2 diffusion to the substomatal cavity was maintained.

Fig. 6 Gas exchange parameters of *C. mucunoides* and *C. ensiformis* at different Ni soil concentrations (mg dm^{-3}): **(A)** Net photosynthesis (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); **(B)** Transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$); **(C)** Water use efficiency (WUE, $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$); **(D)** Internal CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$); **(E)** Stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$). Number of observations: 12. Different letters indicate significant differences by Tukey's test ($p < 0.05$)

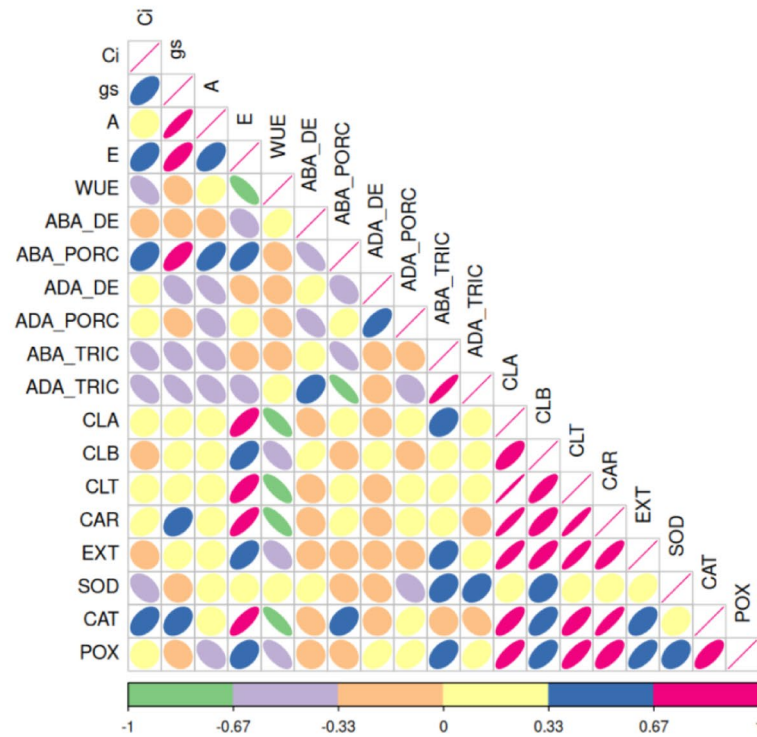


Stomatal conductance (g_s) in *C. mucunoides* showed no significant treatment effect. Conversely, *C. ensiformis* exhibited a pronounced increase in g_s under T240 (175.25 ± 30.10) relative to T0 (50.50 ± 7.90) and T120 (25.50 ± 5.29 ; $p < 0.05$) (Fig. 6E). This elevated stomatal

aperture likely underlies the observed increases in both E and net photosynthesis in *C. ensiformis* under moderate Ni stress.

Pearson correlation analysis in *C. mucunoides* (Fig. 7A) demonstrated several significant interrelationships among

