

Research Article

The role of organic acids in the uptake and storage of nickel in hyperaccumulator plant, *Brackenridgea palustris* ssp. *foxworthyi* (Elm.) P.O. Karis

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Abstract: The role of low molecular weight organic acids (LMWOAs) in the Philippine nickel (Ni) hyperaccumulating plant, *Brackenridgea palustris* ssp. *foxworthyi* (Elm.) P.O. Karis is not yet fully understood. Using High Performance Liquid Chromatography (HPLC), the presence of organic acids such as oxalic, citric and malic acids were determined. Average nickel concentration in the plant tissues followed the ascending order: roots>stem>leaves with values of 7,294.73 µg/g, 7,412.30 µg/g and 9,866.46 µg/g, respectively. Among the organic acids analyzed, only oxalic acid was detected in all the plant tissues at considerable concentration. Linear correlation between oxalic acid and Ni concentrations in 0.025 M HCl plant extracts generated a positive *r*-value of 0.0437 indicating that as Ni content increases, oxalic acid also increases. This paper suggests that oxalic acid can be synthesized by *B. palustris* ssp. *foxworthyi*, therefore, it may acts as a ligand that chelates Ni and other metals to the aboveground tissues where it gets compartmentalized. To our knowledge, this will be the first report on the presence of organic acids in the Philippine endemic Ni hyperaccumulator plant, *B. palustris* ssp. *foxworthyi* whose potential was discovered more than thirty (30) years ago.

Keywords: *chelator, compartmentalize, hyperaccumulator, molar ratio, organic acids*

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Introduction

Hyperaccumulators are plants with unique ability to absorb and detoxify metals in their aboveground tissues without developing any physical and physiological abnormalities (Baker and Walker, 1990; Reeves, 2003; van der Ent et al., 2012). Plants belonging to this group usually thrive in substrate enriched with siderophilic elements such as magnesium (Mg), iron (Fe), chromium (Cr), cobalt (Co), nickel (Ni) and copper (Cu) (Baker, 1981; Proctor 2003). The ability to hyperaccumulate metal is believed to be species-specific and not a trait to be expected from the members of certain plant family hence, it is

possible that unrelated species will exhibit the same capacity (Proctor, 2003; van der Ent et al., 2013). Among the known hyperaccumulators, however, the majority is associated with Ni (van der Ent et al., 2012; Reeves et al., 2017). One plausible reason for this is the availability of a field test kit that can readily detect the presence of Ni in the foliar tissues using a piece of filter paper impregnated with 1% dimethylglyoxime (DMG) dissolved in 95% ethanol. High concentration of Ni is indicated by the formation of pink or (magenta) red stain in the filter paper after rubbing a piece of the foliar tissue (Reeves, 2003; Fernando et al., 2013). To date, more than 500 plant species belonging to Brassicaceae, Euphorbiaceae,

Phyllanthaceae, Asteraceae, Flacourtiaceae (now mostly Salicaceae), Buxaceae and Rubiaceae are now classified as Ni hyperaccumulators (van der Ent et al., 2013; Reeves et al., 2017) but this is expected to increase in the near future. The interest received by hyperaccumulators is associated to their unique potential for the clean up of heavy metals in the areas left behind by mining activities and for phytomining (Reeves 2006; Claveria et al., 2010; van der Ent et al., 2013). However, to successfully utilize these plant resources, the basic molecular, biochemical and physiological mechanisms involved in hyperaccumulation must be fully understood and explored (Anjum et al., 2015; Pence et al., 2000).

Mechanism of metal-tolerance and detoxification in plants can be categorized into two ways: external exclusion and internal tolerance (Baker, 1981; Hall, 2002). The former involves the formation of stable metal-ligand complexes to change the mobility and bioavailability of metals thereby preventing the entry of the metals inside the plant roots while the latter involves the chelation of metal in the cytosol, where ions can be compartmentalized and transformed into less toxic form (Clemens, 2001; Hall, 2002; Dresler et al., 2014). It has been observed that most of the trace elements naturally form complex with ligands containing oxygen-, nitrogen-, and sulphur as part of their tolerance mechanism (Frausto da Silva and Williams, 1991; Centofanti et al., 2013).

