RESEARCH ARTICLE

Polymetallic (zinc and cadmium) hyperaccumulation in the Australian legume *Crotalaria novae-hollandiae* compared to *Crotalaria cunninghamii*

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Abstract

Purpose Hyperaccumulators are plants with the ability to tolerate and accumulate high concentrations of potentially phytotoxic metals. The Australian legume *Crotalaria novae-hollandiae* accumulates remarkably high concentrations of zinc (Zn), cadmium (Cd) and copper (Cu) in its shoots when growing on metalliferous (Zn-Cd 'calamine') soils. This study aimed to investigate zinc-cadmium tolerance in *C. novae-hollandiae* and to compare it with the closely related, but non-metalliferous, *C. cunninghamii*.

Methods *Crotalaria cunninghamii* and *C. novae-hollandiae* were exposed to Zn (3–1000 µM) and Cd (0–60 µM) treatments in hydroponics culture. At the end of the experiment, harvested plants were segmented into roots, old and young stems, old and young leaves for elemental analysis with Inductively coupled plasma atomic emission spectroscopy (ICP-AES). Laboratory-based micro-X-ray fluorescence (µXRF) analysis was used to elucidate elemental distribution in a shoot and in leaflets.

Results *Crotalaria cunninghamii* accumulated up to 1210 µg Zn g^{-1} and 47.6 µg Cd g^{-1} in its leaves, with a 75% reduction in biomass in the Zn treatment. *Crotalaria novae-hollandiae* accumulated up to 16,600 µg Zn g^{-1} and 1250 µg Cd g^{-1}, with a 70% increase in biomass when exposed to Zn. The species both exhibited chlorosis and stunted growth in the Cd treatments, while only *C. cunninghamii* exhibited toxicity symptoms in Zn treatment.

Conclusions *Crotalaria novae-hollandiae* has limited tolerance for Cd and based on the accumulation and distribution of foliar Zn and Cd it is suspected that *C. novae-hollandiae* has different uptake and tolerance mechanisms when compared to other widely studied Zn-Cd hyperaccumulators (such as *Noccaea caerulescens* and *Arabidopsis halleri*).

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Introduction

Some trace elements, such as zinc (Zn), are micronutrients that are essential for plant growth, whereas others such as cadmium (Cd) are not essential and can be phytotoxic, even at low concentrations (Foy et al. 1978). As a micronutrient, Zn plays a key role as a cofactor in numerous enzymes, most notably a structural role in proteins when interacting with nucleic acids (Clemens 2019). Highly elevated Zn and Cd concentrations in soils can be

harmful to plants, causing physiological symptoms such as foliar chlorosis, and ultimately death (Foy et al. 1978). Most plants exhibit Zn toxicity at foliar concentrations of >500 µg Zn g^{-1} (Chaney 1993). Non-essential transition metals, such as Cd, cause significant cellular damage once their limited binding capacity is exhausted, as they are not guided to sites used by the homeostatic system (Chen et al. 2017). Metallophytes can tolerate higher concentrations of potentially phytotoxic metals than 'normal' plants (Baker 1981; Baker 1987). Due to their immobility plants adapt their physiological function in response to environmental factors over generations (Antonovics et al. 1971; Baker et al. 2010; Ernst 2006). Plants that have evolved specialised tolerance mechanisms over millennia in natural metalliferous soils, such as gossans and ultramafic soils, are so-called 'obligate or true metallophytes'. Whereas 'pseudo-metallophytes' are metal tolerant strains of common species that have evolved limited resistance to metals after exposure to (man-made) metal enriched soils (Whiting et al. 2004). The majority of metallophytes are 'facultative' that thrive on both metalliferous soils and non-metalliferous soils, whilst 'obligate metallophytes' are restricted to metalliferous soils (Baker 1981; Baker and Brooks 1989; Pollard et al. 2014).

A subset of metallophytes are hyperaccumulators which can accumulate metal or metalloid concentrations at orders of magnitude greater levels than non-accumulators (van der Ent et al. 2013). The hyperaccumulation threshold depends on the metal/metalloid, in the case of Zn the hyperaccumulation threshold is 3000 µg Zn g^{-1} whilst for Cd it is 100 µg Cd g^{-1} (Krämer 2010; van der Ent et al. 2013). Hyperaccumulation is a rare occurrence which occurs in approximately 0.2% of angiosperms (Cappa and Pilon-Smits 2014). Of the ~700 globally known hyperaccumulators ~3% (~20 species) are Zn hyperaccumulators and~1% (~7 species) are Cd hyperaccumulators (Reeves et al. 2017). A more recent study identified 10 potential Zn hyperaccumulators in the families Celastraceae, Cunoniaceae, Salicaceae, Rubiaceae and Proteaceae from Papua New Guinea, with *Mitragyna speciosa* (Rubiaceae) accumulating the highest Zn concentrations (22,990 µg Zn g^{-1}) (Do et al. 2020). However, this was discovered via herbarium X-ray Fluorescence scanning, and still needs to be confirmed by field sampling. About half of the Zn hyperaccumulating species are in the Brassicaceae family (Balafrej et al. 2020; Reeves et al. 2017), including the two most intensively studied model herbaceous species *Arabidopsis halleri* and *Noccaea caerulescens* (Assunção et al. 2003; Lochlainn et al. 2011; Mishra et al. 2017; Nouet et al. 2015; Szopiński et al. 2020; Zhao et al. 2000). *Arabidopsis halleri* and *N. caerulescens* can accumulate

extremely high concentrations of Zn and Cd (in *A. halleri* up to 53,900 µg Zn g^{-1} and up to 3640 µg Cd g^{-1}; Stein et al. 2017) and/or Ni (in *N. caerulescens* up to 16,200 µg Ni g^{-1}; Reeves et al. 2001) when growing on Zn-Pb-Cd-enriched calamine soils or on Ni-Co-Mn-enriched ultramafic soils. *Sedum plumbizincicola* (Crassulaceae) is another Zn-Cd hyperaccumulator, which can accumulate 7010 µg Cd g^{-1} and 18,400 µg Zn g^{-1} in its aerial tissues (Cao et al. 2014). *Noccaea caerulescens,* as well as *A. halleri* have the ability to hyperaccumulate Zn when growing on soils with only 'background Zn concentrations' (e.g., <300 mg Zn kg^{-1}) (Reeves et al. 2001). The only other known Zn hyperaccumulator which hyperaccumulates Zn on soils with background Zn concentrations is *Dichapetalum gelanioides* subsp. *sumatranum* which is capable of attaining up to 10,730 µg Zn g^{-1} in its leaves on a soil with just 20 mg Zn kg^{-1} (Nkrumah et al. 2018).

The legume (Fabaceae) genus *Crotalaria* has ~600 species occurring mainly in (sub)tropical regions of Africa and Australia with 36 species recorded from the latter (Holland 2002). Some African species of *Crotalaria* are metallophytes that tolerate high soil cobalt (Co) and Cu concentrations (>5 µg Co g^{-1} and/or >50 µg Cu g^{-1}) in the African 'Copperbelt' on metalliferous soils (Brooks et al. 1977; Brooks 1979). *Crotalaria cobalticola* is a Cu-Co obligate hyperaccumulator found in the DR Congo (Brooks et al. 1977; Brooks 1979). In Australia, *C. novae-hollandiae* was originally discovered to be a Zn hyperaccumulator accumulating up to 8975 µg Zn g^{-1} from the Dugald River Zn-Pb-Cd gossan in central Queensland (Farago et al. 1977). Recent investigations on the Dugald River site have revealed that *C. novae-hollandiae* is a polymetallic Zn-Cu-Cd hyperaccumulator that can accumulate up to 16,200 µg Zn g^{-1}, 545 µg Cu g^{-1} and 170 µg Cd g^{-1} (Tang et al. 2021). As a species, *C. novae-hollandiae* occurs across the Northern areas of Australia, and an assessment of over 200 *C. novae-hollandiae* specimens at the Queensland Herbarium (BRI) using portable X-ray fluorescence (pXRF) for foliar metal concentrations was conducted to identify other instances of metal hyperaccumulation. All samples, bar two, had <20 µg Zn g^{-1} and the two samples were above the Zn hyperaccumulation threshold (>3000 µg g^{-1}), both of which were subsequently identified to be growing on Zn-Pb gossans 250 km apart (Tang et al. 2021). One of these sites is the Dugald River gossan which is a polymetallic metalliferous outcrop with extremely high soil concentrations of Zn, Pb, Cd and Cu from which a range of metallophytes and hyperaccumulators is known (Farago et al. 1977; Tang et al. 2021). Polymetallic hyperaccumulation

of metals is rare, and most hyperaccumulators only accumulate one metal, while some hyperaccumulate two or more metals simultaneouslym such as Zn and Cd (Jhee et al. 2006; McGrath et al. 2001; Yang et al. 2004). It is possible that metal tolerance evolved as an ancestral trait and that many (Australian) species of *Crotalaria* share the same genetic trait for metal tolerance, but this remains to be tested experimentally.

