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PII: S0098-8472(14)00235-4
DOI: http://dx.doi.org/doi:10.1016/j.envexpbot.2014.10.001
Reference: EEB 2871

To appear in: *Environmental and Experimental Botany*

Received date: 12-3-2014
Revised date: 3-10-2014
Accepted date: 13-10-2014

Please cite this article as: Dinh, Ngoc T., Vu, Dang T., Mulligan, David, Nguyen, Anh V., Accumulation and distribution of zinc in the leaves and roots of the hyperaccumulator Noccaea caerulescens.Environmental and Experimental Botany http://dx.doi.org/10.1016/j.envexpbot.2014.10.001

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Accumulation and distribution of zinc in the leaves and roots of the hyperaccumulator *Noccaea caerulescens*

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Highlights

- Zn uptake by *N. caerulescens* tranlocated to leaves.
- Great ability of young *N. caerulescens* plants to accumulate Zn in shoots.
- Decrease of Ca and P concentration with increasing amount of Zn treated.
- Zn crystals found in leaf epidermal cells and root cortex of *N. caerulescens*.
- In the plant tissues, P and S co-localized while Ca localized with Zn.

Abstract

Understanding the uptake mechanisms of heavy metals by hyperaccumulators is necessary for improving phytoextraction options to reduce metal toxicities in contaminated soils. In this study, the capacity of Zn uptake by the hyperaccumulator *Noccaea caerulescens* was investigated and compared to the non-hyperaccumulator *Thlaspi arvense.* The plants were grown under hydroponic conditions in a glasshouse. The distribution of Zn in the roots and leaves of these species was investigated by scanning electron microscopy with energy-dispersive X-ray analysis. Compared with the control with no Zn added, it was shown that prolonged Zn treatments decreased the biomass of both *N. caerulescens* and *T. arvense*. Since *N. caerulescens* requires Zn for growth, no Zn toxicity symptoms were observed, even when the Zn

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concentration in shoots reached 2.5% dry mass. *T. arvense* showed serious Zn toxicity only after two weeks of Zn treatment. Zn uptake by *N. caerulescens* was mainly translocated to the leaves while almost all of the Zn taken-up by *T. arvense* was retained in the roots. In *N. caerulescens*, increased concentrations of Zn in the shoots resulted in reductions in Ca and P concentrations by up to 50% and 35%, respectively. Zn-containing crystals were abundant in both the upper and lower epidermal cells of the leaves and in the cortex of the roots during the later growth phase. Co-localization of Ca and Zn, P and S were found in leaf and root tissues. The results suggest that Zn-rich crystals with an abundance of the Zn ligand in the roots and shoots, and co-localization and interaction between Zn and other ions, may have functional significance with respect to conferring particular attributes to *N. caerulescens* that are not present in the non-hyperaccumulator counterpart. An understanding of these species-specific differences has relevance from the perspective of offering some insight into how particular species could contribute to a strategy for the detoxification of Zn-contaminated sites.

Keywords: phytoextraction, hyperaccumulator, *Noccaea caerulescens*, Zn-rich crystal, heavy metals, zinc.

1. Introduction

Phytoremediation through the use of specialized plants and their repetitive harvesting is potentially an efficient detoxification method for metal-contaminated environments including soil, water and sewage sludge (Baker et al., 1994a). It has been considered a suitable technique when environmental health is a priority, and lengthy treatment is acceptable or alternative remediation technologies are prohibitive or unavailable (Monsant et al., 2010). Plant species that accumulate large quantities of heavy metals in their shoot are known as heavy metal-hyperaccumulator plants and are usually referred to as hyperaccumulators (Baker and Brooks, 1989). More than 400 plant species have been recorded as hyperaccumulators and a significant number have the ability to accumulate two or more elements (Baker et al., 2000).

Of the known hyperaccumulators, *Noccaea caerulescens* (formerly known as *Thlaspi caerulescens*) is one of the few model heavy metal hyperaccumulating plants. This species is able to accumulate up to 40000 mg kg^{-1} of Zn and 18000 mg kg^{-1} of Cd in the shoot dry biomass without any toxic symptoms (Saison et al., 2004; Shen et al., 1997), while the normal Zn concentration for most plants is in the range of 30 - 100 mg kg^{-1} dry mass (Marschner, 1995). Grown on contaminated industrial soil, *N. caerulescens* can have higher biomass and lower mortality rates than on agricultural soil (Saison et al., 2004). The properties of Zn and Cd

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accumulation exhibited by *N. caerulescens* make it an excellent experimental species for studying the mechanisms of metal uptake, accumulation, and tolerance relating to metal phytoextraction (Lasat et al., 1996, 1998; Lasat et al., 2000; Monsant et al., 2011) and exploring the ability to exploit these properties for the remediation of heavy metal-polluted sites.

The distribution of metals within plant tissues is considered an important property and an indirect indicator of a detoxification mechanism (Boominathan and Doran, 2003). Zn, Cd, Co, Mn and Ni were readily transported to the shoots, while the higher proportion of Al, Cr, Cu, Fe and Pb remained in the roots (Vazquez et al., 1994). In *N. caerulescens* leaves, Zn is mainly located in the epidermal cells, with smaller amounts found in the mesophyll cells and the leaf veins (Frey et al., 2000; Kupper et al., 1999; Vazquez et al., 1994). It has been shown that 88% of the Zn is in the upper and lower epidermis whereas only 12.4% of Zn remains in the mesophyll cells (Monsant et al., 2010). In contrast to the overall plant distribution of Zn among tissue and cell types, Zn accumulation in the epidermis occurs mainly in the vacuoles, and to a lesser extent in the apoplast. Globular Zn crystals in the vacuoles of epidermal and sub-epidermal leaf cells were observed at a foliar Zn concentration of 13,600 mg kg^{-1} (about 1.3 % dry weight) while only small Zn deposits were found in the epidermal and sub-epidermal cells of roots that contained between 1000 and 18300 mg Zn kg^{-1} dry weight (Vazquez et al., 1994). These crystals have a high Zn/P ratio (Vazquez et al., 1994).

High concentrations of Zn in vacuoles and cell walls (apoplast) of epidermal cells are observed to always associate with high concentrations of Ca (Vazquez et al., 1994), and in leaf cells of *N. caerulescens* it is found that Zn and Ca can co-locate (Monsant et al., 2010). The Zn and Ca concentrations are 36000 and 8600 mg kg^{-1}, respectively, in the upper epidermis in the leaf and 20000 and 6200 mg kg^{-1}, respectively, in the lower epidermis. Zn uptake *per se* has also been shown to be influenced by the presence of other elements. For example, Zn uptake by *N. caerulescens* was significantly reduced when other metal ions such as Pb or Cu were added to the culture solution (Walker and Bernal, 2004), but a high Zn concentration may also reduce the uptake of other elements. It has been reported that Mn uptake, for example, is greatly reduced by increasing Zn concentration, whereas Zn and Cd uptake is possibly reduced by Cu (Keller and Hammer, 2004; Lombi et al., 2001). Zn uptake by *N. caerulescens* was reduced by 15% by adding 50 μM of Cd to the culture solution, but adding 50 μM Zn was shown to inhibit Cd uptake by 30% in protoplasts of *N. caerulescens* (Cosio et al., 2004). Other findings showed that the concentrations of Ca, P and Cl in the epidermal sap generally decreased with increasing Zn concentration (Kupper et al., 1999), while no correlation was found between the uptake of Zn and P, S or Cl (Frey et al., 2000).

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Determining the growth period over which the plant has the maximum capacity for Zn uptake and elucidating the internal Zn distribution strategy that may confer a detoxification capability are necessary for improving and developing technologies for the use of *N. caerulescens* in a role in Zn phytoextraction. This study aims to assess the capacity of Zn uptake and tolerance by *N. caerulescens* for short- and long-term Zn treatment and to benchmark the results relative to the non-hyperaccumulator *Thlaspi arvense*. It also aims to further elucidate the Zn detoxification mechanisms of *N. caerulescens* by characterizing Zn distribution and assessing the influence of Zn uptake on the uptake of other elements in plant tissue. We hypothesized that specific localization of Zn in tissues of *N. caerulescens* would enhance Zn uptake by the plant more effectively than the non-hyperaccumulator. The Zn uptake was also hypothesized to affect the uptake of other elements into the plant.