Chelation is usually accomplished by thiol-compounds such as glutathione (GSH), phytochelatins (PC) and metallothioneins (MT) but the synthesis of non-thiol compounds (also known as low molecular weight ligands) like organic and amino acids was also proven beneficial to the plants (Anjum et al., 2015). By forming a complex with a particular ligand, movement of metals from the roots to the shoot is facilitated (Clemens, 2019). It should be noted, however, that the ability of a ligand to lower the activity of the free metal ion is highly dependent on pH of the substrate (Bhatia et al., 2005; Clemens, 2019). Specifically for organic acids, it usually forms a complex with plant metals growing in an acidic environment to either reach the vacuole in the epidermis of leaf tissues where it will be compartmentalized or stabilize in the apoplast of the root system thereby preventing its upward movement (Brooks, 2000; Hall 2002; Clemens, 2019).

Organic acids (OAs) are weak acid compounds with at least one carboxyl group, thus termed as oxygen-donor metal ligands (Anjum et al., 2015). These metabolites are expected to potentially perform multiple functions in the rhizosphere where it is easily produced either as plant exudates, synthesized by microorganisms or

formed during organic matter degradation (Sposito, 1989; Li et al., 2006). In general, organic acid concentration in the soil varies from 10^{-2} to 5 mM which is relatively higher than any the trace elements present (Sposito, 1989). Within the plant, such metabolites are usually produced during the process of Krebs cycle and glyoxylate pathway as well as form part in the carbon fixation among C4 and Crassulacean Acid Metabolism (CAM) plants (Igamberdiev and Eprintsev, 2016; Osmolovskaya et al., 2018). Organic acids are associated in several biochemical activities particularly those that involved in energy production and synthesis of amino acid precursor that eventually led to the modulation of plants' adaptive mechanism to the surrounding environment (Lopez-Bucio et al., 2000; Osmolovskaya et al., 2018).

The involvement of organic acids as chelators among hyperaccumulator plants has already been proven experimentally (Homer, 1991; Brooks, 2000; Bhatia et al., 2005; Centofanti et al., 2013, Clemens, 2019). However, among the identified Philippine Ni hyperaccumulators, this aspect has not yet been fully explored and very little is known on their role in the uptake and detoxification process. *Brackenridgea palustris* Bartell ssp. *foxworthyi* (Elm.) P.O. Karis is a Ni hyperaccumulator plant whose potential was discovered in 1986 in the island of Palawan, Philippines. Detailed laboratory analysis confirmed that the species has the capacity to concentrate Ni in the foliar tissue with values ranging from 6,000-8,000 $\mu\text{g/g}$, on a dry weight basis (Baker et al., 1992). Apparently, no subsequent work was done to fully understand its unique uptake mechanism including the ligands that chelate Ni in the aboveground tissues, hence this study.

This present work was done to identify the specific organic acid(s) synthesized by *B. palustris* ssp. *foxworthyi* (Elm.) P.O. Karis that are likely responsible for the chelation and detoxification of Ni. It is expected that the information generated from this study will add to the limited body of knowledge known about the Philippine Ni hyperaccumulator plants necessary to promote its potential for future phytoremediation activities.

Materials and Methods

Collection of plant materials

Wildings of *B. palustris* ssp. *foxworthyi* (Elm.) P.O. Karis collected in an ultramafic formation of Sitio Magarwak, Brgy. Sta. Lourdes, Puerto Princesa City, Palawan was used in the study. These wildings were harvested within the 1-meter circumference of the ten (10) selected individuals

in the study site. The mean height of the collected wildlings is 19.6 cm with the tallest reaching to 28 cm and the shortest at 14cm. Taxonomic identity of the wildlings was verified using Merrill's Enumeration of Philippine Flowering Plants (1923) and Flora Malesiana Series (1995-2012). Prior to sample extraction, wildlings were thoroughly washed with distilled water to ensure that no soil and small particulates adhere to the epidermal layer. Also, plant samples were collected for Ni concentration analysis.

