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Zinc and lead accumulation characteristics and *in vivo* distribution of Zn^{2+} in the hyperaccumulator *Noccaea caerulescens* elucidated with fluorescent probes and laser confocal microscopy

Ngoc Dinh[a], Antony van der Ent[b,c], David R. Mulligan[b], Anh V. Nguyen[a,*]

[a] School of Chemical Engineering, The University of Queensland, Brisbane, Queensland, 4072, Australia
[b] Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, Queensland, 4072, Australia
[c] Université de Lorraine – INRA, Laboratoire Sols et Environnement, UMR 1120, France

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ABSTRACT

Tolerance and accumulation of multiple trace elements simultaneously in hyperaccumulator species enable these plants to grow on sites contaminated with these elements. In this study, accumulation and tolerance to zinc (Zn) and lead (Pb) by the hyperaccumulator *Noccaea caerulescens* (Brassicaceae) was investigated using different Zn^{2+} and Pb^{2+} treatments in hydroponic culture. The results confirmed that *N. caerulescens* has a high capacity for Zn^{2+} accumulation while having high levels of Pb^{2+} tolerance. The younger plants were more tolerant to Zn^{2+} and Pb^{2+} than the older plants. The accumulation of Zn^{2+} in shoots or roots was not significantly affected by treatment regime or plant age. Pb accumulated mainly in the roots (0.16–0.23 wt% dry mass), confirming substantial tolerance to Pb. The concentration of phosphorus (P) in older plant shoots decreased ~25% in the plants treated with Zn^{2+}, but enhanced ~26% in the plants treated with Zn^{2+} + Pb^{2+}. The high ratio of Zn to P in both fresh and dry leaves is suggestive of the formation of insoluble Zn-salts. The Zn^{2+} distribution in living cells was examined using three selective fluorescent probes (Zinpyr-1, Newport Green DCF and Phen Green SK). The fluorescent probes showed that Zn^{2+} was mainly located in the apoplastic space of the leaf epidermal cells. Selective fluorescent probes in combination with laser confocal microscopy proved a useful tool for elucidating cellular and tissue-level distribution of Zn^{2+} in living plant cells at high resolution. However, the expected vacuolar sequestration of Zn^{2+} was not observed, which may be explained by insufficient penetration of the fluorophores.

1. Introduction

More than 400 plant species have been identified as trace element hyperaccumulators, including some that are capable of accumulating multiple trace elements simultaneously (Baker et al., 2000; Brooks, 2008). *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) is a well-known zinc (Zn) and cadmium (Cd) hyperaccumulator plant (Cosio et al., 2004; Küpper and Kochian, 2010; Pence et al., 2000; Vázquez et al., 1992), which can accumulate up to 40 000 μg g^{-1} of Zn and 4000 μg g^{-1} of Cd in its shoots without exhibiting any symptoms of toxicity (Chaney, 1993; Ebbs et al., 2002; Shen et al., 1997). *Noccaea caerulescens* can also tolerate soils with high concentrations of nickel (Ni) (Mari et al., 2006) and lead (Pb) (Baker et al., 1994).

Hyperaccumulation of Zn and tolerance to Pb enables *N. caerulescens* to persist in Zn and Pb enriched or contaminated metalliferous environments. Robinson et al. (1998) studied the potential of *N. caerulescens* for phytoextraction of Pb/Zn base-metal mine waste and found that tissue-level Pb and Zn accumulation significantly correlated with both total and extractable soil concentrations, but that no specific tolerance to Pb was evident. Pb was found to accumulate mainly in the roots of *N. caerulescens*, with little translocation to the shoot (Martínez et al., 2006; Walker and Bernal, 2004), while Pb uptake did not significantly affect Zn accumulation or plant growth (Walker and Bernal, 2004). Our previous work on *N. caerulescens* showed that Pb^{2+} treatment inhibited plant growth and Zn accumulation, and that the Pb accumulated in the roots with limited translocation, this being a typical "Excluder" response (Baker, 1981; van der Ent et al., 2012). The only credible report for significant Pb accumulation in a *Noccaea*-species is

Abbreviations: N. caerulescens, *Noccaea caerulescens*; SEM-EDS, scanning - energy dispersive X-ray spectroscopy; μM micro molar, M molar; Wt, weight; TEM, transmission electron microscopy

* Corresponding author.
E-mail address: anh.nguyen@eng.uq.edu.au (A.V. Nguyen).