2. Material and methods

2.1 Plant growth

Seeds of *N. caerulescens* and *T. arvense* that had been held in cold storage were brought to room temperature (23^{o}C) 24 h prior to use. Seeds were rinsed with 0.5% NaClO for 5 min for sterilization and then rinsed again in tap water (Monsant et al., 2010). The seeds were then germinated on wet filter papers in an incubator (TRISL-490-1VW, Thermoline Scientific, Australia) under dark conditions and with temperature set at 23^{o}C. One-week-old uniform seedlings were transplanted into foam strips which kept the plants in direct contact with a basal nutrient solution made up of the following (in μM) (Monsant et al., 2010): 20 KH_2PO_4; 600 K_2SO_4; 200 $MgSO_4$; 100 $CaCl_2$; 10 FeEDDHA; 10 FeNaEDTA; 5 H_3BO_3; 1 $MnSO_4$; 0.2 $CuSO_4$; 0.03 Na_2MoO_4; and 600 $Ca(NO_3)_2$ as a nitrogen source. The plants were cultured in the incubator for a further 10 days with a 16h/8h day/night cycle. The plants were then transferred to a temperature-controlled glasshouse (22 ± 3°C) with natural light. Seedlings of *N. caerulescens* and *T. arvense* were selected for uniformity and then grown in groups of four plants per pot in closed-cell foam strips inserted into rigid plastic discs placed on top of 5L black polyethylene pots in a glasshouse. Each pot contained nutrient solution, which was continuously aerated with pin-hole air outlets.

A factorial experiment consisted of five Zn concentrations: control (no Zn added), 200, 300, and 500 μM, with three replicates across two Thlaspi species: *Noccaea caerulescens* (Prayon) and *Thlaspi arvense*. The Zn additions (as $ZnSO_4$ solution) commenced seven weeks

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after seedlings were transferred to the hydroponic culture pots. Nutrient solutions were replaced every three days.

2.2 Plant harvest and analysis

Plants were harvested 2, 5, 7 and 16 weeks following the introduction of Zn. The plants were washed three times in de-ionized water, and shoots and roots were separated at the root-shoot junction. Fresh weights were recorded and the samples were oven-dried in paper bags at 80°C for 24h. Sample dry mass was recorded immediately prior to grinding in a mortar. The whole shoot and root (in the case where shoot and/or root dry mass was under 0.1 g) or 0.1 g subsamples of the shoots and roots were placed into 50 mL digestion tubes and digested in 6 mL concentrated HNO_3 70% (v/v) mixed with 2 mL hydrogen peroxide H_2O_2 (v/v); this was achieved using a microwave open vessel method in a microwave digester (START D Microwave Digestion System, Milestone S.r.I., Italy) for a total of 1 h 25 min (samples were heated for 10 min to reach 80^{o}C and then for 15 minutes to rise to 125^{o}C, held at this temperature for 30 min and finally ventilated for 30 min). The entire process was carried out at an energy setting of 700 W. After cooling the digested solutions to room temperature, the digests were diluted with triple-deionized water and zinc concentrations were measured by atomic absorption spectroscopy (AAS) (AAnalyst 400, Perkin Elmer). Contents of other elements were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES, Optima 7300 DV, Perkin Elmer). As reference materials for *N. caerulescens*, tobacco leaves (ASPAC 120) and apple leaves (NIST 1515) were included in the element analyses for quantitative verification of the results.

2.3 Sample preparation for Scanning Electron Microscope

Elemental distribution and composition of experimental plants was determined from subsamples of freeze-dried root and leaf materials. The fresh roots were washed gently in deionized water, blotted dry and then cut into cross-sections using razor blades. The three-week root samples were cut into 1-2 cm long transverse cross-sections at the zone of cell maturation and elongation. The 16-week root samples were divided at the middle into longitudinal cross sections (4-8 mm wide x 1.2-2.4 cm long) and the whole root sections were freeze-dried. Fresh mature leaves were cut into rectangular sections of approximately 1 cm wide x 2 cm long that included the midrib but excluded the leaf edges. The root and leaf samples were rapidly frozen by plunging into liquid nitrogen, and then placed into pre-cooled Eppendorf tubes and kept under vacuum for 48 hours at -30°C. Before samples were mounted on a scanning electron microscope (SEM) PST IA023 stub (Frey et al., 2000), the freeze-dried root samples were cut into 3-5 mm

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transverse cross-sections and the freeze-dried leaf samples were cut into cross-sections of 0.5 mm wide x 1 mm high. For the SEM 16-week root samples, both longitudinal and traverse cross-sections at the cell maturation, elongation and division zones, as well as the freeze-dried whole root samples, were mounted on stubs for SEM. The Scanning electron microscopy–energy dispersive X-ray spectra and X-ray mapping (SEM–EDS and elemental mapping) technique was used to determine the Zn distribution in the leaves and roots of the plant samples, which allowed for the qualitative estimation of Zn between different tissues at specific areas. It also enabled the composition of Zn-associated elements in the samples to be determined.

2.4 Statistical analysis

One-way and two-way analysis of variance (ANOVA) of dry mass and Zn content were performed using Genstat 4.2 (VSN International, UK) and SPSS 19 (SPSS Inc., USA). Sample means and standard errors were calculated using Microsoft Excel 2010. Results were considered significant at the 0.05 probability level ($p < 5\%$). Bivariate correlations, *r*, were calculated with SPSS using Pearson's correlation coefficient and a 1-tailed test of significance at the 0.01 and 0.05 levels.

3. Results

3.1 Effects of Zn treatment to plant growth

Two weeks after the introduction of Zn, shoot dry mass of the hyperaccumulator *N. caerulescens* increased by approximately 30% in the 200 μM and 300 μM Zn treatments in comparison with the control (no added Zn) (Fig. 1a). However, the dry mass of both roots and shoots was reduced by 25% in the 500 μM treatments, compared to the 200 μM and 300 μM treatments (Fig. 1a). Similarly, the shoot and root biomasses of the non-hyperaccumulator *T. arvense* were higher in the 200 μM and 300 μM Zn treatments than in the control. At 500 μM Zn, the root dry mass of *T. arvense* decreased by almost 25%, but a less significant decrease in the shoot dry mass was observed (Fig. 1b). Moreover, at the 500 μM Zn concentration, the non-hyperaccumulator showed severe Zn toxicity symptoms (photo not shown) such as stunted growth, and yellowing and wilting of shoot parts. Under the same Zn growth conditions, the accumulated biomass of the non-hyperaccumulator *T. arvense* was much higher than the biomass produced by the hyperaccumulator *N. caerulescens* (Fig. 1). The average shoot dry mass of the hyperaccumulator *N. caerulescens* ranged from 40 to 65 mg per plant (Fig. 1a), while for the non-hyperaccumulator *T. arvense,* the weight varied from 120 to 220 mg per plant (Fig. 1b).

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N. caerulescens showed greater capacity for Zn tolerance than the non-hyperaccumulator *T. arvense* under the high Zn concentration treatment. *N. caerulescens* kept growing well after five and seven weeks in the Zn treatments (Fig. 2), while *T. arvense* almost died two weeks after Zn introduction. The dry mass of shoots and roots of *N. caerulescens* was higher after seven weeks than five weeks exposure to Zn in all treatments, except for the shoot dry mass in the treatment with 500 μM Zn. The highest value of root dry mass was recorded in the control after seven weeks (an average of 160 mg) (Fig. 2b), while the highest value of shoot dry mass (550 mg) was in the 200 μM Zn treatment (Fig. 2a). Increasing the Zn concentration increased both root and shoot dry masses up to the fifth week, while there was a reverse direction of shoot and root dry masses in the seventh week in which shoot and root dry mass was reduced in all Zn treatments. For the treatment of 500 μM, shoot biomass after five weeks was 50% greater than the control, but only 15% above the control at seven weeks (Fig. 2a). Although plant growth tended to be inhibited after seven weeks, no visible toxicity symptoms (such as purple leaves or stunted rosette) were observed. The optimal Zn concentration for growth of *N. caerulescens* appeared to be in the range of 200 μM to 300 μM.