Tissue extraction

Extraction of different plant tissues was done following the modified procedure of Bhatia et al. (2005) and Kachenko (2008). In particular, plant tissues (roots, stem, leaves) were severed from each other, cut into smaller pieces, approximately <1 cm, freeze-dried at -90°C for 100 h and grounded using mortar and pestle. About 50 mg of each sample was placed in a sterile Eppendorf vial and diluted with 1.5 ml of 0.025 M HCl, sonicated for 10 minutes, vortex for a minute and centrifuged at x 3,000 g for 15 minutes. The procedure was repeated twice to extract a sufficient amount of samples (5 ml) and pooled in a volumetric flask. The supernatant was transferred in sterile Eppendorf tube and stored at -20°C prior to analysis.

Organic acid analysis

Determination of the organic acid present in the supernatant was done using High Performance Liquid Chromatography (HPLC) available at the Chemistry Department, De La Salle University. The HPLC system (Agilent 1200) is equipped with an auto-injector, a column oven and a Water 486 tuneable absorbance detector set at 210 nm. To separate the different organic acids present in the sample, an Aminex HPX-87H column (300 x 7.8 mm internal diameter) protected by Bio-Rad cation H guard cartridge were used with the combination thermostated at 30°C. Using Delta 5.5 Chromatography data system software (Digital Solutions), chromatogram acquisition and processing were carried out. Commercially available standards for citric, malic, oxalic and tartaric acids (SIGMA Brand) were prepared in distilled water at a concentration of 10 mg/L and was used as a reference for the retention peak and times for each of the organic acid analyzed. Detection limit used by Kachenko (2008) for organic acid was used as a reference in this study (Table 1). The frozen plant extracts were initially thawed and filtered through a 0.45 µm syringe prior to injection to the HPLC machine. In both the plant sample and standards used, 10 µl were injected and analysis was carried at 4°C. The mobile phase

(0.004 M sulphuric acid) was degassed, filtered and was used for isocratic elution at a flow rate of 0.6 ml/min.

Table 1. Limit of detection of organic acids used (Kachenko, 2008).

Organic acids	Limit of detection (mg/L)
Citric	2.5
Malic	2.0
Oxalic	2.0

The total run time for each sample and standard was 26 minutes. Presence and amount of the organic acids in the sample were compared based on the retention time and peak areas of the standard used. For the Ni concentration, Atomic Absorption Spectrophotometer (AAS) was used and results were correlated to the organic acid identified.

Results and Discussion

Nickel concentration in plant tissues

The average Ni concentration in the roots, stem and leaves of *B. palustris* ssp. *foxworthyi* range from 7,294.73 µg/g, 7,412.30 µg/g to 9,866.46 µg/g, respectively (Figure 1). The data shows that Ni is highly concentrated in the leaves compared to other plant tissues, thereby confirming the earlier report on its Ni hyperaccumulation potential. Statistical analysis, however, showed that there exists no significant difference in the concentration of Ni across plant tissues implying that Ni present in the entire plant is relatively the same ($P=0.71$). Assessment of the Transfer Factor (TF) also proved the efficient movement and compartmentalization of Ni to the aboveground tissue with a generated value of 1.35 (≥ 1 indicate efficient movement from root to shoot).