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for *N. rotundifolium* subsp. *cepaeifolium* which accumulated 8200 μg g^{-1} Pb from a mine site in Italy (Reeves and Brooks, 1983). Hydroponic experiments with *N. caerulescens* suggest that there may be some propensity for Pb hyperaccumulation in *N. caerulescens* (Mohtadi et al., 2012), albeit exclusively in specific populations, similarly to Cd or Ni hyperaccumulation in this species (Assunção et al., 2003).

Interaction between Zn and P in plant tissues was reported to impact Zn^{2+} uptake and detoxification in some hyperaccumulator plants by the formation of Zn-phosphate precipitates (Küpper et al., 1999; Vazquez et al., 1994; Vollenweider et al., 2011). Cakmak and Marschner (1987) found that high concentrations of P in plant tissues reduces the physiological availability of Zn^{2+}, and the level of water-soluble Zn may be considered as an indicator for P-induced Zn-deficiency. However, Zn hyperaccumulation was reported not to induce P-deficiency (Zhao et al., 1998). Our previous study on *N. caerulescens* showed that Zn-deficiency promoted P-accumulation both in shoots and roots, and that high Zn^{2+} treatment increases the shoot P-concentration with 35% (Dinh et al., 2015). Zinc and P-rich globular precipitates have been observed in the leaf epidermal cells of *N. caerulescens* (Vazquez et al., 1994), but this co-precipitation of Zn with P was shown to be important in the roots, not in the shoots (Zhao et al., 1998). Our previous study also elucidated the formation of Zn-rich crystals in the root and leaf epidermal cells in *N. caerulescens*, but only the Zn-rich crystals in the roots contained significant quantities of P (Dinh et al., 2015).

Elucidating the cellular and tissue-level distribution of trace element ions, such as Zn^{2+}, is challenging due to the inherent limitations of many analytical techniques. Methods based on electron probe X-ray microanalysis (SEM-EDS) include scanning electron microscopy (SEM) or transmission electron microscopy (TEM) coupled with energy-dispersive x-ray spectroscopy (EDS), and other techniques such as proton-induced x-ray emission (PIXE) and secondary-ion mass spectrometry (SIMS), all require substantial pre-treatment of samples prior to analysis such as freezing or freeze-drying (Galway et al., 1995; Hodson, 1995; Lombi and Susini, 2009). The use of SEM-EDS suffers from insufficient sensitivity and resolution for analysing Zn^{2+} ions at the subcellular level due to the size of the effective 'probe volume' of Zn characteristic X-rays (Hodson, 1995; Lombi and Susini, 2009). None of these techniques are suitable for fresh or living plant tissue samples due to the high vacuum conditions required. Although living plant tissues may be imaged using synchrotron radiation including X-ray fluorescence microscopy (XFM), there are limitations to sample preparation, dehydration, and radiation damage, and the method is accessible only via highly competitive synchrotron beam time applications. The use of laser confocal microscopy in combination with selective fluorescent probes ('fluorophores') to map trace element ions (such as Zn^{2+}) could overcome some of these challenges (Pollastri et al., 2012). To date, however, few published studies apply fluorescent probes to investigate trace element distribution in hyperaccumulator plants. Nevertheless, ion-selective fluorophores in combination with laser confocal microscopy have been used to image the distribution of Ni and Zn in hyperaccumulator plants, for example Newport Green ($C_{43}H_{30}Cl_2N_4O_8$) has been used for imaging Ni^{2+} in the cells of *Alyssum murale* (Agrawal et al., 2013) and *A. lesbiacum* (Ingle et al., 2008). The chelating dye Dimethylglyoxime ($C_4H_8N_2O_2$) which forms a crimson complex with Ni^{2+} and has been used as a histochemical stain for the localization of Ni within tissues of hyperaccumulator plants (Mizuno et al., 2003; Bhatia et al., 2004). Zinpyr-1 ($C_{46}H_{36}Cl_2N_6O_5$) has been used for *Noccaea caerulescens* for staining Zn^{2+} (Seregin and Kozhevnikova, 2011; Seregin et al., 2011; Kozhevnikova et al., 2014) and in elucidating Zn^{2+} homeostasis in *Arabidopsis* roots (Sinclair et al., 2007). Zinpyr-1 is highly selective for Zn^{2+} over other divalent cations, such as Ca^{2+} and Mg^{2+} with a dissociation constant, K_d, for Zn^{2+} of 0.7 nM (Burdette et al., 2001). Other studies include the application of fluorescent probes to investigate cellular tolerance to Zn^{2+}, Ni^{2+} and Cd^{+2} in *N. caerulescens* and *Arabidopsis halleri* (Marquès et al., 2004). Leadmium Green was used to map Zn^{2+} and Cd^{2+} in the hyperaccumulators *Sedum alfredii* and *Picris divaricata* (Lu et al., 2008; Hu et al., 2012). Similarly, Zincon ($C_{20}H_{15}N_4NaO_6S$), which forms a blue complex with Zn^{2+}, has been used for staining Zn in *N. caerulescens* (Macnair and Smirnoff 1999; Kozhevnikova et al., 2014). Although fluorophores have a number of merits, including high selectivity, the ability to be used in live tissues (in time-resolved visualization), and high-resolution with (laser) light microscopy, there are also limitations related to the unknown penetration into cells and binding to target metal(loid) ions (Gei et al., 2017).