3.2 Zinc accumulation in *N. caerulescens* and *T. arvense*

N. caerulescens responded rapidly to the changes in the concentration of Zn in solution and within two weeks, Zn concentrations in shoots and roots of this species increased markedly (Fig. 3). Without Zn treatment (the control), Zn concentration was around 0.02% in the shoots and 0.012% in roots (dry weight basis). These values increased to 1.5-2.5% and 0.5-1% in shoots and roots, respectively, when grown in culture solution with Zn added at concentrations of 200 μM to 500 μM (Fig. 3a). In contrast, the non-hyperaccumulator *T. arvense* had a much lower overall capacity for Zn accumulation, with most of the Zn taken up by *T. arvense* remaining in the roots (Fig. 3b). Zn concentration varied from 0.4 to 0.6% in roots and was only 0.03-0.05% in shoots when the Zn treatment increased from 300 μM to 500 μM in *T. arvense* (Fig. 3b). Without adding Zn, the concentration of Zn in both root and shoot tissues of *T. arvense* was about 0.015%, which was similar to that in *N. caerulescens*. The Zn content in *N. caerulescens* was 3 to 5 times higher in *T. arvense* at equivalent concentrations, depending on the concentration (Fig. 3c),

The Zn concentration in the roots and shoots of *N. caerulescens* was highest for the 500 μM Zn treatment, ranging from 1.9% to 2.3% in shoots, and 0.9% to 1.1% in roots (Fig. 4a). The control Zn concentration at two weeks of approximately 0.02% in the shoots had increased to 0.3% at seven weeks, suggesting substantial cross-contamination. Apart from the analysis at

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seven weeks following the introduction of Zn, the Zn concentrations in the roots and shoots of the 200 μM and 300 μM Zn treatments were not significantly different. Shoot to root Zn concentration ratios represent the gap between Zn concentrations in roots and shoots (Fig. 4b). In general, these ratios changed from 2 to 3.5, except for the control after seven weeks and the treatment of 300 μM after two weeks. Upon fixed Zn treatment, the shoot to root Zn concentration ratio tended to decrease over the treatment length (two weeks to seven weeks), from 2.6 to 2.0 at the 200 μM Zn treatment, from 4.1 to 2.1 at the 300 μM Zn treatment and from 2.3 to 2.0 at the 500 μM Zn treatment, respectively (Fig. 4b). This decrease might be related to the large amount of Zn remaining in the roots (Fig. 4a). The shoot:root ratio of Zn in the 200 and 300 μM treatments was also higher than in the 500 μM Zn treatment at the same harvest times. In general, the total Zn content of shoots and roots increased with increasing concentrations of Zn and harvest times (Fig. 4c).

The concentrations of Zn supplied appear to have some effect on the concentrations of other elements such as Ca, Mg, Fe, K and P in the shoots and roots of *N. caerulescens* (Fig. 5 and Table 1 in Appendix). In particular, Ca and P concentrations decreased by 50% and 35% compared to the control in both roots and shoots, respectively, of those plants exposed to 500 μM Zn. Concentrations of Mg and S did not significantly change in shoots, but significantly decreased in roots. The Fe concentration in roots increased with an increase in the Zn concentration of the treatments, but there appeared to be a small reduction of Fe in the shoots of the same plants or treatments, while the Zn concentration in shoots and roots of *N. caerulescens* showed strong positive correlations with Zn concentration of treatment ($r = 0.92$ and 0.966 at $p < 0.01$, respectively). In contrast, Ca concentrations in shoots and roots had negative correlations with the Zn concentration of treatments ($r = -0.815$ and -0.925 at $p < 0.01$, respectively). Similarly, the uptake of P negatively correlated with the concentration of Zn in the solution culture: $r = -0.576$ in shoots ($p < 0.05$) and $r = -0.724$ ($p < 0.01$) in roots. Uptake of Fe by roots positively correlated with an increase in the Zn concentration of treatments ($p < 0.01$). The K concentration in the plant was not significantly affected by Zn treatments.

There was a correlation between elemental concentrations in *N. caerulescens* roots and shoots. A Pearson's correlation analysis between concentrations of elements (Table 1 in Appendix) showed that Ca had the most significant negative correlation with Zn accumulation both in shoots ($r = -0.934$) and roots ($r = -0.939$). Similarly, Mg and P were also negatively correlated with Zn accumulation. In contrast, Fe and Zn had the highest positive correlation in shoots ($r = 0.864$, $p < 0.01$). The uptake of Ca positively correlated with Mg and P accumulation, but negatively correlated with Fe uptake in shoots ($p < 0.01$). There was no significant

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correlation between Ca and P in shoots. Fe negatively correlated with Mg (r = - 0.764, p < 0.01) and S (r = -0.605, p < 0.05), but was not correlated with P (r = - 0.528). There was no significant correlation between S and Zn. P uptake had a significantly negative correlation with Zn accumulation (r = - 0.789, p <0.01), whereas Ca and P showed a strongly positive relationship in the roots (r = 0.793, p < 0.01).

3.3 Zn distribution in root and leaf tissues of *N. caerulescens* and *T. arvense* after short-term Zn treatments

Elemental maps of Zn, Ca, P and S across freeze-dried fractures of leaf sections of *N. caerulescens,* as determined using SEM, are shown in Fig. 6. Distributions of elements in the leaves of *N. caerulescens* were compared for the highest concentration of Zn added (500 μM) and the control (0 μM) at 3 weeks. The maps show that there was a large difference in Zn distribution between the 500 μM Zn treatment and the control. Zn occurred at high levels in the epidermal cells of leaves in the 500 μM Zn treatment, but there was a low occurrence in leaves of the control. Ca was abundant across leaf sections and favored the epidermal cells over the mesophyll cells. Distributions of P and S were similar. They were abundant and co-localized in leaf tissues. Both P and S were distinctly higher in the leaf veins. However, there was an invisible elemental distribution difference in the 3-week root sections of *N. caerulescens* (data not shown).

Fig. 7 shows the Zn, Ca, P and S maps of frozen fractures of root sections taken from the non-hyperaccumulator *T. arvense*. Interestingly, in *T. arvense,* Zn-rich crystallization was found abundantly in the root sections, while it was absent in leaves (data not shown). The Zn-rich crystallization was distributed mainly in the cortex and the highest distribution was observed at the ridge between the stele and the cortical cells of the roots. Ca accumulation was mainly in the cortex and the epidermis, and the distribution of S was quite similar to that of Ca across the root section, but at a higher concentration than Ca. P was found to be distributed widely but accumulated mainly in the ridge between the cortex and stele. The crystals contained a large amount of P and a significant amount of S (data not shown). According to quantitative Zn analysis (data not shown), the Zn concentration in the roots and shoots of the non-hyperaccumulator was approximately 6000 mg kg^{-1} and 1000 mg kg^{-1}, respectively. These results would suggest that Zn crystals could be found in the roots of the non-hyperaccumulator *T. arvense* when the *T. arvense* shoots showed Zn toxicity symptoms and the root Zn concentration was around 6000 mg kg^{-1}.

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3.4 Zn distribution in root and leaf tissues of *N. caerulescens* after long-term Zn treatments

In order to further examine the Zn distribution and partitioning strategies observed in the seventh week, it was decided to sample *N. caerulescens* plants that had been kept continually supplied with Zn (and treated as described in the primary experiment) until the 16th week, by which time this species was also showing severe Zn toxicity symptoms.

Localization of Zn and Ca was determined for leaves after 16 weeks of exposure to the Zn concentrations of 0 μM, 300 μM and 500 μM using the SEM-elemental mapping (Fig. 8). Interestingly, Zn-rich crystals of the globular shape were found to be abundant in the leaf epidermal cells of the 300 μM and 500 μM Zn treatments. The population of Zn-rich crystals in the epidermal tissues treated with 500 μM Zn was higher than that of the 300 μM Zn treatment. Both Ca and Zn appeared to localize in the epidermis.

Since Zn-rich crystals were found in the leaves of *N. caerulescens* when the exposure time was extended, their presence in the roots was also examined. The 500 μM Zn treatment root anatomy was observed by SEM-EDS technique (Fig. 9). Zn-rich crystals were found to be distributed heterogeneously along the root cortex and the ridge between the cortex and stele, being more abundant in the cortex of the maturation and elongation cell zones (Fig. 9A). Zn-rich crystals were almost absent in the stele and not observed in the root hairs (data not shown). Ca and Mg were found abundantly and homogenously across the root sections (Fig. 9B). S and P were also detected at high levels in the root sections, although while the distribution of P was scattered, S was found to mainly occur in the ridge between the cortex and stele.

