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# Biogenic Separation, Accumulation and Cellular Distribution of Cu, Co, and Ni in *Medicago sativa* under Idealized Conditions

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# Biogenic Separation, Accumulation and Cellular Distribution of Cu, Co, and Ni in *Medicago sativa* under Idealized Conditions

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**The limits of uptake of Co, Ni, and Cu by the common metallophyte, *Medicago sativa*, were assessed using hydroponic growth and metal uptake experiments. The influence of the growth substrate metal concentration (500 and 1000 ppm) and exposure time, i.e., the time plants were exposed to the metal solution (24, 48, or 72 h) was investigated. The combined roots and shoots of *Medicago sativa* accumulated up to 2.2 wt-% Co, 2.0 wt-% Ni, and 3.5 wt-% Cu, when exposed to aqueous solutions containing 1000 ppm Co for 48 h, 1000 ppm Ni for 72 h, and 1000 ppm Cu for 72 h, respectively. The distribution of the sequestered metals was assessed using proton induced that X-ray emission spectroscopy (μ-PIXE), which indicated that translocation mechanism was most likely xylem loading. However, the rate of translocation of the metal from the roots to the plant stem was different for each metal, suggesting differing mechanisms for each. Collectively, these results suggest the separation and removal of the heavy metals Cu, Co, and Ni from contaminated substrates using *Medicago sativa* is a viable technology.**

**Keywords** cobalt (Co); copper (Cu); *Medicago sativa*; metallophyte; nickel (Ni); phytoremediation

## INTRODUCTION

Industrialization has led to an increase in land and water pollution, particularly due to heavy metals. Consequently heavy metal remediation techniques are required. One of these is rhizofiltration, a remediation technique involving absorption, accumulation, and precipitation of metals from a contaminated water source using plants. It is a subset of a larger field of research known as phytoextraction, i.e., the process of using plants to beneficially remediate or sequester mineral species from soils, sediments and aqueous systems (1–3). Other applications include phytoremediation, where non-naturally occurring contaminants are recovered for disposal or reuse, phytostabilization, where contaminant species are immobilized in situ via plant action, and phytomining which involves the recovery of metals using plants for commercial gain (1–4). There are numerous examples of plants being used to treat contaminated environments containing Cd, Cu, Co, Hg, Pb, Ni, Tl, Ar, Se, $CN^-$, hydrocarbons, explosives residues, and radioactive compounds (3–5).

Most often the technical and economic feasibility of phytoextraction is assessed using plants that are metal hyperaccumulators, i.e., those which exhibit enhanced uptake of specific metals compared with non-accumulators (2). Hyperaccumulator plants have two common characteristics (4):

i) They exhibit a bio-concentration factor, defined as the ratio of metal concentration in plant shoots to that in the soil, greater than one. In some cases bio-concentration factors up to 100 have been observed (3,4). The bio-concentration factor is a measure of the ability of a plant to take up and transport metals to the shoots, which are the parts that can be most easily harvested.
ii) They possess enhanced tolerance (hypertolerance) to metals, both at the cellular level and in the environment, indicative of a strong mechanism for coping with high metal concentrations. For example, a "normal" plant will accumulate between 10 and 100 mg/kg Ni on a dry weight basis; however, a Ni hyperaccumulator will accumulate to >1000 mg/kg (4).

Unfortunately, the majority of hyperaccumulators also exhibit low biomass yields. For example, in the case of Co, uptake levels in the above ground shoots of *Haumaniastrum robertii* were reported to be up 10,200 μg/g but at a typical biomass yield of only 4 tons $ha^{-1}$ $yr^{-1}$ (6–9), while for Cu, *Haumaniastrum katangense* was reported to

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accumulate up to 8356 μg/g at a typical biomass yield of 5 tons $ha^{-1}$ $yr^{-1}$ (10) and *Aeollanthus biformifolius* up to 13,700 μg/g (6,7,11,12). For Ni there have been several hundred reports of hyperaccumulation, with the two most well known being *Alyssum bertolonii* (13,400 μg/g) at a typical biomass yield of 9 tons $ha^{-1}$ $yr^{-1}$ and *Berkheya coddii* (17,000 μg/g) at a typical biomass yield of 18 tons $ha^{-1}$ $yr^{-1}$(10). Other Ni hyperaccumulators include *Phyllanthus xpallidus*, with a reported uptake of between 15,390–60,170 mg/Kg dry weight in the combined plant tissues (13,14), *Sibertia acuminata* (26%-wt dry basis in the leaves) (6,15,16) and *Hybanthus austrocaledonicus* (27wt-% Ni in the ash following combustion) (17).