Crotalaria novae-hollandiae can provide unique insights into polymetallic uptake and hyperaccumulation. As a 2 m tall ligneous perennial legume species that grows across subtropical savannahs on metalliferous and non-metalliferous soils, it is vastly different to the majority of Zn-Cd hyperaccumulators which are small herbaceous plants from temperate climate regions (Holland 2002). This study aimed to investigate the Zn-Cd uptake response of *C. novae-hollandiae* and a closely related Australian *Crotalaria* species with similar morphology and similar habitat distribution, *C. cunninghamii*, to gain insights into: (i) the distribution of Zn-Cd within plant tissue; (ii) the physiological impacts of Zn-Cd; and (iii) the inter-elemental relationships within plant tissue under different treatments.

Materials and methods

Plant material sources and propagation

Crotalaria novae-hollandiae subsp. *novae-hollandiae* (New Holland Rattlepod) is a native 2 m shrub widespread across the northern half of Australia, growing in sandy soils in warm regions (Fig. 1). *Crotalaria cunninghamii* (Regal Birdflower) is native and widespread across inland northern Australia. The species is a perennial shrub which can grow up to 3 m in height. *Crotalaria novae-hollandiae* seeds were collected at the Dugald River gossan (Zn-Pb-Cu), western Queensland, Australia in the second half of June 2018. Upon collection, the seeds were dried at 40 °C for two days and stored in airtight ZIP-lock bags in a desiccator cabinet until used. Seeds of *C. novae-hollandiae* were grown to mature plants in the glasshouse. Cuttings were then produced by cutting 10–15 cm stem lengths, reducing leaves, and striking in Clonex Red Root Hormone (8 g/L Indole Butyric Acid, Yates, DuluxGroup Pty) and rooted in moistened 50% fine perlite and 50% vermiculite over 30 days. The rooted cuttings were then introduced into hydroponics culture. *Crotalaria cunninghamii* seeds were purchased from the Nindethana Seed Company (King River WA, Australia). *Crotalaria cunninghamii* was germinated in a mix of perlite, and vermiculite, and seedlings watered with demineralized water once per day two weeks prior to being transplanted into the hydroponic system. The *Crotalaria* species were exposed to treatment once plants grew to an average height of 17.5 cm (15–20 cm).

Fig. 1 *Crotalaria novae-hollandiae* (panel **A**) growing in its natural environment in central Queensland. *C. novae-hollandiae* sourced from the wild with cuttings grown in hydroponics treated with Zn (panel **B**)

Hydroponics and metal dosing experimentation

The hydroponic system consisted of four separate rectangular containers (21 cm height × 30 cm width × 40 cm length; 25 L each) filled with a nutrient solution that contained: K (2.5 mM as KNO_3), Ca (2.5 mM as $Ca(NO_3)_2 \cdot 4H_2O$), P (0.1 mM as K_2HPO_4), Mg (1 mM as $MgSO_4 \cdot 7H_2O$), Cl (75 μM as KCl), B (10 μM as H_3BO_3), Mn (4 μM as $MnSO_4 \cdot H_2O$), Cu (0.5 μM $CuSO_4 \cdot 5\ H_2O$), Mo (0.2 μM as $Na_2MoO_4 \cdot 2\ H_2O$), and Fe (20 μM as $C_{20}H_{20}N_2O_6FeK$ (FeHBED)) based on Brown et al. (1995). The solution was kept aerated using a 25 cm long air stone at the bottom of each container. The treatment was divided into two phases. The initial phase was to assess the Zn tolerance and uptake of the species based on diethylenetriaminepentaacetic acid (DTPA) extractable Zn soil concentrations found at the Dugald River gossan (min: 1.26 μg Zn g^{-1}; max: 1030 μg Zn g^{-1}; median: 31.6 μg Zn g^{-1}) (Tang et al. 2021). The second phase was to assess Cd tolerance, uptake, and its interaction with Zn at optimum Zn concentrations. The nutrient solution for *C. novae-hollandiae* was spiked with four levels of Zn treatment using $ZnSO_4 \cdot 7H_2O$ at concentrations of 3 μM, 30 μM, 300 μM and 1000 μM. Once the optimum had been identified, a new experiment consisting of four Cd treatments using $CdSO_4$ with optimum Zn concentrations for each species was conducted. The Cd dose rates were: 0 μM, 5 μM, 20 μM and 60 μM. As *C. cunninghamii* is not of metalliferous origin, a reduced concentration of treatment was used (Phase 1 (Zn): 3 μM, 30 μM, 60 μM, 300 μM; Phase 2 (Cd): 0 μM, 5 μM, 10 μM and 20 μM). To calculate free ionic activity of Zn and Cd a simulation was performed using the software GEOCHEM-EZ 1.0 (available at http://www.PlantMineralNutrition.net). Depending on the treatment, the results show that 67–85% of Zn^{2+} was present as the free ion, whereas 85% of Cd^{2+} was present as the free ion and the remainder complexed with HBED or SO_4^{-} (Supp Tables 1, 2 and 3). The pH was automatically maintained at the set value of pH 5.8 ± 0.1 with 0.1 M KOH solution using a Bluelab Pro Controller with PeriPod M3 dosing pumps (Bluelab Corporation, Tauranga, New Zealand). The nutrient solutions were changed completely once a week. In each treatment, 12 plants were grown for *C. novae-hollandiae,* and 8 plants were grown for *C. cunninghamii* (to reduce overcrowding due to its larger size), in 5 cm round plastic baskets with a foam disk to allow the roots to be immersed in the nutrient solution. *Crotalaria novae-hollandiae* were grown for 30 days with a 12-12 hr. light-dark photoperiod, under high intensity LED lights Phytomax 400 (Black Dog LED, Niwot, CO, USA), with a photosynthetic photon flux density of 550 μmol m^{-2} s^{-1} (measured with an Apogee MQ-500 instrument) at 22/20 °C day/night. *Crotalaria cunninghamii* had 2-week growth period at pre-treatment levels until plants were of comparable size to *C. novae-hollandiae* cuttings (approximately 20 cm in height) before being exposed to the treatment followed by a 30-day growth period. At the end of the experiment, the plants were harvested and divided into young and old leaves, young and old stems, pre-treatment stems, and roots. The plant samples were thoroughly rinsed with de-ionised water and dried at 60 °C for 72 hrs in a dehydrating oven.

Laboratory-based μXRF elemental mapping

After 12 weeks of growth in the hydroponics system, elemental maps were obtained on selected leaf samples using μXRF analysis. Leaves of the same age were used as a representative of the plant, the 4th set from the youngest is considered the youngest mature leaf. The University of Queensland micro-XRF facility contains a modified IXRF ATLAS X system with a 50 W XOS microfocus Mo-target tube producing 17.4 keV X-rays (flux of 2.2×10^8 photons s^{-1}) focussing to 25 μm FWM and two silicon drift detectors (SDD) of 150 mm^2 coupled to a XIA Mercury X4 signal processing unit. Samples for analysis were mounted between 4 μm Ultralene thin film stretched over a Perspex frame and sealed airtight to limit evaporation and analysed within 10 minutes after excision. Measurements were conducted atmospheric temperature (~20 °C), using the Mo 25 μm X-ray source at a 50 kV with a per-pixel dwell of 100 ms. The XRF spectra on the UQ micro-XRF facility were acquired using the instrument control package, Iridium (IXRF systems) from the sum of counts at the position of the principal K-line peak for each element. These were each exported into ImageJ as greyscale 8-bit TIFF files and displayed using ImageJ's "Fire" lookup table.

Elemental analysis of plant material samples
(ICP-AES)

Dried plant material samples (100 mg) were weighed
into 6 mL polypropylene tubes, pre-digested for
24 hours using 2 mL 70% HNO_3 and then digested at
70 °C for 1 hour followed by an additional 1 hour at
125 °C. The samples were brought to volume (10 mL)
using ultra-pure water, filtered, and analysed with
inductively coupled plasma atomic emission spec-
troscopy (ICP-AES, Thermo Scientific iCAP 7400) as
described earlier (Tang et al. 2021).