Surprisingly, Zn-rich crystals were not observed in *N. caerulescens* after Zn treatments for three weeks (Fig. 6), even though the Zn concentrations in shoots and roots of the hyperaccumulator *N. caerulescens* were approximately 25000 mg kg^{-1} and 10000 mg kg^{-1}, respectively (Fig. 3a). However, Zn-containing crystals were found in both the leaves and the roots (Fig. 8 & Fig. 9) when Zn concentrations of 60000 mg kg^{-1} in the shoots and 10000 mg kg^{-1} in the roots (data not shown) were achieved by growing on the plants for 16 weeks. It was noted that the Zn concentrations in roots remained at the level of 10000 mg kg^{-1} at the 500 μM Zn treatment from the 2nd week of Zn supply. These results suggest that Zn-rich crystal formation depends not only on Zn content in the root cells, but also on the overloading of Zn sequestration in the leaf tissues.

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4. Discussion

4.1 Plant growth and Zn accumulation

Low biomass is a limitation of *N. caerulescens* for Zn remediation (Knight et al., 1997; Reeves et al., 2001; Robinson et al., 1998). In the current study, shoot biomass of the hyperaccumulator *N. caerulescens* is lower than the non-hyperaccumulator *T. arvense*, by a factor of 2 to 3, at the two-week harvest time (Fig. 1). However, the biomass of *N. caerulescens* shoots increased by 30% with Zn addition in the growth culture compared to the control. This result shows the high Zn requirement for growth by *N. caerulescens*. The result is consistent with previous findings (Robinson et al., 1998; Saison et al., 2004), in that growth of *N. caerulescens* was reduced down to 50% under Zn-deficient conditions compared to plants grown on Zn-sufficient soils.

N. caerulescens showed a high capacity for Zn accumulation compared to the non-hyperaccumulator *T. arvense*. Zn content accounted for over 2.5% (25 g Zn kg^{-1}) of the shoot dry weight after two weeks, without the plant showing any symptoms of toxicity. The Zn concentration in shoots of *N. caerulescens* decreased and was lower in plant tissues in the fifth and seventh weeks than in the second week. This result agrees with the findings from other studies on *N. caerulescens* (Lasat et al., 2000; Martínez et al., 2006), which showed higher Zn concentration in shoots after six weeks than after 12 or 24 weeks. The reason for this difference is most likely Zn dilution in the plant because of the higher biomass of older plants, since metal uptake is usually not proportional to biomass (Lasat et al., 2000; Martínez et al., 2006), or the age of the plant (Martínez et al., 2006). The higher capacity for *N. caerulescens* taking up more Zn at an earlier age possibly relates to Zn influx and Zn translocation from the roots to shoots when the plant is in a state of Zn deficiency. However, this hypothesis requires further study. The observed phenomenon also inspires a new direction for exploiting the plant for Zn phytoextraction at a suitable period of its growth cycle, by integrating with an appropriate fertilizer regime to increase shoot biomass without reducing the concentration of Zn in shoots (Monsant et al., 2008). The capacity of Zn tolerance and Zn accumulation/absorption of *N. caerulescens* growing on Zn-polluted sites strengthens the potential application of *N. caerulescens* in cleaning up Zn-contaminated soils and sediments at concentrations of Zn which may be toxic to other plant species.

4.2 Zn distribution in roots and leaves

The distribution and storage of Zn in the roots and shoots of *N. caerulescens* found in this study is consistent with previous findings which have shown a high ability of the hyperaccumulator *N. caerulescens* to translocate Zn from the root to the shoot while the non-

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hyperaccumulator *T. arvense* stores almost all of the excess Zn in the root cells and minimizes Zn transportation to shoots (Knight et al., 1997; Martínez et al., 2006). In the hyperaccumulator *N. caerulescens,* after 3 weeks, Zn was found to show a high distribution in leaves (Fig. 6A), while it was almost absent from roots (data not shown). Preferential sites of Zn accumulation in the hyperaccumulator were the leaf epidermis and root cortex, regardless of the duration of exposure to Zn. The difference in Zn distribution within plant tissues is an important property that could act as an indirect indicator of detoxification mechanisms which possibly protect metabolically active cellular compartments from the toxicity of high Zn concentration (Martínez et al., 2006).

There were different compositions in the Zn-rich crystals in the non-hyperaccumulator and the hyperaccumulator. In the current study, crystals were found in the roots of the non-hyperaccumulator species, even when Zn content was relatively low and in circumstances when Zn toxicity symptoms were evident. These crystals contained high amount of P and S (data not shown), indicating a high level of Zn-phytate ($C_6H_{18}O_{24}P_6$) and Zn-cysteine. Both Zn-associations are reported to have an important role in the detoxification of metal in the form of Zn-association in the root cells of the plant (Kopittke et al., 2011; Vazquez et al., 1994). However, the Zn concentration at which Zn crystals formed, and the composition of those Zn-rich crystals in the hyperaccumulator *N. caerulescens*, could lead to different conclusions and interpretations. On the basis of the data acquired to date, it is difficult to determine the range of Zn concentrations in shoots or roots at which Zn-rich crystals can be formed. The previous studies show that Zn-rich crystals in the epidermal and sub-epidermal leaf cells appeared at the Zn concentration of 13600 mg kg^{-1} dry weight (1.3%), and Zn deposits in the root cells appeared at the measured Zn concentration from 10000 to 18000 mg kg^{-1} (Lasat et al., 1998). According to our results, however, Zn-rich crystals were not observed in *N. caerulescens,* even when the Zn concentration was roughly equal to 25000 mg kg^{-1} (2.5 %) in the shoot and 10000 mg kg^{-1} (1%) in the roots (Fig. 3A). In the present study, Zn-containing crystals in the leaf and root tissues occurred when Zn concentrations in the shoot were measured at approximately 60000 mg kg^{-1} and at approximately 10000 mg kg^{-1} in roots. This crystal formation occurred after 16 weeks of exposure to elevated Zn, and, hence, it is suggested that the formation of Zn-rich crystals in *N. caerulescens* depends not only on the Zn content in plant cells, but also on the overloading and accumulation of Zn in the leaf as a whole.

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4.3 Uptake of Zn and Ca, Mg, P, Fe and S

In the literature, there is evidence of reciprocal interactions between Zn and other elements such as Ca, Mg, P and S for different types of plant cells. Some evidence shows that a high concentration of Zn is associated with high concentrations of Ca and Mg (Saison et al., 2004). Limestone application was reported to decrease Zn concentration in root tissues, while P was found to reduce Zn concentration in some crop species (Pierzynski, 1993). Other studies have found no relationship between Zn uptake and elements such as P and S, either at the tissue or cellular levels, or in *N. caerulescens* specifically (Frey et al., 2000; Kupper et al., 1999). However, concentrations of Ca, Mg, P and K in the epidermal sap of *N. caerulescens* were found to decrease with increasing Zn applications (Kupper et al., 1999; Saison et al., 2004). Zn uptake in protoplast was inhibited by 20-40% with the addition of 500 μM Zn (White-Monsant and Tang, 2013). Zn and Ca were reported to co-localize and co-increase in leaf cells of *N. caerulescens* (Monsant et al., 2010). Phosphoric acid was found to negatively correlate with Zn content in the shoots, but this correlation did not occur in the roots or xylem (White-Monsant and Tang, 2013).

According to our results, a higher concentration of Zn in shoots was associated with lower concentrations of Ca (r = -0.934, $p < 0.01$) and P (r = -0.649, $p < 0.05$). The concentration of Ca and P was reduced by 50% and 35%, respectively, when the Zn supply was increased from 0 μM to 500 μM, whereas the Zn concentration in shoots increased by 160-fold. Our results support the hypothesis of competition between Zn and Ca for plant uptake because of their similar charges and similar hydrated radii and the lack of specificity to metal transporters (Saison et al., 2004). Past reports suggest that sharing the same transport pathway may also account for the competition (Cosio et al., 2004; Saison et al., 2004). Mg, S and K contents in our experiments were approximately the same for all Zn treatments, showing that these elements may not be affected by Zn uptake, although an increase in Mg concentration has been considered as a possible defense mechanism of the plants to reduce potential heavy metal substitution on chlorophyll (Küpper et al., 1996). Fe was reported to decrease in the shoots of *Arabidopsis halleri* when elevated Zn was applied (Küpper et al., 2000), while the present study found that Fe content in the shoots of *N. caerulescens* was not affected significantly by any of the Zn treatments. The concentration of Fe in the roots was much higher than that in the shoots and this result agrees with the literature (Baker et al., 1994b), in that Zn was readily transported to the shoots, while large amounts of Fe remained in the roots.