Consequently, plants most suited for phytoextraction exhibit a combination of high biomass yield (typically greater than 15 tons $ha^{-1}$ $yr^{-1}$) and metal tolerance (but not necessarily hyperaccumulation). Plants exhibiting metal tolerance are known as metallophytes (18,19). In this work, the phytoextraction potential of Co, Ni, and Cu was assessed using the model metallophyte, *Medicago sativa* (*M. sativa*) grown in aqueous solutions to assess the maximum limits of metal accumulation under idealized conditions. The typical biomass yield of *M. sativa* is >20 tons $ha^{-1}$ $yr^{-1}$, it is highly metal tolerant (20) and grows in most climates making it especially suited to phytoextraction. In this work, the influence of exposure time, substrate metal ion concentration, and chelating agent addition were investigated. To assess the practical limits of metal uptake, a large concentration driving force was used, by exposing the plants to substrates containing up to 1000 ppm, as $M_2SO_4$ (where M = Co, Ni, or Cu). We also investigated the influence of exposure time, i.e., the time a live plant was in contact with the metal rich solution and the addition of a chelating agent (EDTA). It has been demonstrated previously that chelating agents can improve the bioavailability of metals in a soil matrix (21–23) and hence we postulated that if a metal is bound to a chelating agent it may pass through the root membrane more easily than if it remained unbound.



## EXPERIMENTAL

Plant samples were prepared according to Bali et al. (24) as follows: *M. sativa* seeds were surface sterilized in a solution of 1.0% hydrogen peroxide for 15 min to avoid fungal contamination, washed with deionized water, and then germinated on wet paper towels for 48 h in an incubator (without illumination) at 25°C. Seedlings were then transplanted into glass jars containing 250 ml Hoagland's media. All experiments were performed in the controlled environment of a plant growth chamber (Contherm Scientific Ltd) with a 12/12 light/dark cycle (25°C/18°C). Seedlings were harvested between two and three weeks following germination and transferred to Petri plates containing aqueous metal salt solutions of Cu, Co, or Ni at pH 4.12, 4.75, and 5.10 respectively. The effect of metal ion concentration (500 and 1000 ppm), exposure time (24, 48, or 72 h) and plant organ (roots and shoots) were investigated during the course of experiments. Four replicates of each experiment were used; reproducibility between samples across all conditions was within +/− 15%. From these results, outliers were removed using a standard least-median-of-squares algorithm, followed by calculation of the mean and standard deviation of the metal concentration.

After exposure, plants were harvested, washed with demineralized water, dried for 48 h at 105°C, weighed, and ashed in air at 500°C for 4 h. The ash was then digested in nitric acid using microwave-assisted digestion at 105°C for 15 min (Milestone ETHOS SEL). The sample volume was raised to 10 ml with the addition of 1 M HCl prior to analysis by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Calibration standards were prepared using commercial ICP standards (Sigma, 1000 mg/L). All calibration curves had a correlation coefficient of >0.99.

To assess the mechanism of translocation, μ-PIXE analysis was undertaken using facilities at the Australian Nuclear Science and Technology Institute (ANSTO), comprising a high energy heavy ion microprobe which uses a 10 MV Tandem accelerator, providing a 3 MeV proton beam with typical spot size of between 3 and 5 μm. At this spot size, beam currents between 0.1 and 0.5 nA can be achieved, which is sufficient for μ-PIXE analysis of plant samples. A high-purity Ge detector was used with a 100 $mm^2$ active area, located 33 mm from the sample. A 100 μm Mylar foil was used to reduce low energy x-rays and thus pile-up in the μ-PIXE spectrum (25). μ-PIXE analysis has previously been used to quantify Ni in the stems and leaves of *H. floribundus* (27), Cd in *Brassica juncea* and Co in *Solanum lycopersicum* (tomato) and *Triticum* (wheat) (28,29).