Data processing and statistical analysis

Data obtained from ICP-AES was cleaned and organ-
ised in Excel (Microsoft Corporation 2021) and
interpreted in R (R Core Team 2020). All values
are reported on a dry weight (DW) basis. Results
below the Limit of Detection (LOD) were substituted
using $LOD/\sqrt{2}$ in preparation for statistical analysis
if +80% of the data were above the LOD (Hornung
and Reed 1990). Kruskal-Wallis Chi-squared test was
used to identify whether there are any differences in
Zn/Cd uptake in plant parts between treatments. A
Dunn's test with Bonferroni correction was used as a
post-hoc test to identify where the differences occur.
Translocation factor was used to test the efficiency
of translocating Zn and Cd from roots to leaf tissues
using the calculation: translocation factor $_{metal}$ = old
leaf $_{metal}$ / root $_{metal}$. A Wilcox rank sum test was used
to test the differences in translocation factor between
species and treatment. A Principal Components Anal-
ysis using the prcomp() function in R was conducted
to test the inter elemental relationships of metals
between treatments.

Results

Zinc and Cd dose effects on overall plant health

Representative images of the *Crotalaria* species
in all treatments are shown in Fig. 2. For *C. novae-
hollandiae*, the root morphology changed substan-
tially in the 1000 μM Zn treatment compared to the
control, showing signs of thickened stubbed lateral
roots. There were no clear signs of chlorosis in the Zn
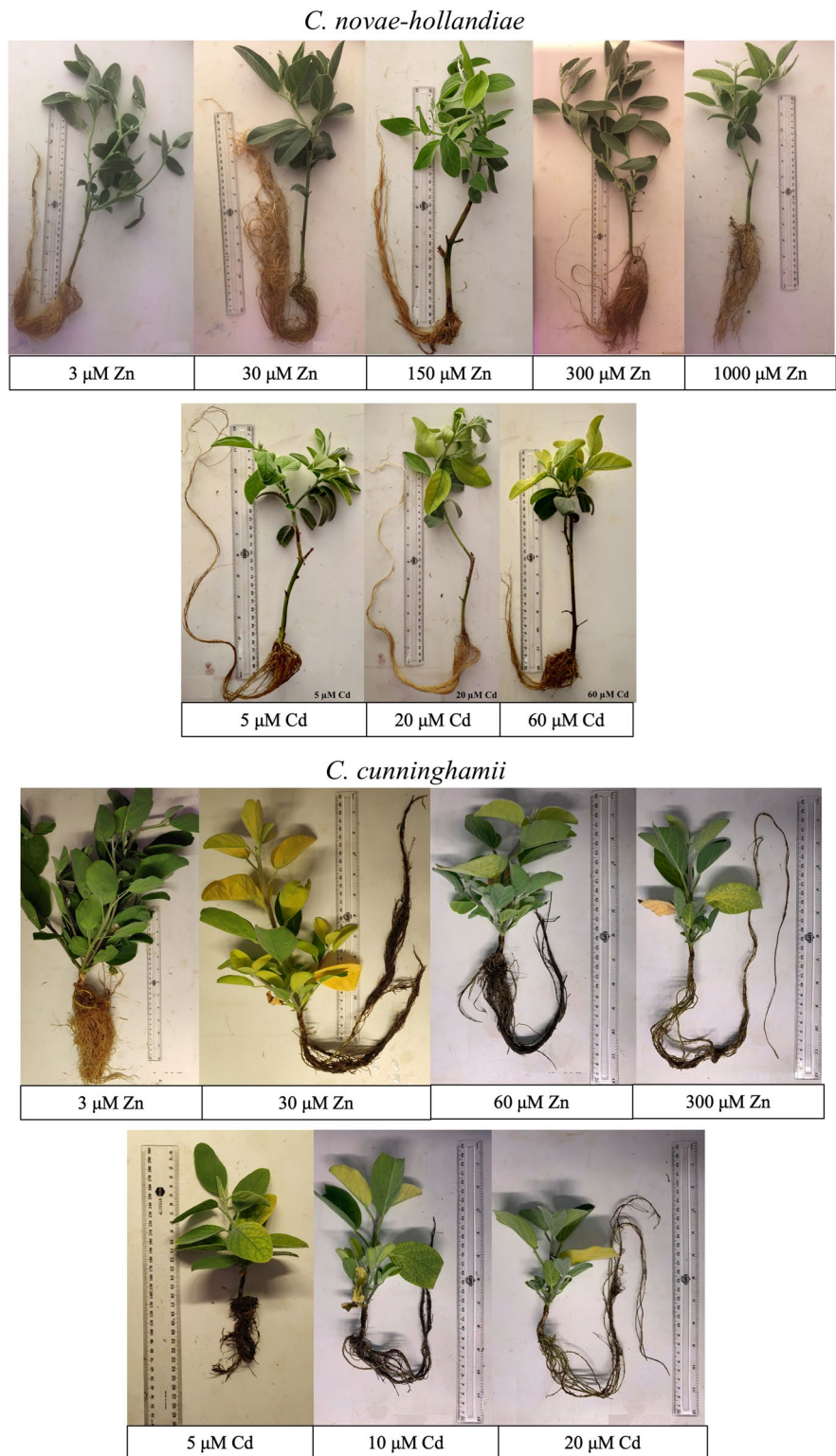treatments; however, there were fewer leaves at the

highest Zn concentration dose level. When exposed
to Cd, the leaves became chlorotic, increasingly with
an increase in treatment level. Plants were stunted
with curled leaves in the highest Cd treatment level.
For *C. cunninghamii*, there was a major decrease in
fine lateral roots in both the Zn and Cd treatments. In
the 30 μM Zn treatment, all new foliage was chloro-
tic. When exposed to Cd, *C. cunninghamii* exhibited
stunted growth and chlorosis in the leaves.

Biomass production, and Zn and cd concentrations in
plant tissues

The biomass and metal concentrations in plant tis-
sues are given in Figs. 3, 4 and 5, Supp Tables 4, 5, 6
and 7 and Supp Fig. 3. In the control Zn treatment, *C.
cunninghamii* grew 320% more biomass than its start-
ing weight compared to *C. novae-hollandiae* which
only increased 63%. However, the foliar biomass of
C. cunninghamii decreased by 80% and 75% in the
Cd and Zn treatments, respectively, compared to the
control. The majority of Zn accumulated in the root
(~5270 μg g^{-1}), followed by old stem (~2120 μg g^{-1}),
old leaves (~1210 μg g^{-1}), young stem (~560 μg g^{-1}),
and young leaves (~795 μg g^{-1}). Zinc concentra-
tions in leaves were well below the hyperaccumula-
tion threshold of foliar 3000 μg Zn g^{-1}. For Cd treat-
ment, Cd was efficiently translocated from root to
shoot while Zn was accumulated in the roots before
being translocated to the shoots in the Zn treatment.
Cadmium accumulation was primarily in the roots
(~240 μg g^{-1}) followed by the old stem (~200 μg g^{-1}),
young stem (~110 μg g^{-1}), old leaves (~47.6 μg g^{-1}),
and young leaves (~19.4 μg g^{-1}). Zinc accumulation
was significantly lower in the roots (−70%) and old
leaves (−80%) but higher in the old stem once Cd was
introduced (difference in median to the control for old
stem Zn uptake: +60%, +100%, +130%; 5, 10, 20 Cd
μM, respectively).