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Our study found Zn-rich crystals available in the roots and shoots, but there were different types of elemental compositions between the Zn-rich crystals in these two locations. This finding concurs with reports from other studies (Vazquez et al., 1994; Zhao et al., 1998). The Zn-containing crystals may be the evidence supporting the hypothesis of the detoxification mechanism that acts via the precipitation of insoluble salts. The Zn-rich crystals were reported to be metal-phytate (inositol hexakisphosphate IP6 - $C_6H_{18}O_{24}P_6$) for inactivating Zn in plant vacuoles (Vazquez et al., 1994), but other reports have concluded that it is not Zn-phytate (Kupper et al., 1999). The Zn crystals have been suggested as Zn-phytates (Zn_3-phytate $Zn_3(PO_4)_2$, Zn_2-phytate $ZnPO_4$) in the root tissues of *N. caerulescens*, but not in the shoots (Zhao et al., 1998). This apparently points to P concentration being related to Zn concentration. P concentration was reported to negatively relate to Zn concentration in shoots, but positively relate to Zn concentration in roots, especially between insoluble P and insoluble Zn (Zhao et al., 1998). Phytate has been known to make some micro-nutrient minerals such as Zn and Fe and, to lesser extent, some macro-nutrient minerals such as Ca and Mg, non-absorbable. Recently, Zn-phytate was recorded at a low proportion (5-25%) in the shoots but high in the roots (45-49%) in *N. caerulescens* (White-Monsant and Tang, 2013). Our results show that P concentration was reduced by 50-60% in shoots exposed to 500 μM Zn for 16 weeks, and Zn-rich crystals in the epidermal cells of *N. caerulescens* leaves contained low concentrations of P, and significant amounts of Ca and Mg (data not shown). Therefore, we suggest that the Zn-crystals in leaves of *N. caerulescens* may not be Zn-phytate. Significant amounts of S and P were found in Zn-containing crystals in the roots and hence metallothioneins (Zn-cysteine ligands: Zn-$C_3H_7NO_2S$) and Zn-phytate are possibly both involved in the Zn crystallization in the roots.

5. Conclusions

The hyperaccumulator *Noccaea caerulescens* has a much higher Zn tolerance and Zn requirement for growth than the non-hyperaccumulator *Thlaspi arvense*. The exposure of these species to additional Zn for a short-term period led to a significant increase in biomass of both *N. caerulescens* and *T. arvense*, but prolonged Zn treatments decreased the biomass. Most of the Zn uptake by *T. arvense* was retained in the roots, while a large amount of the Zn taken-up by *N. caerulescens* was translocated to the leaves. The optimal Zn concentration for *N. caerulescens* growth when grown in solution was found to be from 200 μM to 300 μM. The uptake of Zn affected the concentrations of other elements such as Ca, P and S. The Ca concentration in *N. caerulescens* was significantly reduced when Zn uptake increased, and Ca was found to co-localize with Zn in the plant tissues. Zn-rich crystals appeared in the roots of the non-

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hyperaccumulator *T. arvense* three weeks after the introduction of Zn to the solution. However, the crystal formation did not occur in the hyperaccumulator *N. caerulescens* until later (up to 16 weeks), and not until the plant had accumulated high Zn concentrations and begun to display Zn toxicity symptoms. The composition and element ratios in the Zn-rich crystals between the roots and shoots, especially P and S, requires further studies to answer the question about whether P and S are involved in the formation of Zn compounds to detoxify Zn. Overall, it was postulated that once a threshold of Zn concentration is exceeded, Zn crystals are formed and stored in the less sensitive tissues to diminish toxicity by isolation from those tissues critical for plant metabolism. The different compositions of the Zn-rich crystals in the roots compared to the shoots, especially in regards to the levels and roles of P and S, suggest different Zn detoxification mechanisms in the roots and shoots in *N. caerulescens*. This finding stimulates additional studies into the Zn detoxification capabilities of *Noccaea caerulescens* and its applications.

Acknowledgements

The authors thank Dr. Damien Callahan from Melbourne University for providing the *Thlaspi* seeds. We are grateful to Dr. Marc Hampton for technical support in using SEM-EDS. The first author gratefully acknowledges the 165 scholarship program of Vietnam and The University of Queensland for the top-up scholarships.

Appendix

Table 1

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Fig. 1. Dry mass of shoot and root of the hyperaccumulator *N. caerulescens* (a) and non-hyperaccumulator *T. arvense* (b) two weeks after Zn treatments. Hereafter, bars represent standard errors (SE) of the means of the treatments (n = 3) with the same species if not otherwise stated.

Fig. 2. Dry mass of shoot (a) and root (b) of *N. caerulescens* harvested after five weeks (5W) and seven weeks (7W) at different Zn concentrations.

Fig. 3. Effect of Zn treatments after two weeks on (a) Zn concentration (mg kg^{-1}) of the hyperaccumulator *N. caerulescens*, (b) Zn concentration (mg kg^{-1}) of the non-hyperaccumulator *T. arvense* and (c) Zn content (mg $plant^{-1}$) (Zn content = Zn concentration * dry mass of shoots or dry mass of roots).

Fig. 4. Effect of Zn treatments on (a) Zn concentration of shoots and roots (mg kg^{-1}), (b) shoot-to-root Zn concentration ratio and (c) Zn content in shoots and roots of the hyperaccumulator *N. caerulescens* at different harvest times: two weeks (2W), five weeks (5W) and seven weeks (7W) after Zn treatments applied.

Fig. 5. Concentration of Zn and other elements: Ca (a), Mg (b), P(c), S(d), Fe (e) and K (f) (mg kg^{-1} dry mass) in roots and shoots of *N. caerulescens* after five weeks of Zn treatment (n = 3). Concentrations assigned different letters (A, B, C for element concentration in shoot; a, b, c for element concentration in root) show significant differences between the means ($p < 0.05$).

Fig. 6. SEM elemental maps of *N. caerulescens* leaf across sections of the Zn treatment of 500 µM (A) and control (0 µM) (B) after three weeks. Hereafter, the color scale shows X-ray intensity from low (black, blue) to high (red, white). **A:** Zn is dominant in epidermal layers (both the upper and lower epidermis), but almost absent from the mesophyll and vein cells. Ca is abundant and distributed homogeneously. P and S are higher in the mesophyll and vein cells than in the epidermal cells. The white horizontal scale bars are 50 µm. **B:** Low Zn is present in the leaf sections. There is abundant Ca but this is heterogeneously distributed between the upper and lower epidermises. P is high in the areas, where Ca is low. The white horizontal scale bars are 100 µm.

Fig. 7. SEM images and elemental maps of frozen-fracture across root section of the non-hyperaccumulator *T. arvense* after the 500µM Zn treatment for three weeks. Zn-rich crystallization was abundant in the root sections, and its distribution was dominant in the cortex. The highest distribution occurred close to the root stele. Ca was abundant and its distribution was homogeneous in the cortex. P was high and its distribution was mainly in the junction between the cortex and stele. The white horizontal scale bars are 5 µm.

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Fig. 8. SEM images and elemental maps of frozen-fractures leaf sections of *N. caerulescens* after Zn treatments for 16 weeks. Zn-rich crystals were dominant in epidermal cells (both the upper and lower epidermis) at the Zn treatments of 300μM and 500μM. Ca was high in both the epidermal cells and the mesophyll cells. Ca was significantly higher in the upper epidermis than in the lower epidermis. For the control (0 μM), Zn distribution was insignificant and scattered in the leaf. Ca was abundant and its distribution was dominant in the upper epidermal cells and the mesophyll cells. Zn and Ca were co-localized in the epidermis of the leaf. The white horizontal scale bars are 100 μm.

Fig. 9. SEM images and elemental maps of freeze-dried root sections *N. caerulescens* after 16 weeks of the 500 μM Zn treatment. (A): SEM images of (a) a longitudinal root section of the cell maturation zone, (b) a transversal cortex region and (c) a Zn-rich crystal in the cortex zone. Zn-rich crystals were found abundant in the cortex. (B): SEM image of a longitudinal section at the root cortex and its adjacent stele of the cell maturation zone and elemental maps of the image showing that Zn-rich crystals were abundant but scattered in the section. Ca and Mg were abundant but Ca was more homogenous than Mg. P was scattered but S was mainly in the cortex.