A *Calopogonium mucunoides*



B *Canavalia ensiformis*

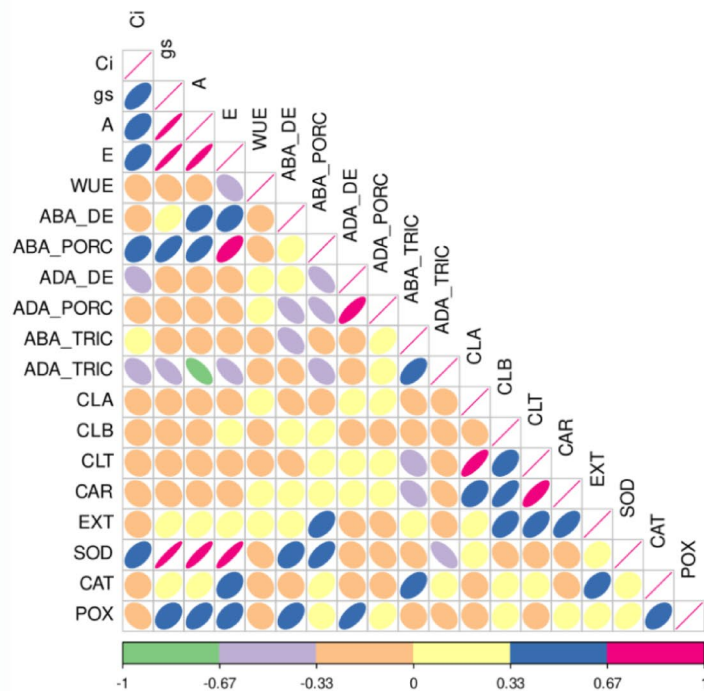


Fig. 7 Pearson correlation for morphophysiological variables of (A) *C. mucunoides* and (B) *C. ensiformis*. Circle size and intensity represent the magnitude of the correlation; color indicates the direction and value of the coefficient (scale: -1 to 1). Colors: negative correlations range from green (strongly negative, $r \approx -1$) to purple (moderately negative); correlations near zero are shown in orange/yellow; positive correlations range from blue (moderately positive) to magenta (strongly positive, $r \approx 1$). Classification of coefficients: $r = 0.10$ – 0.30 (weak); $r = 0.40$ – 0.60 (moderate); $r = 0.70$ – 1.00 (strong) (Dancey and Reidy 2005). Abbreviations: ABA_DE=Abaxial stomatal density; ADA_DE=Adaxial stomatal density; ABA_PORC=Percentage of open stomata on the abaxial surface; ADA_PORC=Percentage of open stomata on the adaxial surface; ABA_TRIC=Trichome density on the abaxial surface; ADA_TRIC=Trichome density on the adaxial surface; EXT=Electrolyte leakage. Numerical values of all correlations are provided in Supplementary Material 3

gas-exchange, pigment, enzyme, and anatomical traits. Intracellular CO_2 concentration (C_i) correlated moderately and negatively with water-use efficiency (WUE) and nonglandular trichome density on both adaxial and abaxial surfaces, suggesting that plants with lower WUE and sparser trichomes tended to accumulate more internal CO_2 . Stomatal conductance (g_s) was strongly and positively associated with net photosynthesis (A) and transpiration (E), and showed a moderate positive correlation with the percentage of open stomata on the abaxial surface; conversely, g_s correlated moderately and negatively with adaxial stomatal density and trichome density on both surfaces. Net photosynthesis (A) exhibited moderate positive correlations with E and with the proportion of open stomata, and moderate negative correlations with peroxidase activity (POX), adaxial stomatal density, and trichome densities. Transpiration (E) correlated strongly and negatively with WUE, and positively with catalase activity (CAT) and pigment concentrations (chlorophyll *a*, total chlorophyll, and carotenoids). In turn, WUE displayed strong negative correlations with CAT and all measured pigments, and moderate negative correlations with chlorophyll *b*, electrolyte leakage (EL), and POX activity—indicating that greater oxidative and pigment-based stress responses coincide with reduced water-use efficiency. In *C. mucunoides*, abaxial stomatal density correlated moderately and negatively with the percentage of open stomata on both surfaces, whereas adaxial stomatal density correlated moderately and positively with the percentage of open stomata on the adaxial surface. The percentage of open abaxial stomata exhibited a strong negative correlation with adaxial trichome density, while adaxial and abaxial trichome densities themselves were strongly and positively interrelated (Fig. 7A).

Chlorophyll *a* (Chl *a*) displayed strong positive correlations with Chl *b*, total chlorophyll, carotenoids, electrolyte leakage, catalase, and peroxidase activities. Chl *b* correlated strongly with total chlorophyll and carotenoids, and moderately with catalase and peroxidase. Total chlorophyll was

strongly associated with carotenoids, electrolyte leakage, catalase, and peroxidase. Carotenoids (CAR) showed strong positive correlations with electrolyte leakage, CAT, and POX. Electrolyte leakage exhibited moderate positive correlations with CAT and POX. Superoxide dismutase (SOD) showed moderate positive correlations with POX, while CAT was strongly positively correlated with POX (Fig. 7A). These interdependencies underscore how pigment accumulation and oxidative-stress responses are tightly linked in *C. mucunoides* under Ni stress.

In *C. ensiformis*, Pearson correlation analysis (Fig. 7B) identified several notable trait interdependencies under Ni stress. Intracellular CO_2 concentration (C_i) correlated moderately and positively with stomatal conductance (g_s), net photosynthesis (A), transpiration (E) and superoxide dismutase activity (SOD), while exhibiting a moderate negative correlation with adaxial trichome density (ADA_TRIC). Stomatal conductance (g_s) was strongly and positively associated with A, E and SOD, and moderately positively correlated with the percentage of open stomata on the abaxial surface (ABA_PORC); it also showed a moderate negative correlation with ADA_TRIC. Net photosynthesis (A) demonstrated strong positive correlations with E and SOD, moderate positive correlations with peroxidase activity (POX), abaxial stomatal density (ABA_DE) and ABA_PORC, and a moderate negative correlation with ADA_TRIC. Transpiration (E) correlated strongly with SOD, moderately with POX and ABA_PORC, and inversely with water-use efficiency (WUE) and ADA_TRIC.

Abaxial stomatal density (ABA_DE) exhibited moderate negative correlations with ADA_PORC and ADA_TRIC, whereas the percentage of open abaxial stomata (ABA_PORC) correlated moderately negatively with ABA_DE, ADA_PORC and ADA_TRIC, and moderately positively with electrolyte leakage (EL) and SOD. Adaxial stomatal density (ADA_DE) correlated strongly with ADA_PORC and moderately with POX. Abaxial trichome density (ABA_TRIC) showed moderate positive correlations with ADA_TRIC and catalase activity (CAT), and a moderate negative correlation with carotenoid concentration (CAR). Conversely, ADA_TRIC correlated moderately and negatively with SOD (Fig. 7B).