The biomass of *C. novae-hollandiae* increased
with increasing Zn treatment, with an optimal Zn
concentration being between 30 and 300 Zn μM. The
average foliar biomass was 70% greater than the
control between the 30–300 Zn μM treatment range.
However, foliar biomass decreased significantly at
1000 Zn μM by 30% compared to the control. Second
to roots (~30,600 μg g^{-1}), *C. novae-hollandiae* accu-
mulated most Zn in the old leaves (~16,600 μg g^{-1}),
followed by old stems (~9160 μg g^{-1}), young leaves

Fig. 2 Images of representative plants of *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii* from the Zn and Cd hydroponics dosing experiments. Treatment dose level is indicated in bottom right corner of each image
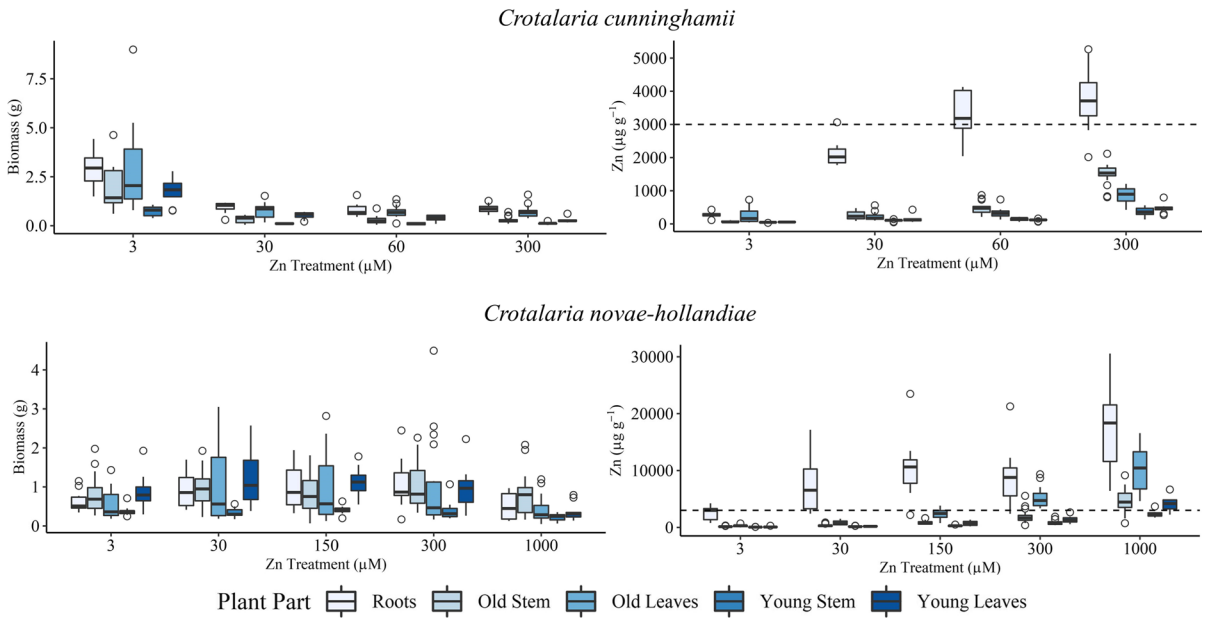
C. novae-hollandiae

| 3 µM Zn | 30 µM Zn | 150 µM Zn | 300 µM Zn | 1000 µM Zn |

| 5 µM Cd | 20 µM Cd | 60 µM Cd |

C. cunninghamii

| 3 µM Zn | 30 µM Zn | 60 µM Zn | 300 µM Zn |

| 5 µM Cd | 10 µM Cd | 20 µM Cd |

Fig. 3 Results of the biomass and Zn accumulation for *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii* from the Zn and Cd hydroponics dosing experiments. The dashed line represents the hyperaccumulation threshold for Zn
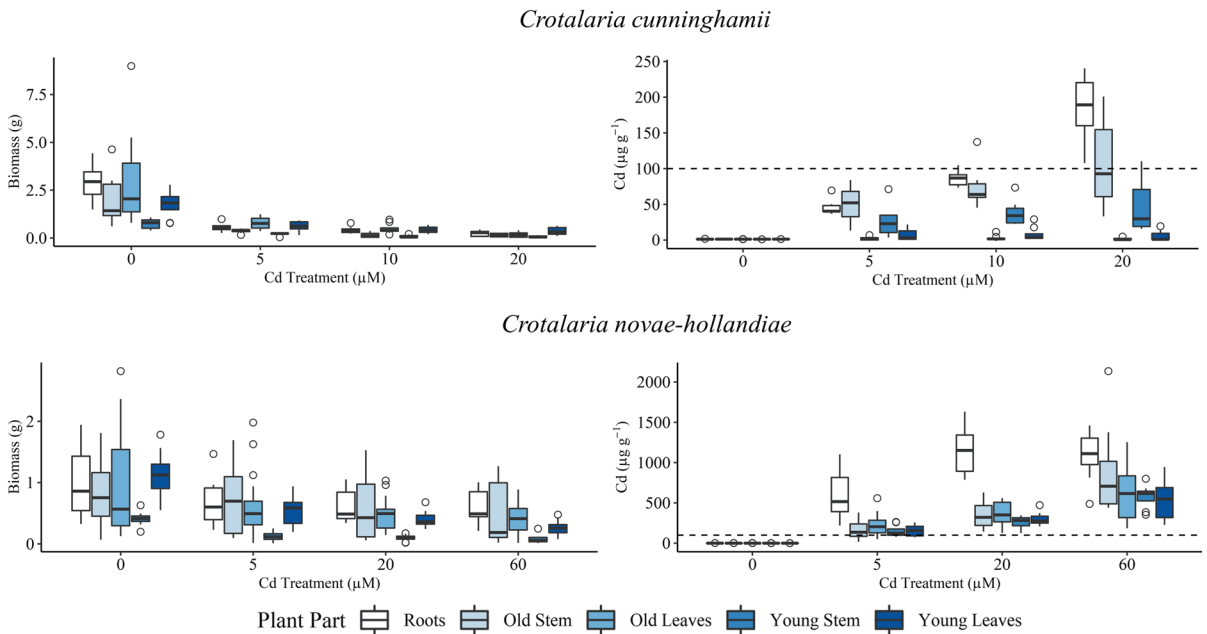
Fig. 4 Cadmium Treatment, biomass, and Cd uptake for *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii*, a dashed line represents the hyperaccumulation threshold for Cd in leaf tissue
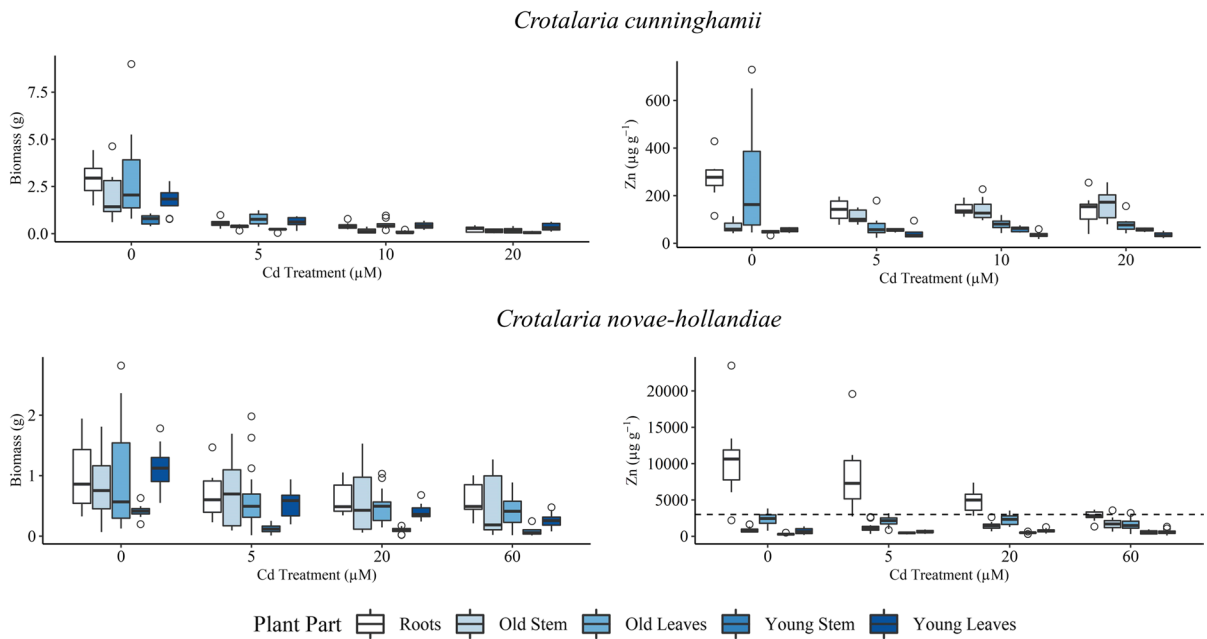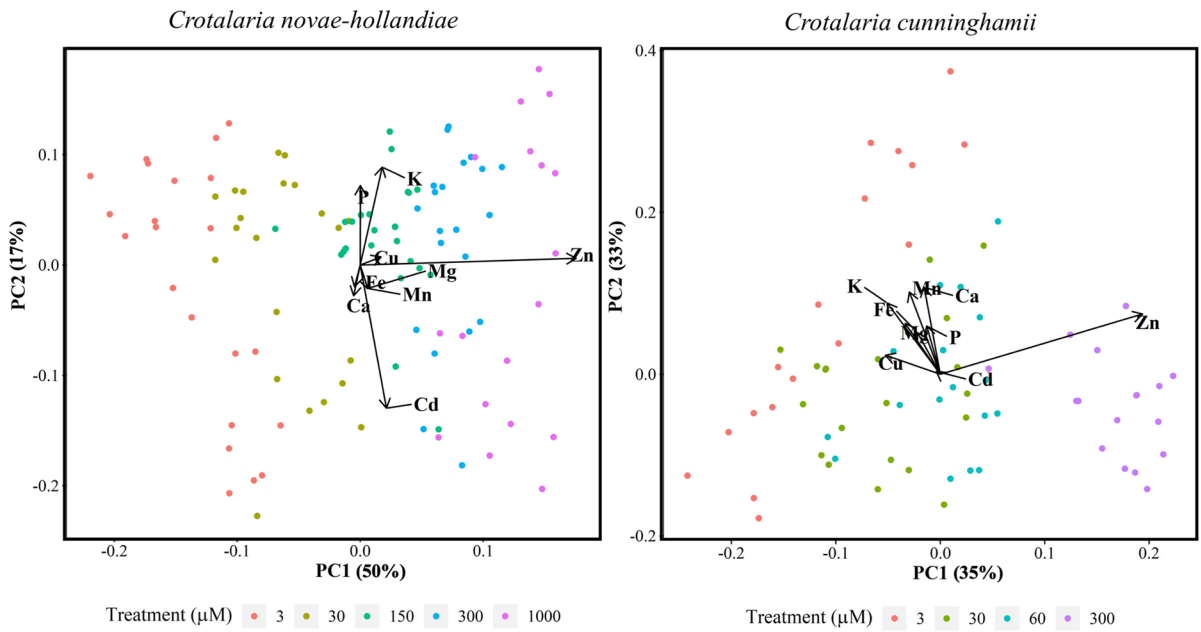
Crotalaria cunninghamii