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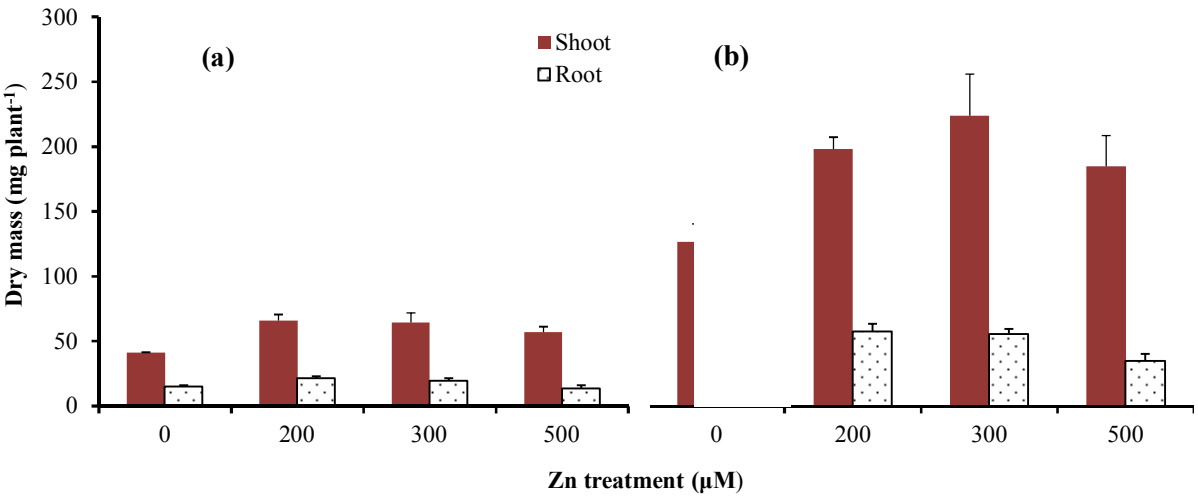

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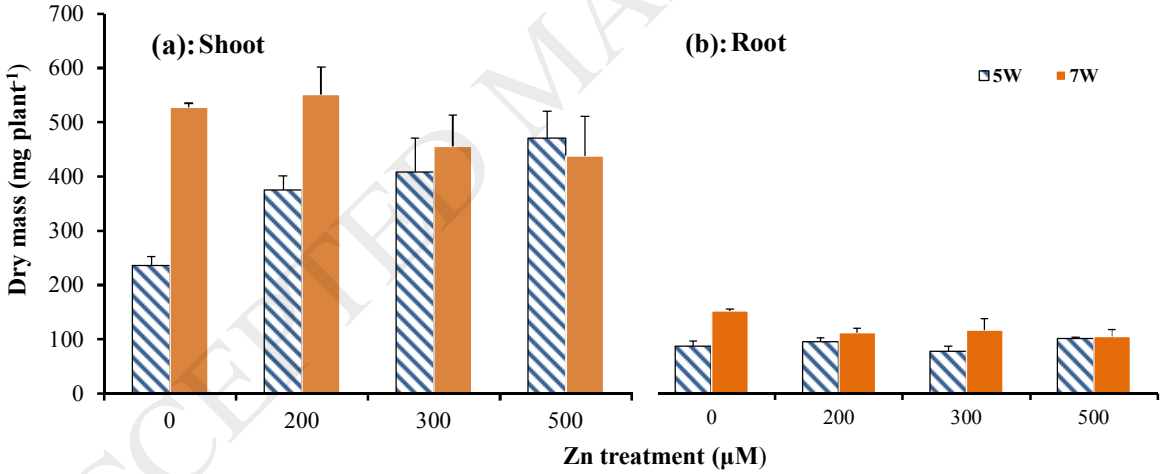

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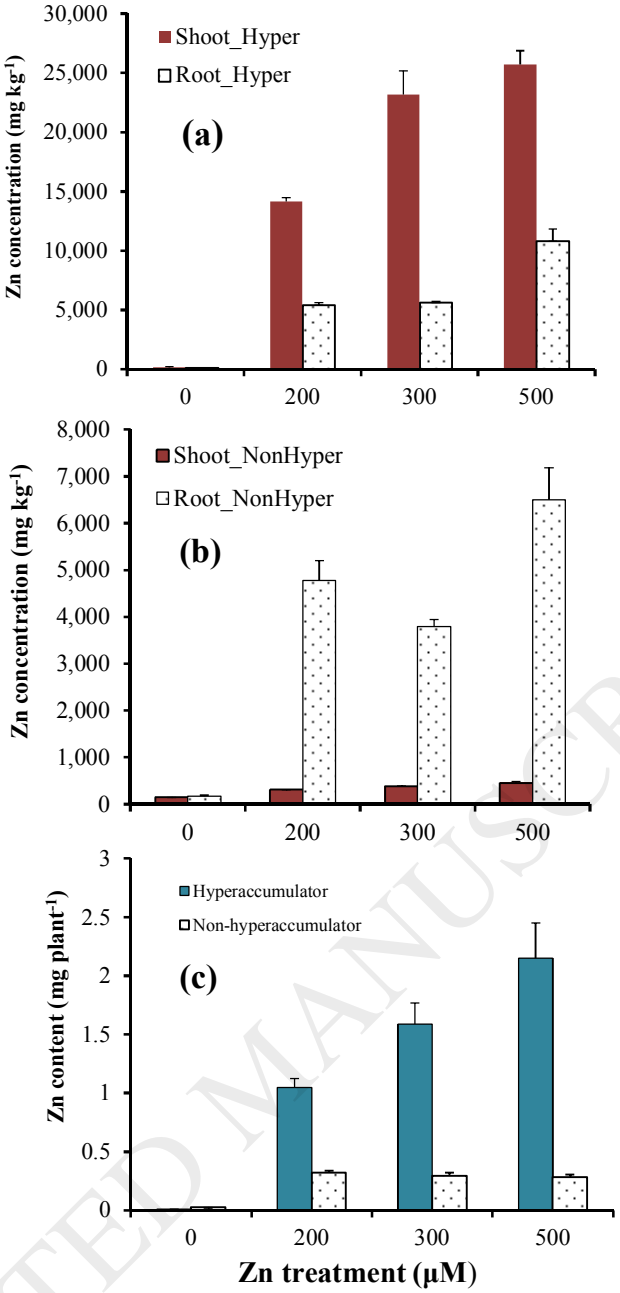

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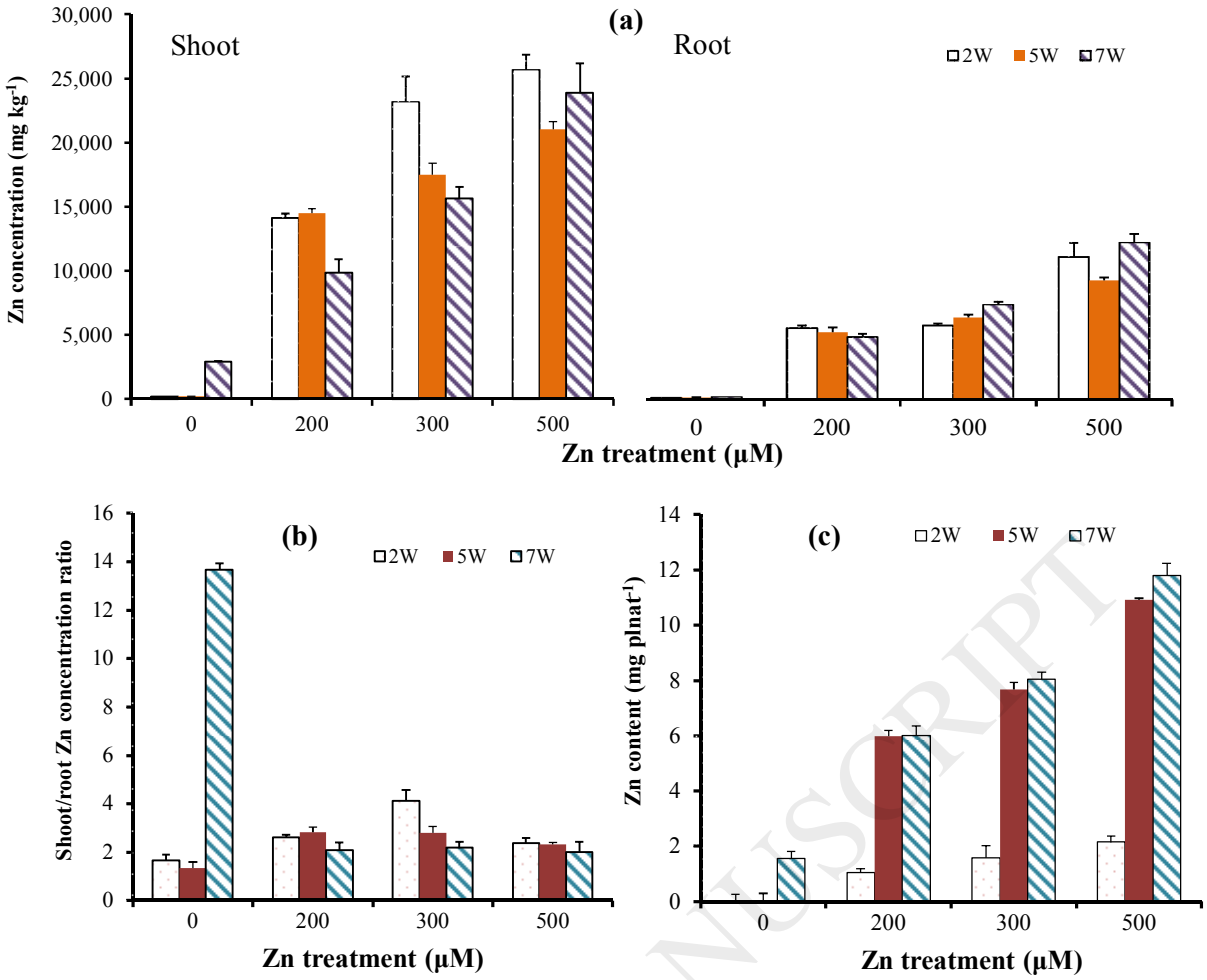