Among pigments, chlorophyll *a* (Chl *a*) correlated strongly with total chlorophyll (Clt) and moderately with CAR. Chlorophyll *b* (Chl *b*) exhibited moderate positive correlations with Clt, CAR and EL. Total chlorophyll (Clt) correlated strongly with CAR and moderately with EL. Carotenoids (CAR) showed a moderate positive correlation with EL. Electrolyte leakage (EL) correlated moderately with CAT, and CAT correlated moderately with POX (Fig. 7B). These patterns suggest that, in *C. ensiformis*, enhanced gas-exchange and antioxidative enzyme activities

are coordinated with morphological adjustments and pigment dynamics under Ni exposure.

Morphological evaluation of leaflet shape (Fig. 8A, C) revealed that both species developed more rounded leaflets under T120 and T240, with *C. mucunoides* additionally showing a clear reduction in trifoliate size relative to the T0 (Fig. 8A). While *C. mucunoides* exhibited no significant change in the number of expanded trifoliates across treatments, *C. ensiformis* experienced a significant decline under T240 (1.25 ± 0.15) compared to both T0 (2.75 ± 0.20) and T120 (2.25 ± 0.18 ; $p < 0.05$) (Fig. 8B).

Fresh mass (FM) of *C. mucunoides* was significantly greater in the T0 treatment (3.01 ± 0.25) compared to T120 (1.20 ± 0.17 g) and T240 (0.67 ± 0.08). Similarly, *C. ensiformis* exhibited the highest FM in T0 (44.38 ± 2.64), followed by T120 (30.50 ± 1.90 g), with T240 (14.23 ± 1.17 g) showing the lowest value (Fig. 8D).

Aerial part length in *C. mucunoides* was significantly greater under T0 (8.66 ± 0.51 cm) than in T120 (4.50 ± 0.49) and T240 (4.33 ± 0.43). In *C. ensiformis*, the T120 treatment (33.16 ± 1.68) led to the greatest length, followed by T0 (26.16 ± 1.41) and T240 (23.00 ± 1.30) (Fig. 8E).

Leaf area (LA) in *C. mucunoides* was highest under T0 (14.28 ± 1.45), significantly decreasing in T120 (3.34 ± 0.54) and T240 (2.63 ± 0.48). In *C. ensiformis*, LA was also greatest in T0 (74.50 ± 4.12), followed by T120 (55.74 ± 3.25) and significantly lowest in T240 (18.31 ± 1.76) (Fig. 8F).

The relative distance plasticity index (RDPI), ranging from 0 (no plasticity) to 1 (maximum plasticity), was calculated for each variable and species according to Valladares et al. (2006). Based on the classification proposed for *Pimenta pseudocaryophyllus* by Mendonça et al. (2019), RDPI values < 0.5 were considered indicative of low plasticity. This criterion was adopted in the present study to