Crotalaria novae-hollandiae

Plant Part ⬚ Roots ⬚ Old Stem ⬚ Old Leaves ⬚ Young Stem ⬛ Young Leaves

Fig. 5 Cd Treatment, biomass, and Zn uptake for *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii*, a dashed line represents the hyperaccumulation threshold for Zn in leaf tissue

(~6660 µg g^{-1}), and young stems (~3680 µg g^{-1}). A significant amount of overlap in Zn uptake occurred in the roots between the treatments with root saturation occurring between 30 and 300 Zn µM (Supp Table 8). All other plant parts accumulated more Zn with increasing Zn treatment (3–1000 µM: 40-fold increase in µg Zn g^{-1}). Hyperaccumulation of Zn was achieved at 150 Zn µM (3830 µg g^{-1}). Similar to *C. cunninghamii*, *C. novae-hollandiae* decreased in biomass when exposed to the Cd treatment (−60%). However, the decrease in biomass was less than in *C. cunninghamii* despite *C. novae-hollandiae* being exposed to 300-fold more Cd (Supp Fig. 8). Cadmium accumulation was approximately 5-fold greater in *C. novae-hollandiae* than in *C. cunninghamii* for the same treatment level (20 µM). Root Cd accumulation was uniform between 20 and 60 Cd µM. However, Cd accumulation in aerial tissues increased significantly between 20 and 60 Cd µM when compared to 5–20 Cd µM (Supp Table 8). Unlike *C. cunninghamii*, *C. novae-hollandiae* achieved Cd accumulation beyond the hyperaccumulation threshold (>100 µg Cd g^{-1}) in its leaves at 5 Cd µM, with up to 1250 µg Cd g^{-1} in old leaves in the 60 Cd µM treatment. Cadmium exposure decreased Zn accumulation in the roots

(0–60 Cd µM: −75% Zn). However, a test of difference shows that Zn concentrations remained relatively stable between the aerial tissues (Supp Table 8).

Inter-elemental relationships in plant tissues

The results of the principal components analysis (PCA) showing inter-elemental relationships and effects of treatment are given in Fig. 6 and Supp Table 10. Groupings primarily influenced by the treatments are clear: for the Zn treatment of *C. novae-hollandiae*, the first principal component (PC) explains 50% of the variation which is driven by Zn, PC2 explains an additional 17% of the variation which is driven by phosphorus (P) and potassium (K). For *C. cunninghamii* in Zn treatment, PC1 is also driven by Zn but only accounts for 35% of the variation, and PC2 explains an additional 33% of the variation which is driven by calcium (Ca)-manganese (Mn)-K. A large proportion of the variance (89%) is explained by Cd for *C. novae-hollandiae*, PC2 accounts for an additional 5% variation which is driven by two groups, Zn and Mg-Mn. Unlike *C. novae-hollandiae*, PC1 in *C. cunninghamii* accounts for 54% for the variation is still driven by Zn but with stronger influences from
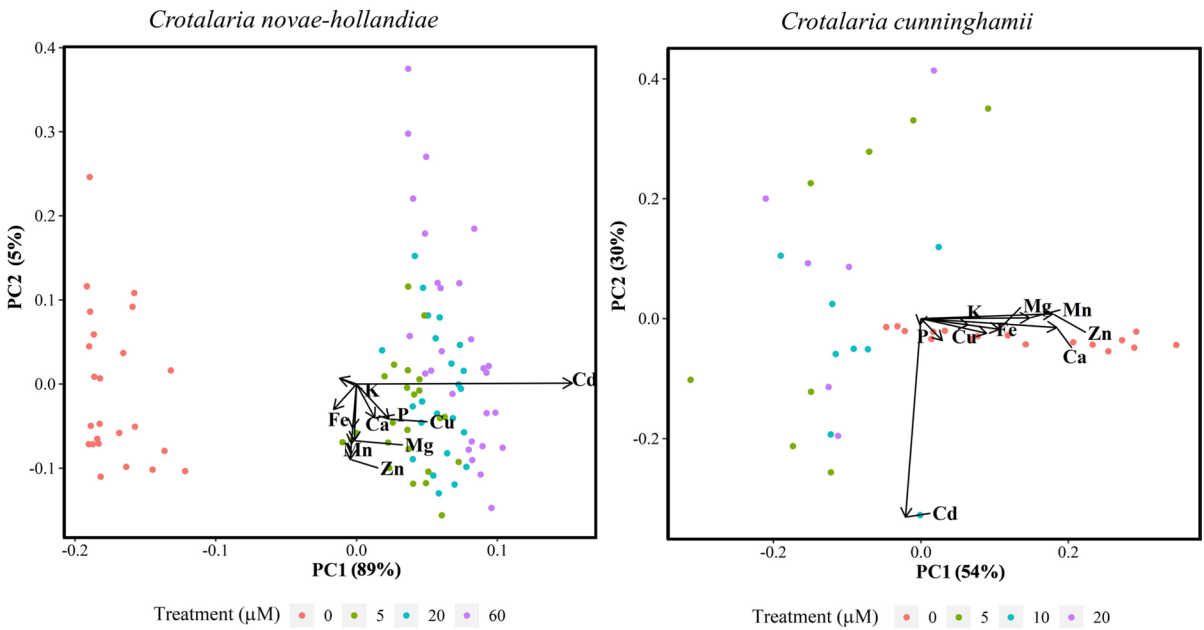
Fig. 6 Principal Components Analysis (PCA) of *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii* leaves for both Zn and Cd treatments

Ca and Mn and PC2 which accounts for an additional 30% variation is driven by Cd.