For preliminary μ-PIXE analysis hand sections of *M. sativa* roots and stems were obtained. These were immediately plunged into liquid nitrogen, followed by freeze drying for 24 h. Freeze dried sections were mounted on carbon tape held within a standard ANSTO aluminium microprobe sample holder. For the remainder of the μ-PIXE experiments the modified method of Bidwell (25) was used. Circular discs of root, stem, and leaf tissue were cut using a hole punch and placed in Au sample holders, few drops of 1-hexadecane were introduced (to exclude gas bubbles) prior to loading into a high pressure freezer (Leica EM HPF). High pressure frozen samples were transferred to a freeze substitution media (diethyl ether, DEE). The frozen leaf sections were then substituted with diethyl ether over a freshly activated molecular sieve for 3 days at −90°C. Samples were then gradually raised to −30°C at a rate of 1°C/h and held for 48 h at this temperature.

The temperature was finally raised to room temperature at 1°C/h. Freeze substituted samples were then infiltrated with increasing ratios of Spurr's resin over 72 h (Spurr's resin: DEE, 2:8 for 24 h, 5:5 for 24 h, and 8:2 for 24 h) before finally being infiltrated with 100% Spurr's resin overnight for 12 h. Once substitution was complete samples were embedded in fresh resin and cured at 60°C for 24 h. Approximately 6–10 μm sections were cut using a dry glass knife microtome (Leica Microsystems), and then mounted onto standard ANSTO aluminium holders ready for analysis.

## RESULTS AND DISCUSSION

Figure 1 shows the accumulated weight (roots and shoots combined) of Co (Fig. 1a), Cu (Fig. 1b) and Ni (Fig. 1c) per dry weight of plant (mg/g) as a function of the exposure time (24, 48, or 72 h) and concentration in the growth substrate (500 ppm and 1000 ppm) for *M. sativa*. The full data set is shown in Table 1. There is a significant increase in accumulated metal in the combined roots and shoots following 24 h exposure (8.8 mg-Co/g plant dry wt and 9.5 mg-Co/g plant dry wt) to 48 h exposure (18 mg-Co/g plant dry wt and 22 mg-Co/g plant dry wt) at 500 ppm and 1000 ppm substrate Co concentrations, respectively. However, at both levels, after 72 h exposure, the plant Co concentration had decreased to 17 mg-Co/g plant dry wt and 20 mg-Co/g plant dry wt, respectively. This suggests there is Co extrusion at these extended exposure times. Co concentrations in non-hyperaccumulators are reported to be as low as 0.1–10 μg/g dry weight (30), and hence the high Co uptake and translocation in our experiments suggests *M. sativa* to be an hyperaccumulator of Co. Hyperaccumulators can accumulate 100 times elemental concentration in the shoots than normal plants growing in the same environment, e.g., the amounts should be more than 1000 mg/kg in As, Pb, Cu, Ni, and Co (31,32). The uptake in the plant roots increased with time at both 500 and 1000 ppm, reaching a