Organic acids present

The potential role of low molecular weight organic acids (LMOAs) in the uptake and compartmentalization of Ni in *B. palustris* ssp. *foxworthyi* (Elm.) P.O. Karis was assessed in this study. A comparison of the chromatogram of the different plant extracts with the standards showed that oxalic acid was the only organic acid visible above the detection limit indicating its possible participation in the chelation of Ni and other metals to the aboveground tissues (Figure 2). The molar ratio of Ni to oxalic acid which ranges from 5.5:1 (roots), 3.7:1 (leaves) and 2.8:1 (stem) further showed that oxalic acid is likely accountable for more than half of the accumulated Ni by the plant. In addition, there is also a linear correlation observed between oxalic acid and Ni concentration

in the 0.025 M HCl plant tissue extracts with an r -value of 0.0437 ($P \leq 0.05$). Citric and malic acids, on the other hand, were detected but only in trace amounts as indicated by lower peaks of chromatograms. The results of our study support the idea that organic acids may perform a significant role in the detoxification process and homeostasis (Anjum et al., 2015; Osmolovskaya et al., 2018; Clemens, 2019; Chen et al., 2020). The presence of oxalate in plant tissues is considered significant as it facilitates the efficient movement of metals to the aerial plant part by forming a stable complex with Ni and other mobile trace elements in the soil (Anjum et al., 2015; Osmolovskaya et al., 2018). Oxalate or C2 dicarboxylic acid anion is a constituent of the plant tissue that is present in a relatively small amount compared to citrate (Anjum et al., 2015). However, since they share

similar chemical structures, it is possible that they perform similar functions in the chelation process and metal uptake (Li et al., 2006). In addition, oxalic acid also known to aids in metal tolerance, transport through xylem sap and detoxification process (Rauser, 1999). Evidences also showed that oxalate plays a significant role in both Al and Cd toxicity (Watanabe et al., 2005; Li et al., 2006; Chaffai et al., 2006; Yang et al., 2008). Similarly, ability of root secreted-oxalate was also noted to reduce the bioavailability and phytotoxicity of Pb in rice cultivars (Yang et al., 2000). This organic acid either reduce the number of ionized bioavailable forms of metals in the rhizosphere or convert it to less bioavailable complex thereby increasing the overall tolerance of plants to unwanted metals (Hall, 2002; Dong et al., 2007; Clemens, 2019).

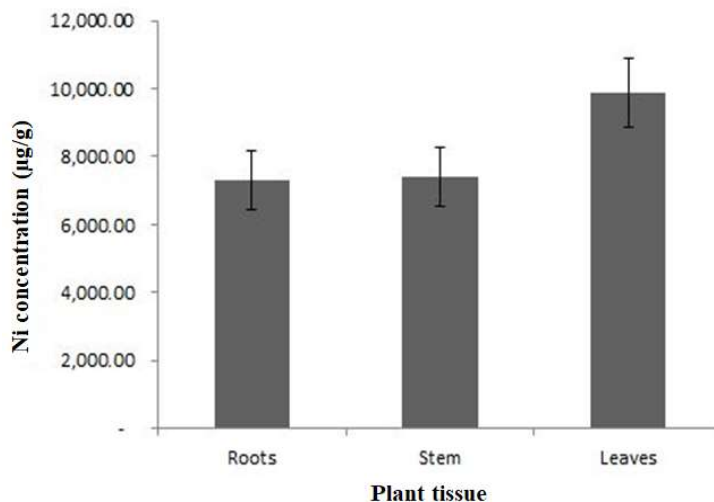


Figure 1. Ni concentration in roots, stem and leaf tissues of *Brackenridgea palustris* ssp. *Foxworthyi*.

Another potential role of oxalate was documented in the vacuolar sequestration as well as its transport of Cadmium in the shoot tissues of *Spartina alterniflora* of (Chai et al., 2012). It is therefore clear that oxalate significantly plays multiple roles in the intracellular detoxification of heavy metals which is central to heavy metal homeostasis (Kochian et al., 2015; Osmolovskaya et al., 2018). Among known hyperaccumulator plants, the significant role of organic acids acting as a potential ligand was also reported (Bhatia et al., 2005; Kachenko, 2008; Reeves et al., 2017). Among Philippine Ni hyperaccumulator plants, *Dichapetalum geloniodes* ssp. *tuberculatum* that was detected for the presence of citrate and malate that may potentially act as chelator (Homer et al., 1999). Oxalate was not reported in that work