Zinc and Cd translocation from roots to shoots

The results for the translocation factors are given in Fig. 7. In the Zn treatment, *C. cunninghamii* had decreasing translocation factors with an increase in Zn exposure, whilst *C. novae-hollandiae* had increasing translocation factors with an increase in Zn exposure. This is reflected by significant differences in the translocation factors between the species at 3 μM Zn (*C. cunninghamii* translocation factor: 0.713; *C.*

novae-hollandiae translocation factor: 0.157) and the 300 μM Zn treatment (*C. cunninghamii* translocation factor: 0.258; *C. novae-hollandiae* translocation factor: 0.808), whilst translocation factors were both relatively low and not significantly different in 30 μM Zn (*C. cunninghamii*: 0.1; *C. novae-hollandiae*: 0.151) (Supp Table 9). For *C. cunninghamii*, the translocation of Cd in Cd treatment remained low (<0.1) and decreased with an increase in Cd treatment (5 μM Cd: 0.0462; 20 μM Cd: 0.00716). There was a significant difference in Cd translocation factors between both species at the respective Cd treatment levels ($p < 0.05$), with *C. novae-hollandiae*

Fig. 7 Translocation of Zn and Cd from Root to Old leaves in *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii*
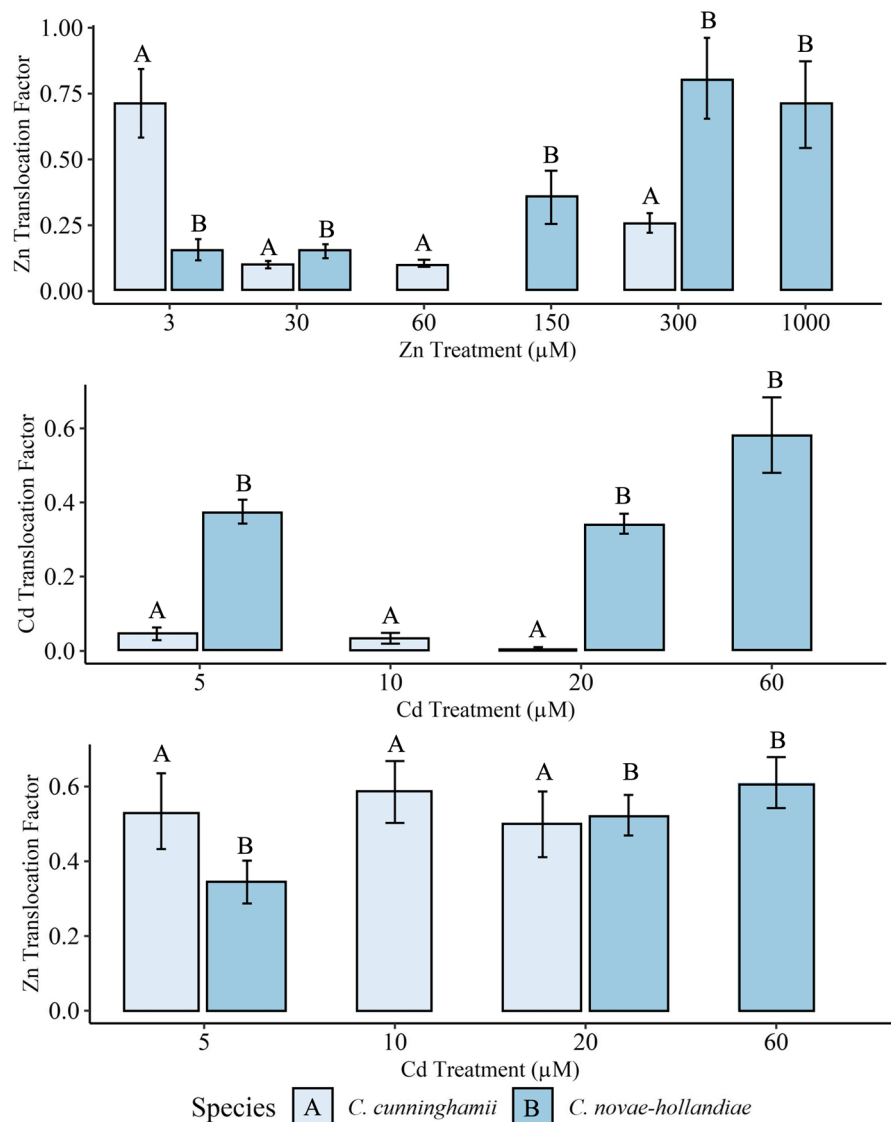
having relatively higher translocation factors (Supp Table 9). There was no difference in the translocation factors of Cd between 5 and 20 Cd μM for *C. novae-hollandiae* but there was an increase in translocation factors between 20 and 60 Cd μM (Supp Table 9). There was no difference in the translocation factors for Zn for both species in Cd treatment, with only a marginally significant difference in 5 Cd μM with *C. cunninghamii* having a higher translocation factor.

Micro-X-ray fluorescence spectrometry elemental mapping

Laboratory-based micro-X-ray fluorescence (μXRF) analysis results for leaves are shown in Figs. 8, 9 and 10. In Fig. 8, it can be seen that *C. novae-hollandiae* had no obvious signs of toxicity in the colour photo (such as yellowing/chlorosis). The elemental maps show that Zn was more enriched in the old leaves compared to young leaves. Zinc was evenly distributed in the leaf of *C. novae-hollandiae* in the 30 μM Zn treatment. However, in the 150 μM Zn treatment (Fig. 9), Zn accumulated in clusters

within the lamina. Zinc was more evenly distributed at 300 μM Zn, however, at a much higher intensity compared to 30 μM Zn and high concentrations in the margin. In the 1000 μM Zn treatment, Zn decreased in the mid-rib followed by the veins, with the highest Zn in the lamina and margin. Under 30–150 μM Zn, Ca was low in the midrib and veins, but high in the lamina. However, at 300–1000 μM Zn, *C. novae-hollandiae* had much lower concentrations of Ca in the lamina. There was no major change in K distribution in the leaf of *C. novae-hollandiae* under any of the Zn treatments. It can be seen in the colour photo (Fig. 9), that *C. cunninghamii* was chlorotic in all of Zn treatments (30–300 μM Zn). In the lower Zn treatments (3–30 μM Zn), Zn concentrations were low throughout the leaf. Zinc was slightly enriched in the midrib, veins, and the lamina at 300 μM Zn. There was significant variability in Ca and K distribution and concentration between the treatments with no clear visible trends.

There were no differences in Zn distribution in the leaf of *C. cunninghamii* in the respective Cd treatments (Fig. 10). Zinc was enriched in the

Fig. 8 Instrumental μXRF elemental maps of *Crotalaria novae-hollandiae* shoot exposed to Zn tailings from the Dugald River Pb-Zn Mine to capture Zn-Ca-K accumulation in a whole plant. Scale bar denotes 10 mm
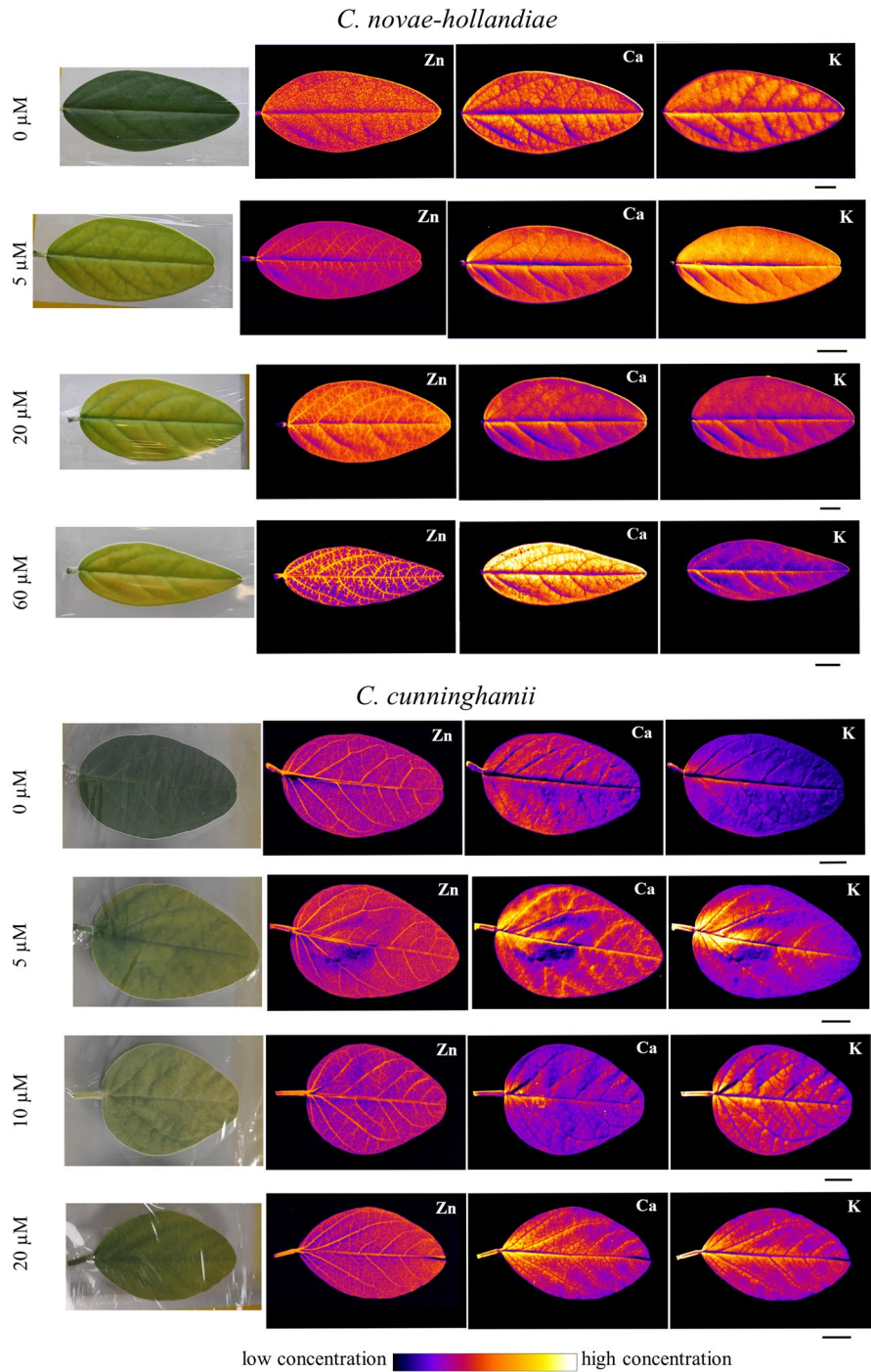
low concentrations ▇▇▇ high concentrations