This study aimed to investigate: (i) the effect of plant growth on Zn and Pb tissue-level accumulation and tolerance; (ii) the interactions between water-soluble fractions of foliar Zn and P, and; (iii) to elucidate the distribution of Zn^{2+} in living cells using selective fluorescent probe and laser confocal microscopy with the aim of evaluating the usefulness of this method.

2. Materials and methods

2.1. Plant culture

The plants were grown hydroponically in a glasshouse using methods detailed in a previous study (Dinh et al., 2015). Briefly, the seeds of *N. caerulescens* (Prayon ecotype) were sterilized with 0.5% NaClO, germinated in the dark, and then 1-week-old seedlings were transplanted into foam strips in contact with basal nutrient solution (Monsant et al., 2010) consisting of the following: 20 μM KH_2PO_4, 600 μM K_2SO_4, 200 μM $MgSO_4$, 100 μM $CaCl_2$, 10 μM FeEDDHA, 10 μM FeNaEDTA, 5 μM H_3BO_3, 1 $MnSO_4$, 0.2 μM $CuSO_4$, 0.03 μM Na_2MoO_4 and 600 μM $Ca(NO_3)_2$. The plants were grown in groups of three per closed-cell foam strip (six strips per pot) placed on top of 5 L black polyethylene pots. The nutrient solution was aerated continuously. The factorial experiment consisted of four treatments: Zn11W (500 μM Zn) and Zn + Pb11W (500 μM Zn + 24 μM Pb) each applied to 11-week old plants for 27 weeks; Zn17W (500 μM Zn) and Zn + Pb17W (500 μM Zn + 24 μM Pb) each applied to 17-week old plants for 27 weeks, all replicated three times. The plants treated from 11 weeks were harvested at 38 weeks and plants treated from 17 weeks were harvested at 44 weeks, hence they are referred to as younger and older plants, respectively. The Zn^{2+} was supplied as $ZnSO_4$ and the Pb^{2+} as $Pb(NO_3)_2$. The nutrient solutions were maintained at the same volume by adding nutrient solution daily and replacing the solution at five-day intervals.

2.2. Plant harvest and analysis

The harvested plants were washed with de-ionized water, blotted dry with tissue paper, and then separated at the root-shoot junction. The fresh shoots and roots were weighed, and then dried in paper bags at 80 °C for 24 h. The dried plant samples were weighed immediately after removal from the drying oven, and then ground using a mortar and pestle. Weighed subsamples of shoots and roots were placed into 50 mL digestion tubes and digested in 7 mL concentrated HNO_3 70% (v/v) mixed with 2 mL H_2O_2 30% (v/v) in a microwave digester (Milestone Start) for a total process time of 1 h 25 min (Dinh et al., 2015). The total concentrations of Zn, Pb and other elements in the digests were determined using inductively-coupled plasma atomic emission spectroscopy (ICP-AES, Varian Vista Pro II).

Water-soluble Zn and P in fresh mature leaf blades were extracted with 1 mM MES buffer at pH 6.0 and the respective concentrations