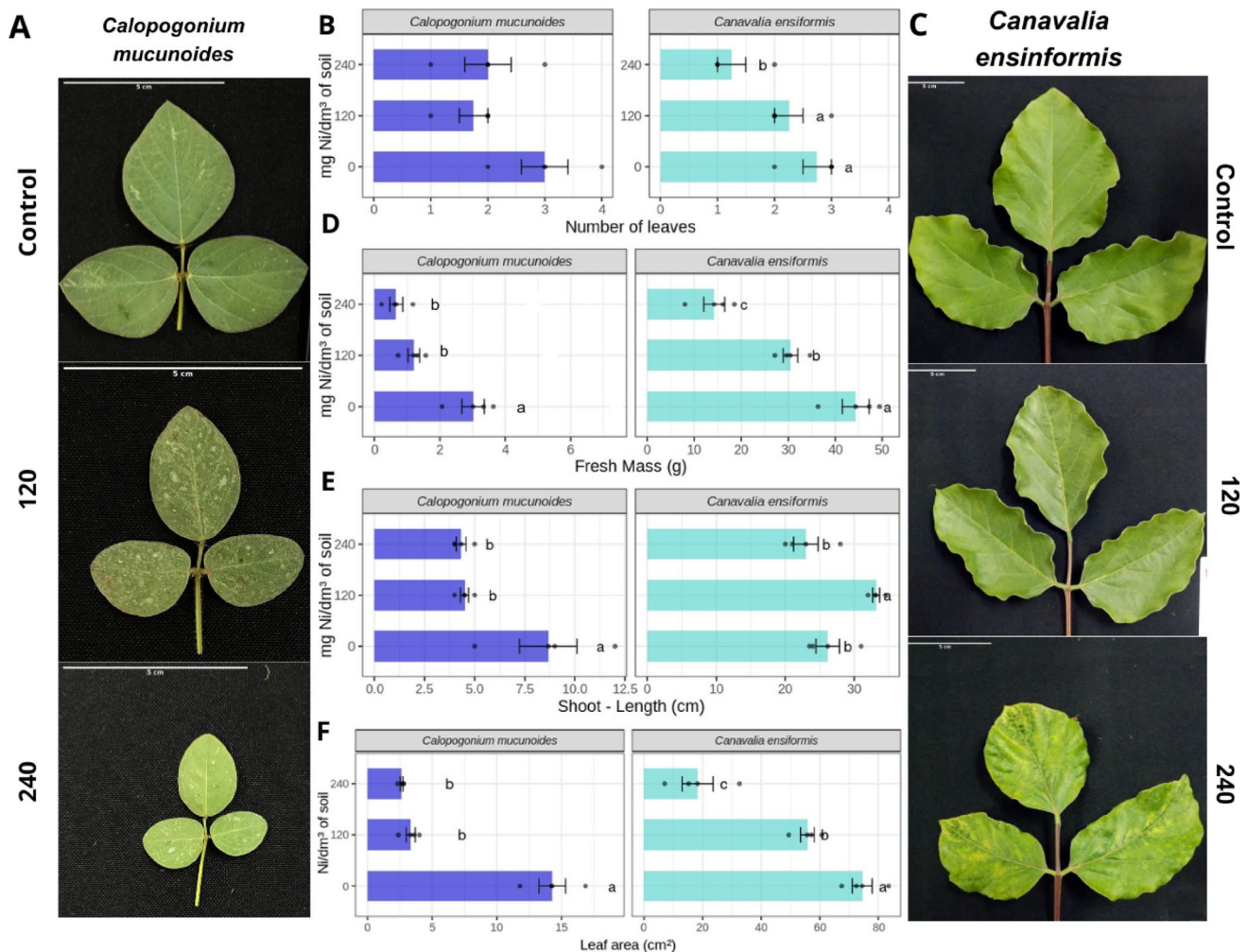
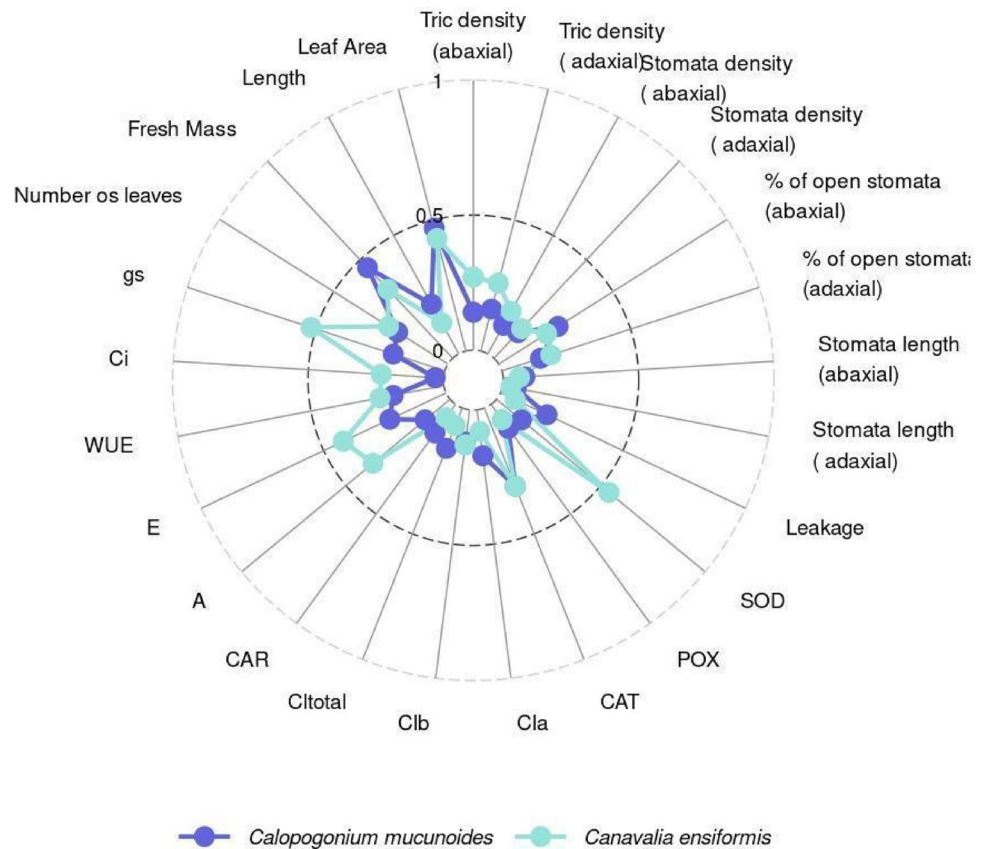


Fig. 8 Trifoliate morphology and growth parameters of *C. mucunoides* and *C. ensiformis* at varying Ni soil concentrations (mg dm^{-3}): (A) Leaf morphology of *C. mucunoides* across treatments; (B) Number of leaves; (C) Leaf morphology of *C. ensiformis* across treatments; (D)

Fresh mass (g); (E) Length of aerial part (cm); (F) Leaf area (cm^2). Number of observations: 12. Different letters indicate significant differences by Tukey's test ($p < 0.05$)

Fig. 9 Relative Distance Plasticity Index (RDPI) for morphological, physiological, and growth traits of *C. mucunoides* and *C. ensiformis* under different Ni soil concentrations. High plasticity defined as RDPI > 0.5. Treatment codes as in previous figures



interpret plasticity levels under nickel stress for both *C. mucunoides* and *C. ensiformis*.