1cm

Fig. 9 Instrumental µXRF elemental maps of *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii* leaves under different Zn treatments. Scale bar denotes 10 mm
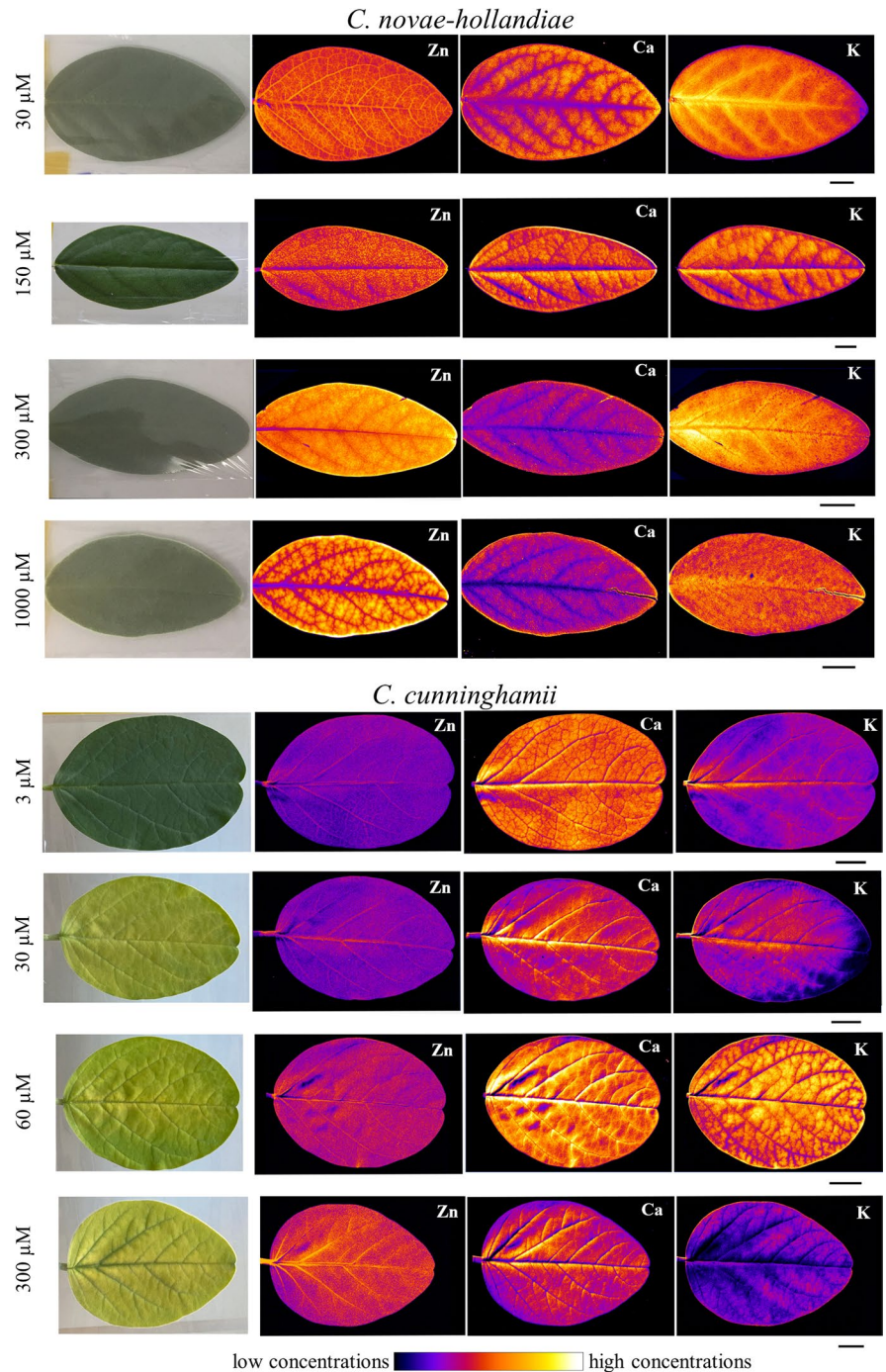
veins, but relatively depleted in the lamina. Leaf Ca and K were enriched in the base and areas adjacent to the veins when exposed to Cd treatments. For *C. novae-hollandiae*, Zn accumulation in the lamina decreased but increased in the midrib and veins when exposed to Cd treatments. In the highest Cd treatment (60 µM Cd), Ca was highly concentrated in the lamina but low in the mid-rib and veins whereas K was very low in the lamina and higher in the mid rib. In the lower Cd treatments (0–20 µM Cd), Ca and K had similar enrichment patterns.

Fig. 10 Instrumental µXRF elemental maps of *Crotalaria novae-hollandiae* and *Crotalaria cunninghamii* leaves under different Cd treatments. Scale bar denotes 10 mm

Discussion

In hydroponics, Zn accumulates primarily in the old leaves of *C. novae-hollandiae*. This may be an adaptation to reduce Zn toxicity, by reducing the total concentration of Zn within the plant. The non-metallophyte *C. cunninghamii* had a different response to Zn; despite Zn accumulation being restricted to the roots with foliar concentrations below the level of toxicity, *C. cunninghamii* still showed signs of toxicity

(e.g., chlorosis and stunted growth). Cadmium did not stimulate biomass growth in *C. novae-hollandiae*. However, *C. novae-hollandiae* accumulated very high foliar Cd concentrations. Like *C. cunninghamii, C. novae-hollandiae* decreased in biomass when exposed to Cd treatment, though at a much lower rate, which may be indicative of some level of tolerance. For *C. cunninghamii*, Cd accumulation was largely restricted to the stems with lower foliar concentrations.

Crotalaria novae-hollandiae accumulated foliar Zn (up to 16,600 µg Zn g^{-1}) similar to what was found in nature at the Dugald River gossan (~16,200 µg Zn g^{-1}) (Tang et al. 2021). However, Cd shoot concentrations well exceeded in-situ concentrations (maximum foliar concentration at Dugald River gossan: 170 µg Cd g^{-1} and maximum foliar concentration in hydroponics: 1250 µg Cd g^{-1}) due to Cd exposure being significantly greater in the experimental treatment than at the Dugald River (soil DTPA-extractable Cd at Dugald River: 6.59 µg Cd g^{-1}; hydroponics dose rate: 60 µM Cd) (Tang et al. 2021). *Crotalaria novae-hollandiae* had a much higher tolerance to Zn (30–1000 µM Zn), whereas *C. cunninghamii* exhibited signs of toxicity with chlorotic leaves in the 30 µM Zn treatment. *Crotalaria cunninghamii* is generally a faster growing species than *C. novae-hollandiae*; however, Zn treatment reduced growth, whilst it strongly stimulated growth in *C. novae-hollandiae*. Stimulatory effects on plant biomass caused by (toxic) metals have been observed in other metallophytes, but the physiological causes are still uncertain (Tang et al. 2009). One hypothesis postulated for the stimulatory response of metallophytes/hyperaccumulators to metals is an extreme form of hormesis (Roosens et al. 2003; Tang et al. 2009; Salinitro et al. 2021).

