# Accumulations of heavy metals in *Clethra barbinervis* -characteristics and mechanisms-

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## Summary

### Introduction

The heavy metal is defined as a metal element having a specific gravity of 4 or more, such as cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), vanadium (V), and zinc (Zn). Essential heavy metals in plant nutrition are mainly used for metabolic functions such as enzymatic activity and oxidation reduction in plants. Fe, Cu, and Mn play crucial roles for redox systems, with Cu and Mn being important activators of various enzymes. Zn is involved in membrane integrity and the synthesis of proteins. Ni is essential for the function of plant ureases. On the other hand, Co is an interesting element. Co has not been recognized as an essential element for higher plants, but it is regarded as a beneficial nutrient through enhancement of the growth of root symbiont in $N_2$ fixation and through retardation of leaf senescence via inhibition of ethylene biosynthesis. However, elevated accumulation of these heavy metals in plant tissue causes toxic effects by the formation of reactive oxygen species or deficiency of other essential elements due to their similar chemical properties.

In contrast to normal plants, which absorb various metals at concentrations necessary for metabolism, hyperaccumulator plants are able to accumulate specific heavy metals at extremely high concentrations in their shoots. These plants have been studied for use in phytoremediation. *Clethra barbinervis* can inhabit metalliferous areas, such as serpentine and mining areas, and is known as a species that accumulates Ni, Co, Cd, Mn, and Zn in its leaves, and particularly accumulates Ni and Co at high concentrations. An interesting observation regarding the accumulation of Ni and Co was reported following an analysis of *C. barbinervis* leaves taken from two different geological sites. Whereas *C. barbinervis* accumulated Ni in serpentine soil, the tree accumulated Co in schist soil at high concentrations; although the concentrations of both Ni and Co were higher in the serpentine soil than in the schist soil. Because of the similarities of Ni and Co, I questioned whether *C. barbinervis* could distinguish between Ni and Co during absorption and accumulation. Additionally, the properties and mechanisms of heavy metal accumulation in *C. barbinervis* itself are hardly elucidated. Therefore, this study aims to clarify the characteristics and mechanisms in accumulation of heavy metals, especially Co, in *C. barbinervis*.

**Distinguish between cobalt and nickel in uptake and translocation of *Clethra barbinervis***

I hypothesized that *C. barbinervis* cannot distinguish between Ni and Co because of the similar chemical properties of these two elements. To confirm this hypothesis and understand the role of these elements in *C. barbinervis*, I conducted a hydroponic split-root experiment using Ni and Co solutions. As the results, the leaves of *C. barbinervis* treated with single and multiple metal solutions accumulated Ni or Co at high concentrations. I found that the bioconcentration factor (BCF; metal concentration of each tissue/metal concentrations of each treatment solution) of Ni and Co did not significantly differ in the roots, but the BCF for Co in the leaves was higher than that for Ni. I also found the simultaneous accumulation of Ni and Co by the multiple heavy metal treatments (Ni and Co) at high concentrations similar to those for the single treatments (Ni or Co). Elevated sulfur concentrations were observed in the roots and leaves of Co-treated seedlings but not in the Ni-treated seedlings. This result indicates that S was related to Co accumulation in the leaves. These results suggest that *C. barbinervis* distinguishes between Ni and Co during transport and accumulation in the leaves but not during root uptake.

**Accumulation of cobalt and nickel in tissues of *Clethra barbinervis* in a metal dosing trial**

I investigated the accumulation properties of Co from viewpoints of the analogy with Ni and the other essential heavy metals in the Co accumulator tree, *C. barbinervis*. Seedlings of *C. barbinervis* were grown in single and mixed metal treatment solutions containing Co, Ni, or both at three different concentrations (5, 50, and 500 μM). For roots, stems, and leaves, the biomass as well as the concentrations of Co, Ni, Cu, Fe, Mn, and Zn were determined. As results, biomass of the plants treated with 500 μM Ni reduced, but not in the Co-treated plants. The Co concentration in leaves was higher than those in other tissues, and the maximum Co concentration in leaves was 2010 μg $g^{-1}$. Accumulation of Co competed with Zn transport and stimulated Cu transport. These results indicate that *C. barbinervis* is a Co hyperaccumulator and is more tolerant to Co than to Ni. The Co hyperaccumulation capacity is likely achieved by Zn transport systems involved in root to shoot translocation, and Co accumulation has some competitive and facilitative interactions with the other heavy metals.