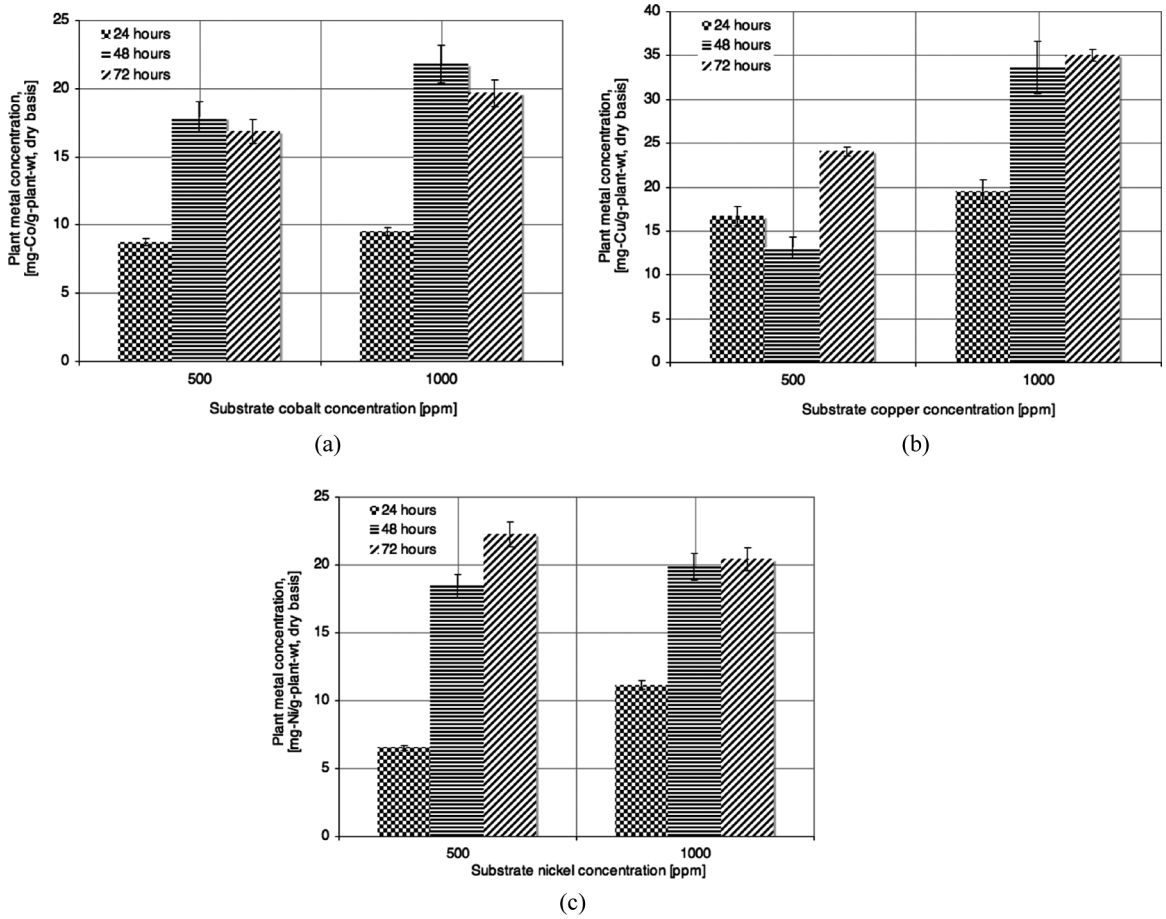
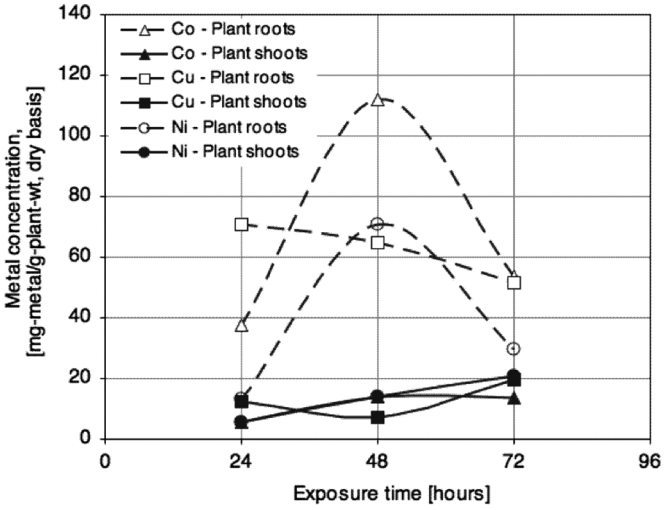