Analysis of the relative distance plasticity index (RDPI) for morphological traits revealed species-specific patterns in response to nickel treatments. In *C. mucunoides*, RDPI values were low across all evaluated variables: 0.14 for trichome density on the abaxial surface and 0.16 on the adaxial surface; 0.12 for stomatal density on the abaxial surface and 0.13 on the adaxial surface; 0.26 for the percentage of open stomata on the abaxial surface and 0.15 on the adaxial surface; and 0.08 for stomatal orifice length on the abaxial surface and 0.05 on the adaxial surface.

In contrast, *C. ensiformis* exhibited higher plasticity for most traits: 0.27 for trichome density on the abaxial surface and 0.26 on the adaxial surface; 0.18 for stomatal density on the abaxial surface and 0.15 on the adaxial surface; 0.21 for the percentage of open stomata on the abaxial surface and 0.19 on the adaxial surface; and 0.06 for stomatal orifice length on the abaxial surface and 0.03 on the adaxial surface (Fig. 9).

Regarding physiological traits, *C. mucunoides* exhibited RDPI values of 0.19 for electrolyte leakage, 0.12 for superoxide dismutase (SOD), 0.11 for peroxidase (POX), 0.31 for catalase (CAT), 0.17 for chlorophyll *a* (CLA), 0.12 for chlorophyll *b* (CLB), 0.16 for total chlorophyll (CLT), 0.13 for carotenoids (CAR), 0.12 for net photosynthesis (A), 0.23

for transpiration (E), 0.19 for water-use efficiency (WUE), 0.03 for intracellular CO₂ concentration (Ci), and 0.20 for stomatal conductance (gs).

Conversely, *C. ensiformis* showed higher RDPI values for several physiological variables: 0.06 for electrolyte leakage, 0.54 for SOD, 0.07 for POX, 0.31 for CAT, 0.08 for CLA, 0.13 for CLB, 0.07 for CLT, 0.07 for CAR, 0.37 for A, 0.42 for E, 0.24 for WUE, 0.23 for Ci, and 0.52 for gs (Fig. 9).

In terms of growth responses, *C. mucunoides* presented RDPI values of 0.22 for leaflet number, 0.46 for fresh mass, 0.21 for shoot length, and 0.47 for leaf area. For *C. ensiformis*, the values were 0.26 for leaflet number, 0.35 for fresh mass, 0.13 for shoot length, and 0.43 for leaf area (Fig. 9).

Discussion

Nickel uptake and translocation within plants occur via a combination of passive diffusion and active transport, followed by movement to aerial tissues through the transpiration stream. This process is mediated by both specific and non-specific transport channels (Ameen et al. 2019). Because leaves are the primary photosynthetic organs, many species employ mechanisms to restrict heavy metal accumulation in these tissues, minimizing damage to

photosynthetically active cells (Yadav et al. 2021). Such mechanisms include metal chelation, vacuolar sequestration, and the induction of antioxidant responses (Ameen et al. 2019), which vary depending on species, exposure time, and metal concentration.

Nickel stress triggers complex responses in plants, influencing nutrient uptake, photosynthesis, and structural integrity (Ameen et al. 2019; Yadav et al. 2021). This study revealed that *Calopogonium mucunoides* and *Canavalia ensiformis* respond to increasing Ni concentrations through distinct acclimation strategies, aligning with our initial hypothesis of species-specific responses involving both structural and biochemical traits.

The epidermis, particularly through structures such as trichomes and cuticle, plays a fundamental role in mediating plant–environment interactions under abiotic stress, including heavy metal exposure (Karabourniotis et al. 2020). Trichomes, in particular, contribute to regulating transpiration, light reflectance, and metal sequestration, thus serving as potential indicators of stress tolerance.

In this study, *C. mucunoides* exhibited stable morphological traits under nickel stress, with no significant variation in trichome or stomatal densities across treatments, and consistently low RDPI values for these traits (≤ 0.16). This pattern suggests a morphologically conservative response to Ni exposure, possibly associated with avoidance or internal detoxification strategies. Conversely, *C. ensiformis* exhibited a significant increase in adaxial trichome density under moderate Ni exposure (T120), followed by a decrease at higher concentration (T240), indicating a plastic response modulated by stress intensity. The RDPI values for trichome density in *C. ensiformis* reached 0.26–0.27, reinforcing its greater morphological sensitivity.