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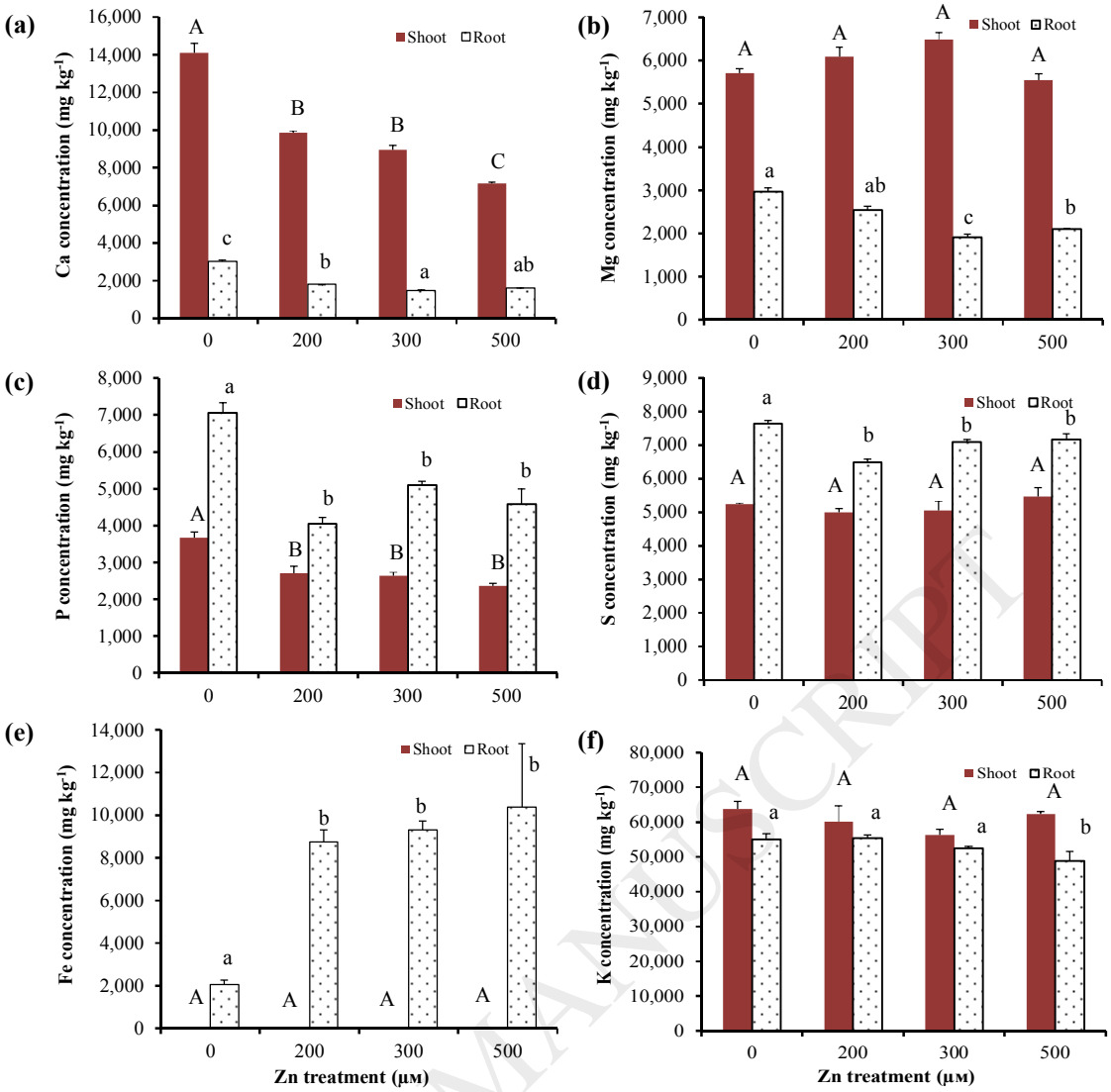

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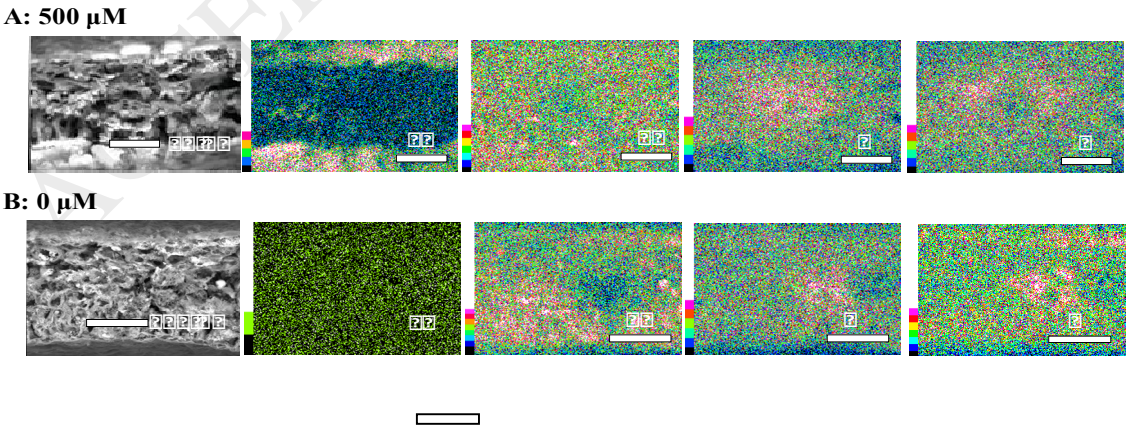

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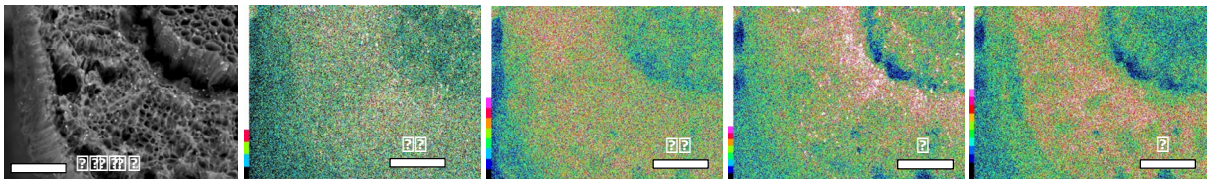