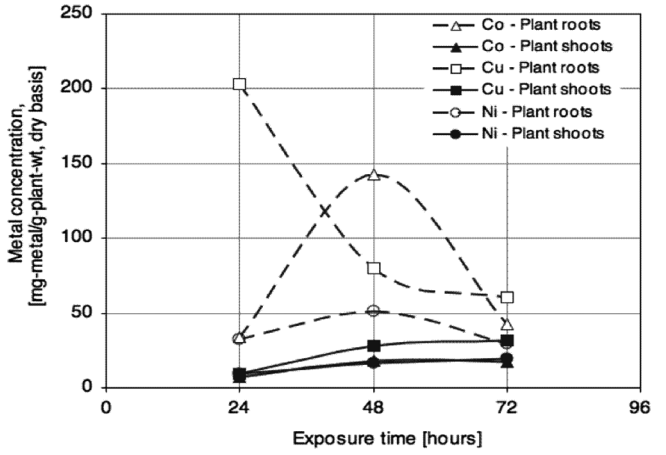

FIG. 1. (a) Cobalt concentration in the plant tissues of Medicago sativa, expressed as mg Co/g plant dry weight as a function of the cobalt ion concentration in the growth medium and the exposure time, (b) Copper concentration in the plant tissues of Medicago sativa, expressed as mg Cu/g plant dry weight as a function of the copper ion concentration in the growth medium and the exposure time, and (c) Nickel concentration in the plant tissues of Medicago sativa, expressed as mg Ni/g plant dry weight as a function of the nickel ion concentration in the growth medium and the exposure time.

TABLE 1
Results of experiments conducted on Medicago sativa showing the influence of exposure time, substrate concentration and plant organ

| Plant organ | Metal | Concentration (ppm) | Exposure (hrs) | Uptake (mg/g) | Translocation factor |
|---|---|---|---|---|---|
| Root | Co | 500 | 24 | 37.8 | 0.15 |
| | | | 48 | 111.9 | 0.12 |
| | | | 72 | 53.6 | 0.26 |
| | | 1000 | 24 | 34.0 | 0.21 |
| | | | 48 | 142.4 | 0.12 |
| | | | 72 | 42.3 | 0.40 |
| | Cu | 500 | 24 | 70.7 | 0.18 |
| | | | 48 | 64.7 | 0.11 |
| | | | 72 | 51.6 | 0.38 |
| | | 1000 | 24 | 203.3 | 0.05 |
| | | | 48 | 80.0 | 0.35 |
| | | | 72 | 60.6 | 0.53 |
| | Ni | 500 | 24 | 13.3 | 0.42 |
| | | | 48 | 71.0 | 0.20 |
| | | | 72 | 29.7 | 0.71 |
| | | 1000 | 24 | 32.4 | 0.30 |
| | | | 48 | 51.2 | 0.32 |
| | | | 72 | 29.6 | 0.65 |
| Shoot | Co | 500 | 24 | 5.7 | |
| | | | 48 | 13.8 | |
| | | | 72 | 13.7 | |
| | | 1000 | 24 | 7.0 | |
| | | | 48 | 17.8 | |
| | | | 72 | 17.0 | |
| | Cu | 500 | 24 | 12.5 | |
| | | | 48 | 7.2 | |
| | | | 72 | 19.7 | |
| | | 1000 | 24 | 9.5 | |
| | | | 48 | 28.3 | |
| | | | 72 | 31.9 | |
| | Ni | 500 | 24 | 5.6 | |
| | | | 48 | 14.0 | |
| | | | 72 | 20.9 | |
| | | 1000 | 24 | 9.6 | |
| | | | 48 | 16.4 | |
| | | | 72 | 19.1 | |




(a)




(b)

FIG. 2. Distribution of accumulated metal between the above and below ground plant tissue, as a function of exposure time in Medicago sativa, expressed as mg metal/g plant dry weight, at a substrate metal concentration of 500 ppm, and (b) Distrubution of accumulated metal between the above and below ground plant tissue, as a function of exposure time in Medicago sativa, expressed as mg metal/g plant dry weight, at a substrate metal concentration of 1000 ppm.

maximum of 112 and 142 mg-Co/g plant dry wt respectively at 48 h and then decreased with a further increase in exposure time. In the shoots, the uptake increased significantly from 24 to 48 h and then reaches a plateau at 72 h in both concentrations studied (Fig. 2a and Fig. 2b). Translocation Factor (TF), defined as the ratio of metal accumulation in the shoots to metal accumulation in the roots is also reported. Across both concentrations and exposure time studied TF varied from 0.12–0.40 time. In general a higher transport of Cobalt to shoots was observed at a longer exposure time of 72 h at both concentrations of 500 and 1000 ppm. In general previous reports suggest higher Ni uptake than Co, e.g., single element pot trials using *Allysum* showed higher Ni uptake (33); but there are exceptions. These include *Rinorea* species where elevated Co concentrations were observed (8). Our results demonstrate higher accumulation of Co than Ni, suggesting Co accumulation to be a plant specific phenomenon.