This modulation in *C. ensiformis* may reflect structural adjustments to mitigate Ni uptake through increased barrier function at moderate concentrations, followed by possible tissue damage at higher doses. Similar patterns have been observed in *Lavandula angustifolia*, where trichome reduction accompanied increasing Fe₂O₃ nanoparticle levels (Talebi et al. 2022), and in *Rumex acetosella*, where high trichome density was associated with metal accumulation and transpiration control in Cu-contaminated soils (Čiamporová et al. 2021). In *Glycine max*, decreased trichome density increased endocytic activity and metal accumulation, including Pb (Ben et al. 2021), indicating that trichome modulation is a species-dependent strategy under metal stress.

Additionally, while stomatal density showed little variation in *C. mucunoides* (RDPI ≤ 0.13), *C. ensiformis* displayed moderate plasticity (RDPI = 0.15–0.18), especially on the abaxial surface. This variation was accompanied by changes in the percentage of open stomata and stomatal orifice length. Notably, in *C. ensiformis*, a reduction

in stomatal orifice length at high Ni concentration (T240) coincided with increased electrolyte leakage and trichome loss, suggesting compromised epidermal regulation.

Altogether, these results highlight divergent morphoanatomical strategies between the two legumes. *C. mucunoides* appears to maintain structural integrity and restrict variation under Ni stress, while *C. ensiformis* demonstrates plastic adjustments in trichome and stomatal traits that may reflect greater physiological responsiveness or sensitivity to Ni accumulation.

The observed modulation of trichome density in *C. ensiformis*, particularly the increase at T120 followed by a reduction at T240, suggests an active acclimation response to nickel exposure. This behavior contrasts with *C. mucunoides*, in which trichome density remained stable across treatments. These species-specific responses may reflect different strategies for coping with metal stress: structural plasticity in *C. ensiformis* versus morphological stability in *C. mucunoides*.

Trichomes have been implicated in metal accumulation and tolerance. In soybean, synchrotron-based X-ray fluorescence (XFM) demonstrated Ni accumulation in trichome pedicels following foliar application, supporting the hypothesis that trichomes can serve both as entry points and temporary sinks for metals (Oliveira et al. 2024). The variation in trichome density in *C. ensiformis* may indicate a regulatory mechanism to control Ni uptake or mitigate its toxicity, consistent with patterns described in *Rumex acetosella* growing on Cu-contaminated soils (Čiamporová et al. 2021).

This adaptive potential is further supported by the moderate RDPI values for trichome density in *C. ensiformis* (0.26–0.27), in contrast to the low plasticity observed in *C. mucunoides* (≤ 0.16). Such plasticity may be linked to genetic pathways involved in trichome development and stress signaling, as proposed by Zhang et al. (2021), although further investigation is needed to clarify these regulatory networks.

Members of the Fabaceae family, including *C. ensiformis* and *C. mucunoides*, are recognized for their trichomes' capacity to accumulate metals due to their anatomical and biochemical characteristics. Metal-binding ligands and transporters, along with the anatomical connection of basal trichome cells to the vascular system, facilitate this accumulation (Li et al. 2023). Elements such as Zn, Mn, Ni, and Cd are frequently reported in trichomes of different species. In *Nicotiana tabacum* HK326, higher densities of glandular trichomes enhanced Cd tolerance through excretion mechanisms (Zhang et al. 2021). Although our study did not quantify Ni in trichomes, the observed variation in density under stress in *C. ensiformis* suggests that this structure may contribute to Ni tolerance in legumes. Future studies should

quantify metal content in these structures to clarify their functional role.

In addition to trichome adjustments, *C. ensiformis* exhibited stomatal plasticity under Ni stress. At T240, the abaxial surface showed an increased percentage of open stomata compared to T120, while the adaxial surface exhibited a significant reduction in stomatal orifice length relative to T0 and T120. These contrasting patterns may reflect a compensatory adjustment aimed at optimizing gas exchange while limiting water loss under high Ni availability. Similar mechanisms have been reported in *Cichorium intybus* exposed to Pb, where reduced stomatal dimensions and partial closure were interpreted as a detoxification strategy (El-Banna et al. 2019). In plants under Al stress, stomatal closure reduces transpiration and limits metal translocation to aerial tissues (Guo et al. 2024), a mechanism that may also be relevant for Ni.

These results suggest that *C. ensiformis* exhibits greater morphological and anatomical plasticity under Ni stress, particularly through trichome and stomatal modulation, which may serve as adaptive strategies to regulate metal uptake and minimize physiological damage. In contrast, *C. mucunoides* maintain low morphological plasticity and may rely on other mechanisms, such as antioxidant defense, to cope with Ni exposure.

The stable net photosynthesis (A) observed in *C. mucunoides* across treatments indicates a tolerance strategy in which photosynthetic capacity is maintained despite increasing Ni levels. In contrast, *C. ensiformis* showed a significant increase in A at T240, suggesting a distinct acclimation mechanism that enhances carbon assimilation under metal stress. This pattern reflects species-specific strategies to cope with nickel exposure, supported by the differing RDPI values for A (0.12 in *C. mucunoides* vs. 0.37 in *C. ensiformis*), with the latter exhibiting greater photosynthetic plasticity.

