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A bisphosphonate increasing the shoot biomass of the metal hyperaccumulator *Noccaea caerulescens*

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HIGHLIGHTS

- The effects of 7 bisphosphonates on the growth by *N. caerulescens* were studied.
- Also the shoot metal removal was investigated.
- The plants were grown in soils spiked with Cd, Ni, Zn and Pb.
- An insoluble aminobisphosphonate was found to enhance the plant growth in Ni-spiked soil.
- Therefore, also the shoot Ni removal was increased by the bisphosphonate.

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ABSTRACT

The feasibility of using the hyperaccumulator plant, *Noccaea caerulescens*, to remove trace elements from contaminated soils has been studied extensively. However, this plant creates too low biomass and an inappropriately slow growth rate for actual use in the field. Soluble bisphosphonates (BPs) are well-known pharmaceutical compounds e.g. affecting the osteoclast function in body through metabolic pathways. We devised an insoluble aminoBP, hydroxyundecylidene-1,1,-bisphosphonic acid with a long alkyl chain to be extremely effective metal chelator, and its possible use in phytoremediation deserves more attention. This article examines the effects of seven BPs on the shoot biomass, shoot metal concentrations and removal (Cd, Ni, Zn and Pb) by *N. caerulescens* in a pot experiment. The soluble BPs were incorporated into the soil in the irrigation water and the insoluble BP as solid after which the soil was spiked with metals. The insoluble aminoBP was found to considerably increase the shoot yield of *N. caerulescens*, especially in Ni-spiked soil, thus showing enhanced shoot Ni removal.

1. Introduction

Phytoremediation, the use of plants and associated microorganisms to remove pollutants or make them harmless in soil, is a putative solution for the ever increasing problems originating from water and soil contamination (Salt et al., 1998; Vangronsveld et al., 2009; Vamerali et al., 2010; Ali et al., 2013; van der Ent et al., 2013). Trace elements, which originate from the anthropogenic activity such as main tailings, use of fertilizers and pesticides, industrial discharge etc., are one of the main pollutants in soil and currently a cause of public concern. Phytoextraction refers to the use of plants to remove metal(loid)s from soil by concentrating them in the harvestable parts of the plants. It is both an ecological and economical way to remove harmful contaminants but it poses certain requirements for the characteristics of the plant: the ideal plant should be able to take up metal ions effectively from soil into the root cells, load them in xylem and transport the metals from roots to shoots. In the leaves, metal ions are stored within the cells and detoxified effectively (Meyer and Verbruggen, 2012).

Noccaea caerulescens (previously named *Thlaspi caerulescens*) is a well-studied plant, hyperaccumulating zinc (Zn), cadmium (Cd) and nickel (Ni) (McGrath et al., 2006; Vangronsveld et al., 2009; Bhargava et al., 2012). Unfortunately, the plant has a low biomass and a slow growth rate, these characteristics virtually exclude the possibility of using the plant in the field for phytoextraction. Generally, the increase of the biomass of plants can be accomplished by using fertilizers. However, N, P and S fertilization has been shown not to affect strongly the shoot biomass of *N. caerulescens* (Sirguey et al., 2006). Another way to increase the biomass could be by genetic engineering (Martínez et al., 2006). The possibility of adding chelating agents into the soil for increasing metal

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bioavailability, uptake and translocation has been extensively studied, with ethylenediamine tetraacetate (EDTA) being the most commonly used chelator due to its high efficiency in extracting several metals (Vamerali et al., 2010). However, EDTA is poorly biodegradable, it can be toxic to the plants and soil microbial communities, and it poses the risk of metal leaching (Grčman et al., 2001). In addition, low-toxic and biodegradable chelators, such as ethylenediamine disuccinate (EDDS), nitrilotriacetic acid (NTA) or low molecular weight organic acids have been investigated as supplements to phytoextraction (Luo et al., 2005; McGrath et al., 2006).

Bisphosphonates (BPs) are chemically very stable molecules containing an O═P—C—P═O structure. They are known to be good metal chelators due to the superior complexing ability of the diphosphonic moiety (Matveev et al., 1998; Gumienna-Kontecka et al., 2002; Matczak-Jon and Videnova-Adrabinska, 2005). BPs have been used for many purposes during the past 50 years (Fleisch, 1995). Originally they were utilized as water softeners, but nowadays they are used as drugs for the treatment of bone-related diseases, a property based on their high affinity for the bone mineral, hydroxyapatite. In addition, they are being investigated, for example, as herbicides (Kafarski et al., 2000). However, despite the wide range of applications by BPs, we were able to find only one publication in the literature considering any other effect of BPs on plants: the study by Manzano et al. on the root growth and chlorophyll formation of *Arabidopsis thaliana* seedlings with BPs in the culture medium (Manzano et al, 2012). Moreover, as far as we are aware, the effects of BPs on the metal uptake of the plants have not been reported previously.

BPs can be divided into two groups based on their mechanism of action: non-nitrogen-containing (NNBP) and nitrogen-containing (NBP) BPs (Benford et al., 1999). NBPs have at least one nitrogen atom in the structure where as NNBPs do not contain any nitrogen. In mammalian cells, NNBPs are incorporated into analogs of adenosine triphosphate (ATP), which induce osteoclast apoptosis, but NBPs act through a different mechanism. They inhibit the farnesyl pyrophosphate synthase (FPPS), which is a key enzyme in biosynthesis of isoprenoids e.g. carotenoids, chlorophylls, tocopherols and phytoalexins. FPPS also has an important role in farnesylation and geranylation of proteins (Dhar et al., 2013). In plants, the role of FPPS has been investigated, e.g. in *Brassica napus*, where the *FPPS* gene was RNAi-silenced resulting in a decrease of the extent of farnesylation of proteins (Wang et al., 2005). A transgenic canola has displayed enhanced sensitivity to abscisic acid (ABA) and as a consequence the plant is drought resistant. This property was attributed to decreased stomatal conductance and water transpiration.