Figure 1b shows concentrations of Cu in *M. sativa* increase in response to an increase in Cu concentration in the growth media. At 1000 ppm, the combined root and shoot concentration increased to a maximum of 35 mg-Cu/g plant dry wt after 72 h. However, at 500 ppm, a slight decrease in concentration from 24–48 h (17 to 13 mg-Cu/g plant dry wt) was observed, followed by a significant

increase in uptake from 48–72 h. Metals enter the plant through the root membrane, via cation absorption from solution, either by selective transport systems or by binding to specific sites on the cell walls. The decrease in metal uptake after 24 h is therefore likely to be the result of non-availability of free metal binding sites or alteration of the membrane permeability. The observed increase in uptake from 13 mg-Cu/g plant dry wt to 24 mg-Cu/g plant dry wt from 48–72 h Cu may be due to a subsequent release of metal ions from the binding sites to the cytoplasm, hence freeing them for metal binding (Fig.1b). Copper transports across root cells as $Cu^{+2}$ (34) and the xylem cell walls are known to contain numerous negatively charged carboxyl groups, which tend to bind to divalent metal ions. Hence, as Cu moves through the plant cells as a divalent ion, it is possible that binding to the xylem cell wall occurs which further impedes transport to above ground tissues. Figure 2a shows that in general uptake in the roots decreased with time. A high rate of Cu uptake at 24 h was observed, to a maximum of 203 mg-Cu/g plant dry wt observed at 1000 ppm (Fig. 2b). In general, the results show higher uptake of Cu in the roots than shoots, a phenomenon previously observed in many other plant species (35,36). However, there is a greater influx of Cu from roots to shoots with time and concentration (Fig. 2a and Fig. 2b). The TF varied from 0.05–0.53 across time and concentration studied. With an increase in time, the shoot is taking more of the metal from the plant roots, depleting the root Cu concentration. Similar observations of higher TFs at longer exposure times were observed in the cobalt experiments. After 72 h of exposure, a maximum TF of 0.38 and 0.53 was observed at 500 and 1000 ppm respectively, corresponding to a maximum of 3.2-wt% Cu in the shoots after 72 h exposure at 1000 ppm. This suggests *M. sativa* to be a hyperaccumulator of Cu under these conditions.

For Ni, the combined root and shoot plant metal uptake rate increased with an increase in substrate metal concentration and exposure time, i.e., the higher the metal concentration in the growth media and the longer the plants were exposed to this, the more metal they accumulated (Fig. 1c). The concentration of Ni was 2–3 orders of magnitude greater at 48 h (11 to 20 mg-Ni/g plant dry wt) compared to 24 h (6.5 to 18 mg-Ni/g plant dry wt) and the Ni concentration varied from 5.6 to 71 mg-Ni/g plant dry wt between roots and shoots at 500 and 1000 ppm concentrations (Fig. 2a and Fig. 2b). Like Cu and Co, the quantity of Ni retained in the root system was substantial. However, the results also suggest the lack of substantial increase in uptake in roots with concentration (Fig. 2a and Fig. 2b). At an exposure concentration of 500 ppm, the highest concentration of Ni in the root tissue was approximately 71 mg-Ni/g plant dry wt after 48 h reducing to 29.6 at 72 h, suggesting activation of an exclusion mechanism. However, the shoots showed an increase in uptake with time and concentration (Fig. 2a), with concentrations between 9.6 to 19 mg-Ni/g plant dry wt with time at 1000 ppm exposure. Amongst the three metals studied, root to shoot transport was highest for Ni, followed by Cu and then Co (Fig. 2a and Fig. 2b). A maximum TF of 0.71 was observed after 72 h of exposure to 500 ppm. In general the TF varied from 0.20–0.71 across concentrations and time studied.

*M. sativa* showed an order of metal uptake Co (1.7%) < Ni (1.9%) < Cu (3.2%) in its shoots. Previous studies have shown that some plants may absorb large quantities of more than one metal (37). The same phenomenon has been observed in *M. sativa*, which showed an ability to absorb large quantities of more than one metal (20,22). However, the uptake abilities of each plant type are reported to differ with metal type, as different mechanisms are responsible in each case. For example, Homer et al. (33) showed a totally different order of tolerance Ni>Co ≈ Cu in *Aurinia saxatilis*.