Laboratory µXRF elemental maps of *C. novae-hollandiae* shoot and leaflets show that old leaves are major Zn sinks, which suggests limited phloem redistribution. One physiological advantage of allocating Zn to the old leaves is the potential for it to remove excess Zn with natural leaf shedding (Ernst et al. 1992). *Crotalaria cunninghamii* is less tolerant to Zn whereas *C. novae-hollandiae* shows no visual signs of Zn toxicity, which suggests an inherent ability to inhibit Zn accumulation in the chloroplast (Bayçu et al. 2017; Sitko et al. 2017; Szopiński et al. 2020). *Crotalaria novae-hollandiae* shows a clearer trend of Zn being distributed into the lamina whilst *C. cunninghamii* restricts the majority of Zn within its veins. Vacuolar compartmentalisation and organic acid complexation are two key mechanisms of Zn tolerance in plants (Chardonnens et al. 1999; Clemens 2001; Ernst et al. 1992; Hall 2002; Verkleij et al. 1998). The Metal Tolerance Protein 1 (MTP1) is highly expressed in Zn hyperaccumulators (*N. goesingensis, N. caerulescens, Sedum alfredii* and *A. halleri*) as a vacuolar transporter associated with Zn tolerance (Gustin et al. 2009; Krämer 2005; Milner and Kochian 2008; Zhang et al. 2011). However, *C. novae-hollandiae* had previously been identified to accumulate Zn in the phloem and cell walls and not in the vacuoles (Farago et al. 1977). In addition, the majority of Zn is in a water-soluble form and present in non-specific sites (70% of total foliar Zn), while the insoluble component is accumulated in carbohydrates associated with the cell wall at non-metal specific sites and can be exchanged with Ca ions (Farago et al. 1977).

Hyperaccumulators are characterised by their ability to accumulate very high concentrations of metal(loid)s into their aerial tissues (Roosens et al. 2003; van der Ent et al. 2013). Therefore, hyperaccumulators have translocation factors >1, which is indicative of efficient root-to-shoot translocation of metals (Baker 1981; Gupta et al. 2016). As expected, the translocation factors for the non-metallophyte, *C. cunninghamii*, were low when exposed to Zn or Cd treatment. However, for the hyperaccumulator *C. novae-hollandiae*, Zn-Cd translocation factors increased with increasing Zn-Cd treatment levels. Nevertheless, the translocation factors for *C. novae-hollandiae* did not exceed 1, suggesting relatively limited Zn-Cd translocation. However, *C. novae-hollandiae* did surpass the Zn hyperaccumulation threshold by 5.5-fold and as such confirmed its status as a Zn hyperaccumulator under experimental conditions (Tang et al. 2021). The increase in Zn translocation factors for *C. novae-hollandiae* in Cd treatments indicates that Cd facilitates Zn translocation. However, the underlying mechanisms are not presently known. The role of ZIP (Zrt-Irt-like Protein) transporters in the uptake of Zn in hyperaccumulating metallophytes has been well studied and may be expressed in *C. novae-hollandiae* (Corso and de la Torre 2020; Merlot et al. 2021; Singh et al. 2019; Sytar et al. 2020). However, unlike other well-known Zn hyperaccumulators (*A. halleri and N.*

caerulescens), *C. novae-hollandiae* has a much lower translocation factor and does not accumulate Zn or Cd when grown on soils or substrates with low Zn or Cd status (Bert et al. 2003; Peer et al. 2006; Zhao et al. 2000).

Crotalaria novae-hollandiae and *C. cunninghamii* are both sensitive to Cd. However, *C. novae-hollandiae* accumulated Cd well beyond the hyperaccumulation threshold although its tolerance threshold may be lower than expected. Unlike Zn, Cd does not have a specific transporter and uptake is via the binding of non-specific transporters of essential elements with great attention being given to ZIP transporters (Huang et al. 2020). Iron transporters (IRT) have also received attention for their possible involvement in Cd uptake in the *N. caerulescens* 'Ganges' population of Southern France. IRT1, which is primarily expressed in the root epidermis, has been found to increase root Fe uptake in the Ganges population when exposed to Cd (Halimaa et al. 2019; Vert et al. 2002). The mechanisms of Cd uptake in *C. novae-hollandiae* may differ as no significant change in root Fe-uptake occurred with exposure to Cd. Zinc and Cd share chemical similarities, though Cd is more chemically similar to Fe which can result in preferential uptake via the same pathways and transporters without altering Fe uptake or the Fe transport system if it is not saturated (Nouet et al. 2015). As Zn is hyperaccumulated by *C. novae-hollandiae*, Cd may also be inadvertently hyperaccumulated and no specific mechanism of Cd detoxification has evolved. However, *C. novae-hollandiae* is still able to accumulate up to 170 µg Cd g^{-1} in its old leaves growing naturally in soils with up to 6.59 mg Cd kg^{-1} bioavailable (DTPA-extractable Cd) with no visible signs of toxicity (Tang et al. 2021). It may, therefore, be classified as a (rather weak) Cd hyperaccumulator; and its tolerance range is clearly not as great as other well-known Zn-Cd hyperaccumulators (with foliar concentrations in *N. caerulescens* of up to 2890 µg Cd g^{-1} and in *A. halleri* with up to 3600 µg Cd g^{-1}) (Bert et al. 2003; Reeves et al. 2017). In *C. novae-hollandiae* leaves, Cd appears to restricts Zn to the mid-rib and veins from entering the lamina, which is most clear in the highest Cd treatment (60 µM in solution) and K concentrations are reduced throughout the leaf (Fig. 10). This contrasts with *A. halleri* where Zn is restricted from translocation to the veins (Morina and Küpper 2020). Cadmium increased Ca in *C. novae-hollandiae* and

has also been shown to alleviate toxicity effects of Zn-Cd in *N. caerulescens* (Bayçu et al. 2017). However, Ca in *C. novae-hollandiae* decreased under Zn treatment which may indicate that the tolerance mechanisms for Zn and Cd differ; but as Cd is able to replace Zn in the roots, the Zn-Cd transport pathways may be the same (Bayçu et al. 2017). As the patterns of uptake and mechanisms of tolerance appear to differ between *C. novae-hollandiae* and other well studied Zn-Cd hyperaccumulators, further research is required to provide insights at the cellular and subcellular levels using synchrotron-based techniques, and molecular approaches to identify putative transporters.

Conclusions

Crotalaria novae-hollandiae is one of the few polymetallic (Zn-Cd-Cu) hyperaccumulators known, and the only known Zn hyperaccumulating ligneous legume species growing in tropical savannahs (although there is a Zn hyperaccumulating legume, *Anthyllis vulneraria,* from southern France; Escarré et al. 2010). The species has wide distribution across Northern Australia and its tolerance to dry climates makes it a strong contender for use in mine rehabilitation and/or phytoextraction. *Crotalaria novae-hollandiae* can accumulate high foliar concentrations of Zn (up to ~16,600 µg g^{-1}) and Cd (up to 1250 µg g^{-1}) in its leaves when growing on metalliferous soils or mine wastes. There is, hence, potential for producing "green" zinc oxide derived from its harvested biomass in a phytoextraction operation. This could be used in a variety of applications including sunscreens, cosmetics, and supplements, and potentially generate an estimated revenue of USD$6000 per ha per year (at $195 per kg for high-purity zinc oxide), excluding production and processing costs. Obviously, the final product should be extremely low in Cd for any such health product applications.

The Zn treatment was found to have a positive impact on growth when compared to the control until toxicity at 1000 µM Zn. The physiological reasons for this response are unknown, as such concentrations are far beyond the essential Zn requirement. Cadmium induces foliar chlorosis even at low dose rates (5 µM Cd in solution) and its tolerance and accumulation in nature (maximum of 170 µg Cd g^{-1} in leaves) are not

as high as other Zn-Cd hyperaccumulators growing in situ (*S. plumbizincicola* 7010 µg Cd g^{-1}; *N. caerulescens* 2890 µg Cd g^{-1}; *A. halleri* 3600 µg Cd g^{-1}). However, *C. novae-hollandiae* can still be classified as a Cd hyperaccumulator as it exceeds the hyperaccumulation threshold of 100 µg Cd g^{-1} when growing in the natural habitat. The research on *C. novae-hollandiae* is still in the early stages and further investigations employing state of the art techniques, including synchrotron µXRF analysis and molecular techniques, are expected to provide more detailed insights into the fundamental mechanisms underlying Zn-Cd hyperaccumulation in *C. novae-hollandiae*.

Data availability The data generated during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no conflicts of interest relevant to the content of this article.

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