Both species showed elevated transpiration rates (E) under the highest Ni concentration (T240), likely as a thermoregulatory strategy to dissipate excess heat and maintain physiological homeostasis. However, while *C. mucunoides* experienced a significant decline in water use efficiency (WUE), *C. ensiformis* maintained WUE across treatments. This decoupling between A and E in *C. mucunoides* suggests a cost in water economy, possibly reflecting an imbalance between carbon gain and water loss. Conversely, the ability of *C. ensiformis* to increase A without compromising WUE highlights a more efficient gas exchange regulation under stress.

Photosynthetic plasticity differed markedly between the species. While *C. mucunoides* maintained stable photosynthetic rates across treatments (RDPI=0.12), *C. ensiformis* significantly increased net photosynthesis under T240

(RDPI=0.37), coupled with sustained WUE. This highlights an adaptive trait in *C. ensiformis*, enabling it to optimize carbon assimilation under Ni stress without excessive water loss.

These findings align with the understanding that xylem transport and stomatal behavior are closely linked to ionic stress. According to Rucińska-Sobkowiak (2016), both stomatal conductance and root pressure modulate the pressure gradient in the xylem, and metal-induced anatomical changes may reduce water transport efficiency. The responses observed here reinforce that *C. ensiformis* exhibits a more integrated and plastic gas exchange system than *C. mucunoides*, maintaining carbon gain without excessive water loss under Ni stress.

Photosynthetic responses are inherently tied to oxidative stress. Reactive oxygen species (ROS) generated under heavy metal exposure can impair essential biomolecules and processes, including enzyme activity, membrane integrity, and nucleic acid stability (Mansoor et al. 2023). To counteract ROS, plants rely on enzymatic antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX), which act in a coordinated manner to detoxify superoxide and hydrogen peroxide (Apel and Hirt 2004; Hasanuzzaman et al. 2020).

In *C. mucunoides*, the T240 treatment induced a significant increase in electrolyte leakage (EXT), along with elevated CAT and POX activities, indicating an active antioxidant response to oxidative damage. The low RDPI for SOD (0.12) and moderate values for CAT (0.31) and POX (0.11) suggest limited enzymatic flexibility, possibly contributing to compromised membrane integrity despite enzyme induction. In contrast, *C. ensiformis* showed no significant change in EXT and a marked increase in SOD activity at T240, consistent with higher plasticity in this enzyme (RDPI=0.54). This coordinated antioxidant response appears more effective in limiting ROS accumulation and preserving cellular homeostasis in *C. ensiformis*.

Overall, the contrasting physiological profiles between the species suggest that *C. ensiformis* exhibits a more adaptive phenotype under Ni exposure, balancing gas exchange and oxidative stress responses. Meanwhile, *C. mucunoides* displays a stress-tolerant but less plastic strategy, maintaining photosynthesis at the expense of increased water loss and membrane damage.

Nickel exposure often affects photosynthesis indirectly, primarily by reducing chlorophyll concentration and limiting stomatal conductance, as observed in *Cajanus cajan* L. (Sheoran et al. 1990). However, contrasting responses were found in *C. mucunoides*, which exhibited a significant increase in chlorophyll *a*, *b*, and carotenoids under high Ni concentration (T240). The elevated chlorophyll *b* levels suggest an adjustment in light-harvesting pigment

composition to optimize photosynthetic efficiency under stress. Carotenoids, especially xanthophylls and β -carotene, play crucial roles in photoprotection and are closely associated with thylakoid membrane stability (Rudenko et al. 2023). This response indicates that *C. mucunoides*, despite its lower photosynthetic plasticity, activates non-enzymatic antioxidant strategies to mitigate photodamage.

In contrast, *C. ensiformis* maintained stable levels of photosynthetic pigments across treatments, suggesting pigment homeostasis under nickel exposure. The absence of chlorosis and the sustained pigment concentration, even at T240, may reflect a more efficient ROS detoxification system involving unmeasured non-enzymatic antioxidants, such as ascorbic acid and glutathione. This stability coincides with an increased photosynthetic rate and transpiration in T240 without compromising water-use efficiency, highlighting a more resilient physiological profile. However, the concomitant reduction in leaf area and fresh biomass at this concentration suggests a decoupling between carbon assimilation and biomass accumulation, possibly indicating constraints on carbon use efficiency or a shift in resource allocation under stress.

By contrast, *C. ensiformis* maintained pigment stability across treatments, which may reflect a reliance on unmeasured non-enzymatic antioxidants (e.g., ascorbate, glutathione). These divergent pigment responses reinforce the view that while *C. mucunoides* attempts to preserve photosynthesis through pigment compensation, *C. ensiformis* maintains stability via upstream oxidative control mechanisms.