The addition of EDTA resulted in no discernable improvement in the ability of *M. sativa* to extract and sequester any of Co, Ni, or Cu. Indeed, its addition impacted negatively upon the health of the plants, and the level

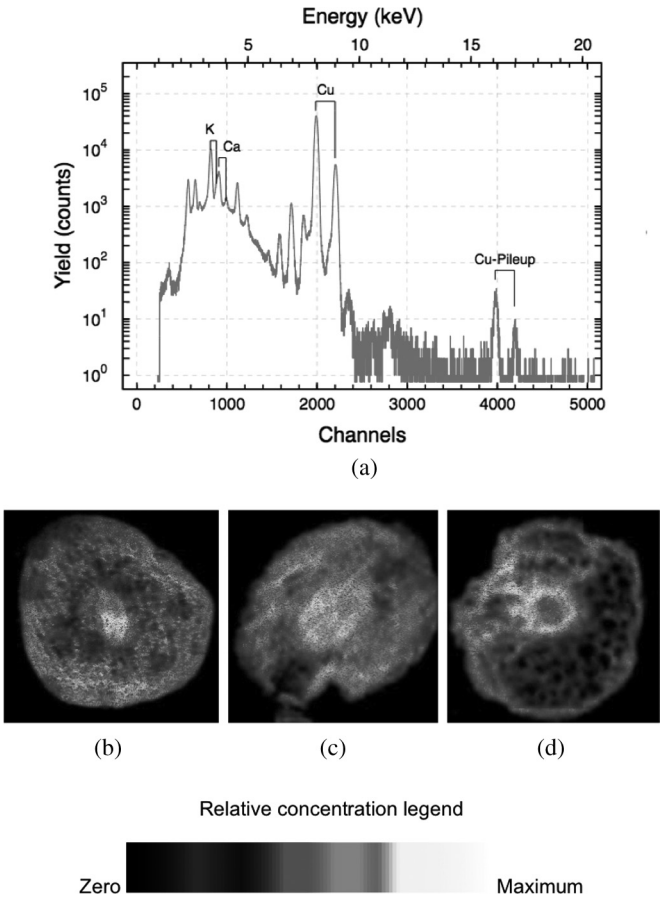



FIG. 3. PIXE; a) Sample acquisition spectrum; b) Co, c) Ni, d) Cu distributions in the cross section of Medicago sativa root suggesting xylem loading as the dominant metal transport mechanism.

of uptake reduced accordingly. Robinson et al. (38) made similar observations for *B. codii* where the Ni uptake reduced markedly with the addition of chelating agents. For the successful uptake of a metal from a system it has to be bio-available. Although the soluble metal content increases with the addition of chelating agents, it also increases the competition with plant Ni binding agents (39) and thus leads to a decrease in metal uptake.

Figure 3a shows a typical spectrum for Cu as well as elemental maps of *M. sativa* root cross sections (derived from these spectra) after 24 h exposure at 1000 ppm showing the respective presence of Co, Cu, and Ni across all the three root tissue systems (epidermal, cortex, and stele) (Fig. 3b). The initial high driving force caused the metal to be present throughout the cross section. However, the elemental maps suggest preferential accumulation of metals in the central vascular cylinder followed by epidermis and then cortex. Most likely, in the vascular cylinder large quantities of metal are accumulated in the xylem (indicative of xylem loading), from where the metal is translocated to the above ground tissues. As suggested above, the epidermal cells are the site of highest metal accumulation for all the metals studies (Fig. 3).



## CONCLUSIONS

The common metallophyte *M. sativa* was able to accumulate up to 1.7% Co, 1.9% Ni, and 3.2% Cu in its shoots, suggesting *M. sativa* to be a hyperaccumulator for all these metals. In general, with an increase in time and concentration, metal uptake in the shoots increased. Of the three metals *M. sativa* was found to accumulate Cu rapidly in its roots and it was able to transport larger quantities to its shoots. The distribution patterns obtained using μ-PIXE clearly suggest a substantial quantity of Co, Cu and Ni is retained in the stele followed by epidermis and cortex.

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