suggesting that its quantity might too small to be detected. It is also highly probable that the concentration of organic acids is tissue-specific to ensure a more efficient uptake and detoxification process (Li et al., 2006). Using Al hyperaccumulator plant, *Melastoma malabathricum*, it was determined that citrate which predominantly exists in the root tissue is responsible only for root to shoot translocation of the metal while oxalate which is relatively higher in concentration is a ligand for Al accumulation in both root and shoot tissues (Watanabe et al., 2005). This is also likely possible to our test plant but this warrant further study using a larger sample size. But regardless of the form, it is clear that organic acids perform critical role in the detoxification and sequestration process of trace elements.

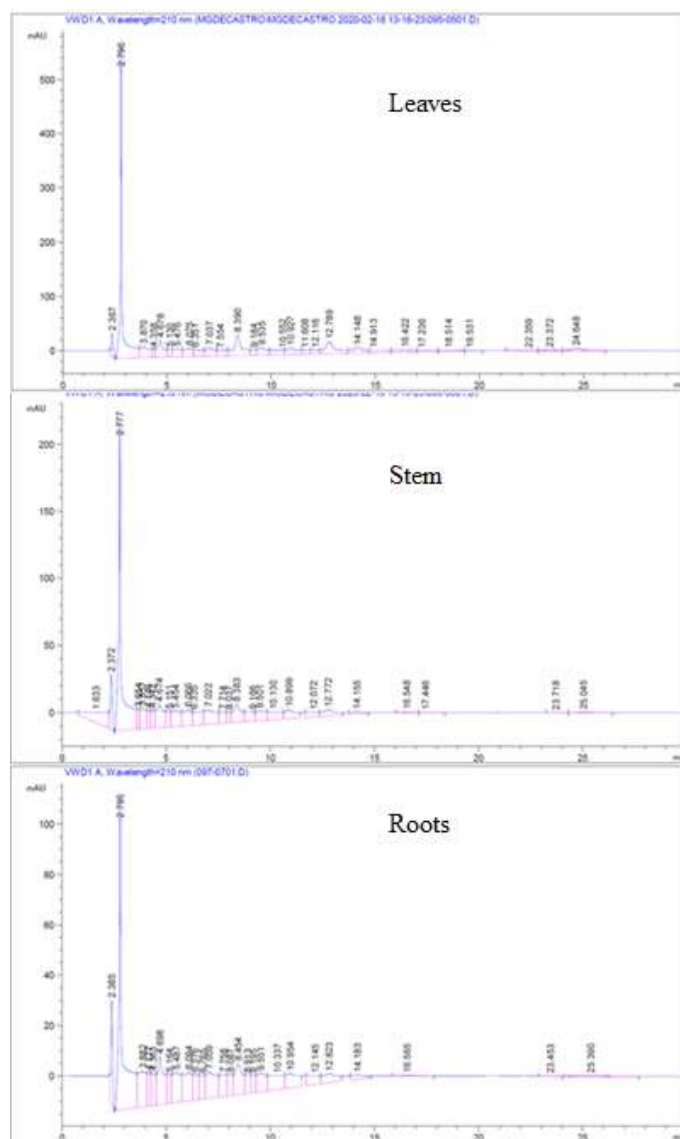


Figure 2. HPLC chromatogram of organic acids in a 0.025M HCl tissue extracts of *B. palustris* ssp. *foxworthyi* wildlings.

Conclusion

The results of the present work proved the ability of *B. palustris* ssp. *foxworthyi* (Elm.) P.O. Karis to naturally synthesize organic acids in its plant tissues. In particular, oxalic acid was detected suggesting its potential as a chelator of Ni and other trace elements. This possibility, however, necessitates additional works in the future. Likewise, the involvement of other low molecular weight ligands like amino acids and thiol compounds in the chelation process must also be verified among Philippine Ni hyperaccumulator plants.

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