This phenomenon has also been observed in *Phaseolus vulgaris* L., where no direct impairment in primary photochemistry was detected via chlorophyll *a* fluorescence under Ni stress, but downstream processes such as carbon metabolism were significantly affected (Krupa et al. 1993). In *C. ensiformis*, the increase in photosynthetic activity at T240 may not translate into growth due to possible metabolic bottlenecks in the Calvin cycle or energy redirection toward defense mechanisms. Further enzymatic and transcriptomic analyses are needed to determine whether carbon fixation enzymes or stress-responsive metabolic pathways are responsible for this apparent trade-off.

The integration of morphoanatomical and physiological responses reveals distinct strategies between the two species. While *C. mucunoides* responded with pigment accumulation and antioxidant enzyme induction (notably CAT and POX), these responses were accompanied by increased membrane damage, indicating limited physiological plasticity and potentially higher cellular stress. In contrast, *C. ensiformis* showed higher plasticity in stomatal conductance and SOD activity (RDPI=0.52 and 0.54, respectively), suggesting an active and flexible adjustment to stress conditions. The Relative Distance Plasticity Index (RDPI), as proposed

by Valladares et al. (2006), offers a robust measure of phenotypic responsiveness without assumptions about data distribution, being especially useful in studies with limited replication.

These findings suggest that *C. ensiformis* adopts a more dynamic acclimation strategy, involving coordinated regulation of gas exchange and ROS detoxification. The low electrolyte leakage and stable pigment levels, even under high Ni exposure, support its potential for greater resilience and may be indicative of early stress perception and more efficient signaling pathways. This ability to fine-tune physiological responses reflects an adaptive phenotype that prioritizes functional stability and resource efficiency under abiotic stress.

In contrast, *C. mucunoides* seems to rely on a more constitutive defense, with lower plasticity in key traits. While this may confer some tolerance, it also appears to impose costs in terms of water-use efficiency and membrane stability under increasing Ni levels. The divergent strategies observed between the two legumes underscore the ecological and agronomic relevance of phenotypic plasticity as a determinant of stress resilience.

Although plasticity allows short-term acclimation, it is important to note that in hyperaccumulator species such as *Alyssum serpyllifolium*, high tolerance is often associated with local genetic adaptation. In these species, polymorphisms in metal transporter genes, such as NRAMP and IREG, underpin the ability to accumulate and tolerate Ni at levels exceeding 1000 $\mu\text{g g}^{-1}$ dry mass (Ferrero et al. 2020). This distinction highlights that both plastic and genetic mechanisms can contribute to metal tolerance, but operate on different temporal and evolutionary scales.

From an ecological and agronomic perspective, these findings are highly relevant. The ability of *C. ensiformis* to regulate gas exchange, control oxidative damage, and maintain pigment stability under Ni exposure supports its potential for phytoremediation and cultivation in contaminated soils. In contrast, the relative rigidity of *C. mucunoides* suggests limited resilience under prolonged or intensified metal stress.

The Fabaceae family presents a unique opportunity to explore these mechanisms. Given the diversity of responses observed here and in other legumes, the integration of phenotypic plasticity data with omics approaches holds promise for identifying molecular markers and genes related to stress resilience (Singh et al. 2021). Moreover, legumes' ecological roles in nitrogen fixation and soil improvement enhance their potential in sustainable agriculture. Their adaptability under metal stress, as shown by the contrasting responses of *C. mucunoides* and *C. ensiformis*, strengthens the argument for their strategic inclusion in phytoremediation and climate-smart agricultural practices (Gualtieri et al. 2020).

This study was conducted under controlled greenhouse conditions, which may not capture field-scale variability in edaphic and climatic factors. We did not perform tissue-specific metal localization or whole-plant metal quantification, and molecular markers/transcriptomic profiling were outside the present scope. Moreover, root system architecture and root morphological traits were not assessed. Future work should integrate field trials, metal partitioning and localization, transcriptome-level responses, and detailed root phenotyping to refine mechanistic understanding and translational potential.

In summary, the differential responses between *C. mucunoides* and *C. ensiformis* reflect species-specific strategies involving pigment composition, gas exchange regulation, antioxidant activity, and growth plasticity. These findings contribute to a deeper understanding of legume adaptation to metal stress and highlight the importance of integrating morphological, physiological, and biochemical traits to assess plant resilience.

These findings advance our understanding of nickel acclimation mechanisms in legumes, highlighting the critical role of phenotypic plasticity in modulating physiological and morphological traits under heavy metal stress. The differential strategies observed suggest species-specific adaptations that can inform targeted selection of legumes for phytoremediation purposes.

Conclusion

This comparative study demonstrated that *Calopogonium mucunoides* and *Canavalia ensiformis* exhibit distinct response strategies to nickel stress. *Canavalia ensiformis* showed notable physiological plasticity, particularly in adjusting stomatal conductance and antioxidant enzyme activities, which likely contribute to its enhanced tolerance to elevated nickel levels. In contrast, *Calopogonium mucunoides* exhibited a more conservative response characterized by activation of antioxidant defenses but accompanied by significant growth limitations, indicating lower acclimation capacity.

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Data Availability The data are contained in the article and Supplementary Materials.

Declarations

Competing Interests The authors declare no competing interests.

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