**Interactions between Co or Ni and other heavy metals accumulation in uptake and translocation of *Clethra barbinervis***

The interaction among heavy metals in uptake and translocation under the existence of several metals in rhizosphere of *C. barbinervis* at high concentrations was researched. Co, Mn, and Co + Mn treatments (Experiment 1) and Zn, Zn + Co, Zn + Ni, and Zn + Co + Ni treatments (Experiment 2) were conducted for growth of *C. barbinervis*. As a result in Experiment 1, although stimulations of Cu and Mn translocation to leaves were caused by Co treatment, the stimulation of Cu translocation disappeared as the Mn accumulations increased. The competition between Mn and Fe was found in root uptake. In Experiment 2, competition among heavy metals accumulation by the Co, Ni and Zn mixing treatment was not confirmed, and influence on the Zn concentration in leaves and stems by Co, Ni treatment was not confirmed. Therefore, competition between Zn and Co or Ni confirmed in the previous study is due to the fact that Co and Ni can utilize the Zn transport systems, and additionally it is suggested that there were high-affinity transport systems specific for Co and Ni. Moreover, no competitive effect was confirmed in other heavy metal accumulation. From these results, several uptake and transport systems with low selectivity to heavy metals (Co, Cu, Mn, Ni, and Zn) are involved in heavy metal accumulation in *C. barbinervis*. Therefore, it takes a competitive relationship in each transport depending on the concentration balance, but new transport systems may be expressed as the overall heavy metal concentration increased.

**Localization and speciation of cobalt and nickel in the leaves of the cobalt-hyperaccumulating tree *Clethra barbinervis***

I investigated the localization and speciation of Co and Ni in the leaves of *C. barbinervis* to reveal the mechanisms of tolerance to high concentrations of these elements. *C. barbinervis* seedlings were grown by the treatment with Co or Ni. X-ray fluorescence (XRF) and X-ray absorption near edge structure (XANES) analyses were conducted to evaluate the distribution and chemical state of Co, Ni, and S. In addition, the leaves were divided into different parts according to the XRF imaging results of Co or Ni and the concentrations of elements, sulfate, and organic acids were determined chemically in each. XRF imaging showed that Co was present at a high concentration in the leaf tip, whereas Ni was mainly distributed around the leaf edge. The results also indicated that sulfate acted as a counter ion for Co, and that Ni combined with succinic acid and/or oxalic acid in the parts where these elements were accumulated. I also found that sulfate tended to be reduced and glutathione was generated in leaf tip where Co existed at a high concentration. These results indicate that Co and Ni have different distributions and speciations in the leaves of *C. barbinervis*.

**Conclusion**

In this study, I found that the Co accumulation ability was higher than that of Ni in *C. barbinervis*. Therefore, *C. barbinervis* can be defined as a Co hyperaccumulator. The absorption and transport of Co supporting hyperaccumulation characteristics are interacted with the transport mechanism of various essential heavy metals such as Cu, Fe, Mn, and Zn. On the other hand, it was observed that the distributions of Co, Ni, Mn, and Ca in leaves were clearly different. From the results related to metal transports, I found that the Co hyperaccumulation capacity is achieved by Fe uptake systems with high capacity and high-affinity transport systems for Co involved in xylem loading and unloading. Additionally, it became clear that transport systems expressed by Co accumulation could increase Cu and Mn transport in root to shoot translocation. From the results about metal accumulation in leaves, I found that Co was present at a high concentration in the leaf tip, whereas Ni was mainly distributed around the leaf edge. I also found that Co and Ni combined with mainly sulfate and succinic and/or oxalic acids, respectively. In addition, it was suggested that surplus sulfate was reduced and glutathione was produced at leaf tip containing high concentration of Co. These findings represent the specific characteristics and mechanisms in accumulation of heavy metals in *C. barbinervis*.