An insoluble aminoBP with an alkyl chain length of eleven carbons has proven to be an effective metal chelator, forming stable complexes with several metals, i.e. Cd, Ni, Zn and lead (Pb) (Alanne et al., 2013). During the chelation process, the BP remains as a solid which makes it readily filterable from the solution. This property could be utilized, e.g., in the waste water purification. In parallel, metal chelation properties of this compound could be exploited to enhance metal solubility in the soil, root uptake and shoot metal removal preventing the negative effects of the contaminant metals on the plant growth and the rhizosphere.

This study investigated the single incorporation of seven BPs and EDTA into the soil and their influence on shoot yield as well as on metal concentration and removal by *N. caerulescens* Ganges ecotype. The metals studied were Cd, Ni, Zn and Pb, and the plants were grown with and without addition of the metals for comparing the effects of the treatments. Moreover, the phosphorus (P) concentrations in the shoots and the metal chelation abilities of the used BPs, as well as soil properties were determined.

2. Materials and methods

Additional parts of the materials and methods are shown in the Supplementary Material section S1.

2.1. Plant growth

The seeds of an inbred accession of *N. caerulescens* Ganges (GA, also called St Laurent Le Minier) (Assunção et al., 2003) were sown in pots filled with 150 g of the mixture (1:4) of sand and Kekkilä Garden Soil (according to producers, containing peat with different degrees of decomposition, very fine sand, and clay as well as fertilizers, lime, and compost) for BPs **1**–**6**. In addition, seeds were sown by broadcasting in the sand–soil mixture and the germinated seedlings were transferred to the pots containing 200 g of the sand–soil mixture including the insoluble BP**7** in concentrations of 73.2 mg kg^{-1} (0.21 mmol kg^{-1}) low concentration (**low**) or 1.464 g kg^{-1} (4.22 mmol kg^{-1}) high concentration (**high**) (see Table S1 in Supplementary Information for concentrations). Other BPs (**1**–**6**) and EDTA as a control were given to the plants in different amounts of irrigation water (10–100 mL) depending on their solubility, to the final concentrations of 0.25 mmol kg^{-1} of soil dry weight (DW) (**low**) and 5 mmol kg^{-1} soil DW (**high**). The concentrations as mass unit kg^{-1} soil DW (**low** and **high**, respectively) were: 73.3 mg kg^{-1}, 1.44 g kg^{-1} for BP**1**; 51.6 mg kg^{-1}, 1.03 g kg^{-1} for **2**; 44.0 mg kg^{-1}, 0.88 g kg^{-1} for **3**; 62.2 mg kg^{-1}, 1.24 g kg^{-1} for **4**; 69.3 mg kg^{-1}, 1.39 g kg^{-1} for **5**; 76.4 mg kg^{-1}, 1.53 g kg^{-1} for **6**; and 92.9 mg kg^{-1}, 1.86 g kg^{-1} for EDTA.

The metals were added in 10 mL of water to the final concentrations: 38.5 mg kg^{-1} soil DW (0.05 mmol kg^{-1}) as $3(CdSO_4) \times 8H_2O$, 13.1 mg kg^{-1} (0.05 mmol kg^{-1}) as $NiSO_4 \times 6\ H_2O$, 287.5 mg kg^{-1} (1 mmol kg^{-1}) as $ZnSO_4 \times 7H_2O$ and 331 mg kg^{-1} (1 mmol kg^{-1}) as $Pb(NO_3)_2$ for BPs **1**–**6** and EDTA. For BP**7** the same salts were used and the concentrations were 22.5 mg kg^{-1} (0.029 mmol kg^{-1}) for Cd, 19.7 mg kg^{-1} (0.075 mmol kg^{-1}) for Ni, 215.6 mg kg^{-1} (0.75 mmol kg^{-1}) for Zn and 248.2 mg kg^{-1} (0.75 mmol kg^{-1}) for Pb (Table S1). Cd and Ni were used for the **low** BPs whereas Zn and Pb for the **high** ones. In addition, zero controls, BP controls (both **low** and **high**) and metal controls were included in the experiment. All the plants were grown in four replicates in a greenhouse for seven weeks (winter time 5.11.12–15.1.13) after application of metals and BPs (18/20 °C night/day, 18 h photoperiod, 200 μmol m^{-2} s^{-1}) in Kuopio Finland (69.75°N, 5.34°E). However, three replicates were considered to be enough for the experiments due to the large amount of samples and slow sample treatment, thus three of the four grown plants were randomly selected for the metal (and other) determinations. Therefore, the fourth plant was grown just to be sure that at least three replicates were usable if there were some clear outliners.

3. Results and discussion

3.1. Metal chelation by bisphosphonates

The BPs used in the experiments are shown in Fig. 1. AminoBPs (**4**–**7**) were selected for the experiments initially because of their metal chelation properties. BP**7** in previous experiment was an excellent chelator for several metals and therefore was suggested as the most interesting BP for phytoremediation experiments (Alanne et al., 2013). In addition, it has a very low solubility in water (58 mg L^{-1} at pH 4.7) which distinguishes it from the other BPs (Alanne et al., 2012). Because of its insolubility, it holds no potential as a drug. Moreover, BP**7** exerted no undesired effects in an acute toxicity study in rats, in Ames-test or in ecotoxicological experiments. Thus, it should not cause any harm for the