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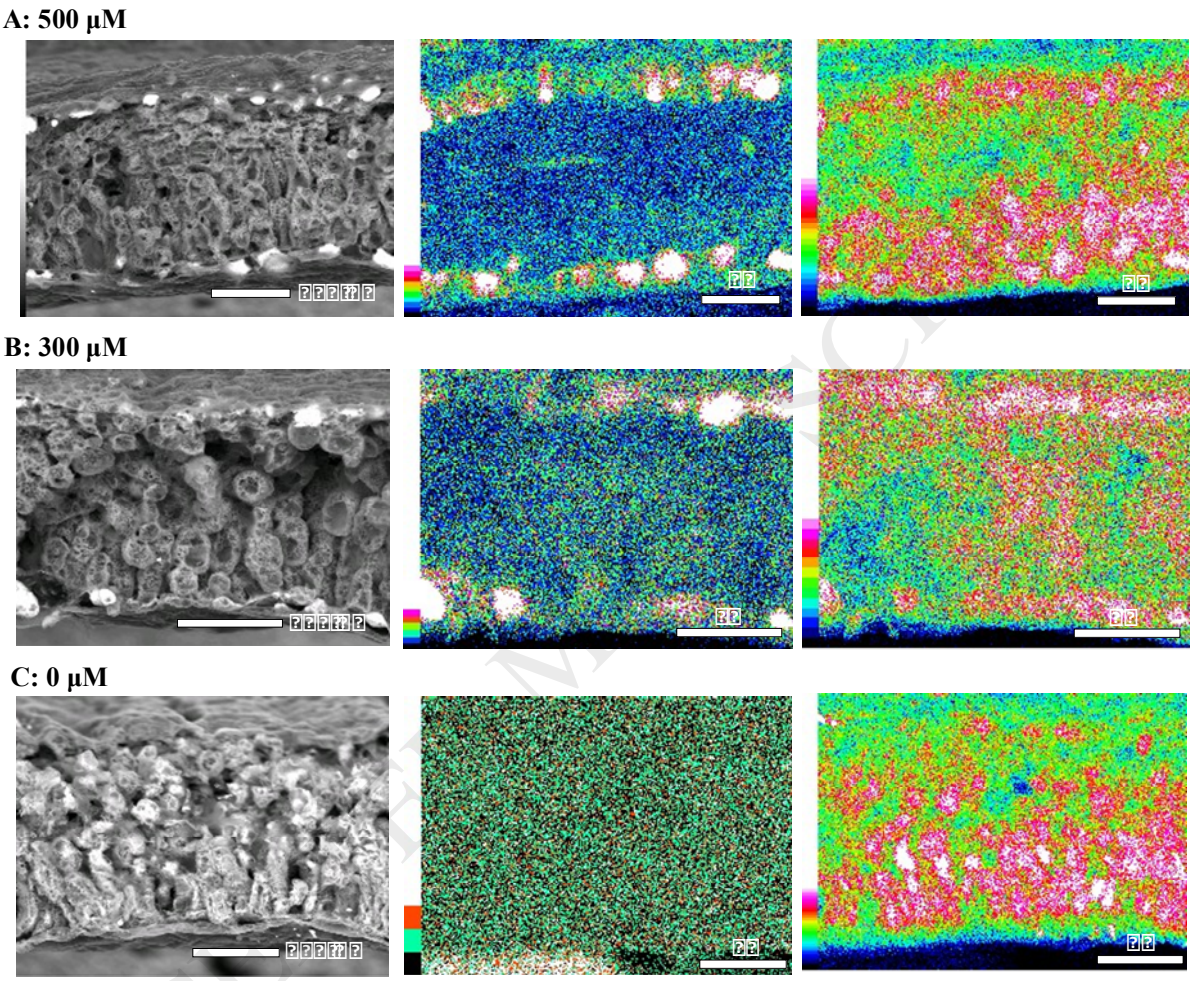

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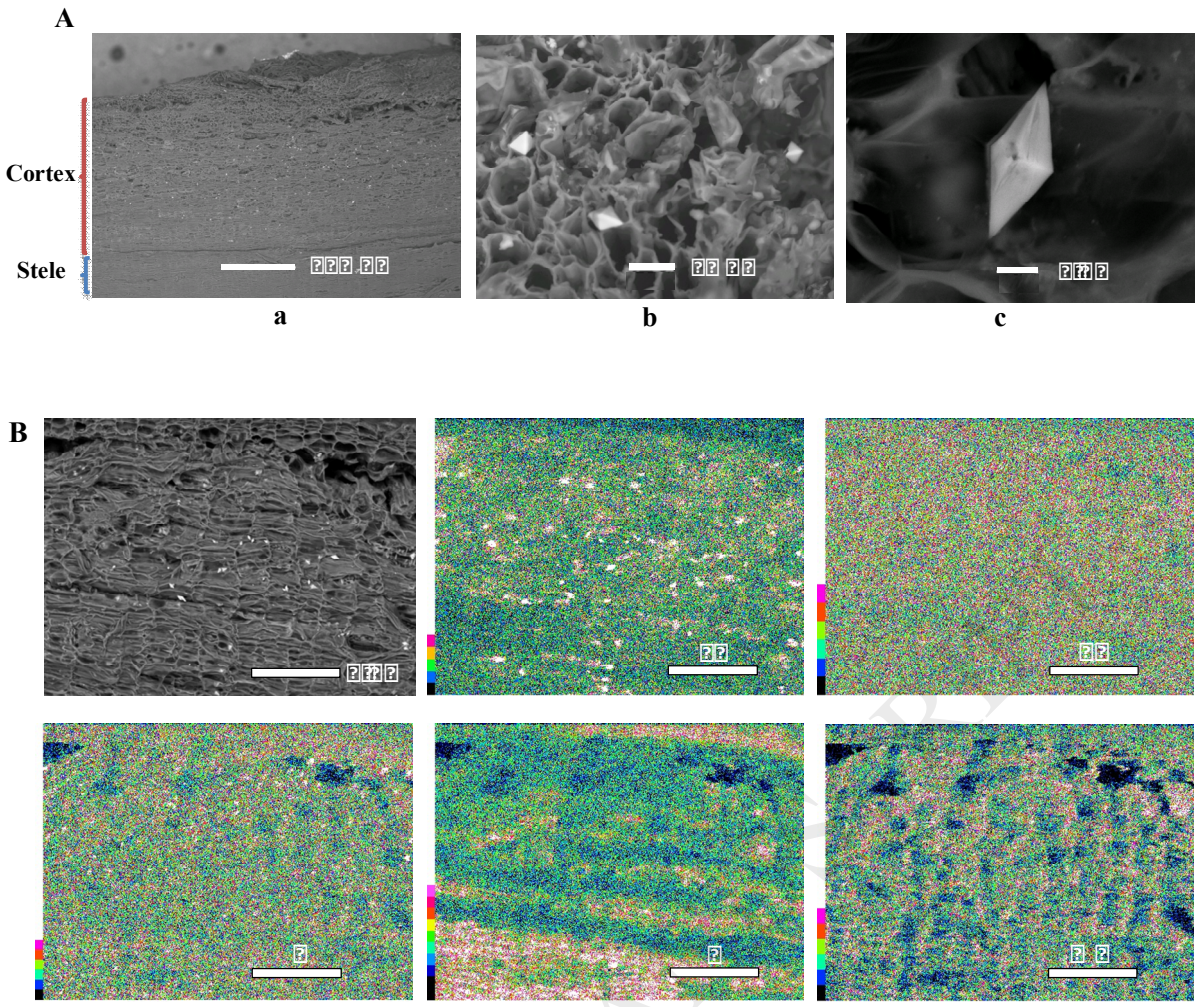

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Table 1. Correlations with statistical significance between concentrations of Zn and other elements in shoots and roots of *N. caerulescens* after five week Zn treatments (n = 12). Pearson's correlation and a two-tailed test of significance at the 0.01 and 0.05 level were applied

Element	Metabolite	Correlation, r	Level of significance
Shoot			
Zn	Ca	-0.934	0.01
Zn	Mg	-0.767	0.01
Zn	P	-0.649	0.05
Zn	S	-0.438	ns
Zn	Fe	0.864	0.01
Ca	Mg	0.849	0.01
Ca	Fe	-0.857	0.01
Ca	P	0.733	0.01
Ca	S	0.493	ns
Mg	Fe	-0.764	0.01
P	Fe	-0.528	ns
S	Fe	-0.605	0.05
Root			
Zn	Ca	-0.939	0.01
Zn	P	-0.789	0.01
Ca	P	0.793	0.01
S	K	0.412	ns

* ns means "not significant"

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