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Soil Amendments Affecting Uptake and Storage of Nickel in the (Hyper) Accumulator Plants *Berkheya coddii* R. and *Helianthus Annuus* L.

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

Signed on 21 September 2021 in Johannesburg

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

21 SEPTEMBER 2021 at JOHANNESBURG

ABSTRACT

Phytoremediation provides one of the most environmentally friendly solutions to pollution. However, the long timeframes for plant growth and slow uptake rates are a deterrent for widespread use of this technique. Heavy metal uptake and accumulation in plants is governed by biosynthesis of organic and amino acids. Understanding their inter-relationship may provide insight into potential soil amendments which can be used to increase phytoremediation capacity of plants, and reduce the timeframe of phytoremediation.The aim of this project was to analyse the relationship of the heavy metal Ni with selected amino and organic acids within two plant species of the Asteraceae family: *B. coddii* R. (*B. coddii*), classified as a South African Ni-hyperaccumulator, and *Helianthus annuus* L. (sunflower), a metal accumulator. Amendments applied to investigate uptake changes included addition of Ni, chelidonic acid (ChA), histidine (His) and a Ni/ChA mixture to soil in which these plants were grown. The impact of addition of His and ChA on organic and amino acids within plants has not previously been analysed. Nickel was quantified by ICP-MS, and organic and amino acids by HPLC-UV. Nickel accumulated predominantly in the shoots of *B. coddii* (1 060 – 7 300 mg kg^{-1}) and sequestration was linked to chelation by chelidonic acid and malic acid. The plants exhibited toxic effects to high concentrations of applied bioavailable Ni. Severe necrosis was observed at a concentration of 7 300 mg kg^{-1}, indicating for the first time, a threshold accumulation capacity for *B. coddii*. Sunflowers accumulated lower levels of Ni (20 – 246 mg kg^{-1}), with sequestration occurring in the roots. The addition of bioavailable Ni induced biosynthesis of amino acids which respond to stress factors in the plant.

Soil amendments with results most applicable to future phytoremediation work were the application of His in both plants and ChA in sunflower plants. Development of both species was enhanced by His enrichment, with an enhanced biosynthesis and metabolism of amino acids (AA). The potential for enhanced Ni uptake through increasing plant biomass may provide a useful tool in improving the amount of Ni that plants are able to tolerate in shorter periods of time. Addition of ChA negatively impacted development of both species of plants. Amino acids were biosynthesised in shoots of *B. coddii* in response to addition of chelidonic acid, a stress response. There was no apparent influence on Ni uptake within *B. coddii*. Sunflowers, however, accumulated ChA, along with Ni from the soil. Malic acid, an organic acid related to metal sequestration, was biosynthesised.

This study has shown that nickel hyperaccumulation is dependent on plant development and biosynthesis of chelating ligands. Addition of His into (hyper)accumulators can be useful to enhance growth and development of plants, while ChA can be used to enhance biosynthesis of the metal chelating ligand, malic acid, within metal accumulators.

In memory of my mother and grandfather

Gloria Lyons

1976 – 2017

Edgar Lyons

1950 – 2021

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ABBREVIATIONS

Organic Acids:

AA	Amino Acid
CA	Citric Acid
ChA	Chelidonic acid
MA	Malic acid

Amino Acids:

Ala	L-Alanine
Arg	L-Arginine
Asp	L-Aspartic acid
Cys	L-Cysteine
Glu	L-Glutamic acid
Gly	Glycine
His	Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serrine
Thr	L-Threonine
Trp	Tryptophan
Tyr	L-Tyrosine
Val	L-Valine

Miscellaneous:

AMQ	6-aminoquinoline
AQC	6-aminoquinolyl-*N*-hydroxysuccinimydyl
CRM	Certified Reference Material
EDTA	Ethylenediaminetetraacetic acid
PTFE	Polytetrafluoroethylene

CHAPTER 1

Introduction

Metals and metalloids with atomic density greater than 4 g cm^{-3} are termed “heavy metals” (Nagajyoti *et al*, 2010). These elements are available in soil and aquatic ecosystems, sourced naturally from processes such as weathering of rock or volcanic activity, and transported by wind, water or glaciation (Thornton, 1981). The presence of heavy metals within the environment has been exacerbated by anthropogenic processes such as mining, urbanisation, production, and power station operations (Sawar *et al*, 2017). Heavy metals tend to accumulate in the environment since they are less biodegradable than organic substances, and have a negative effect on the normal functioning of biogeochemical systems (Ali *et al*, 2013). Traditional remediation methods for metals involve changes to the natural landscape, expensive and harmful chemicals, and large waste disposal challenges and costs (Mesjasz-Przybylowicz *et al*, 2004). Use of greener approaches for environmental clean-up, such as phytoremediation, have been sought out to combat these disadvantages (Pramanik *et al*, 2014).

Phytoremediation is a process in which heavy metal accumulator plants and/or soil microbes, are used to naturally reduce the concentration of heavy metals, radionuclides or organic pollutants in soil and water (Ali *et al*, 2013). Phytoextraction is a type of phytoremediation in which soil is remediated by metal accumulators: plants which accumulate heavy metals above typically toxic concentrations. Two categories of heavy metal accumulator plant species can be utilized for this process: 1) moderately accumulating plant species with high biomass yield, including *Zea mays* L. (maize), *Nicotiana tabacum* L. (tobacco) and *Helianthus annuus* L. (sunflowers), and 2) hyperaccumulator plant species, such as *Brassica juncea* (Indian mustard) and *Senecio coronatus*, which can accommodate significantly increased concentrations of some heavy metals (Chaudhry et al, 2020; Mench *et al*, 1989; Nehnevajova *et al*, 2005; van der Pas and Ingle, 2019).

1.1 Plant Responses to Toxic Heavy Metals Within the Environment

Some heavy metals, including iron (Fe), copper (Cu), nickel (Ni), manganese (Mn), lead (Pb) and Zinc (Zn), are essential to plant metabolism at ultra-trace to trace concentrations (Corzo Remigio *et al*, 2020). An excess of either essential or non-essential metals within plant tissue can inhibit optimal plant functionality, and lead to plant death (Wei *et al*, 2009). Toxic effects include K^+ leakage, cell damaging oxidative stresses, and protein thiol cross-linking, leading

to plant cell damage and impaired plant functioning (Naila *et al*, 2019).Plants which grow in areas with high heavy metal concentrations employ two mechanisms for survival: exclusion or uptake and accumulation (Raskin *et al*, 1994).

1.1.1 Exclusion

Exclusionary plants work to maintain low concentrations of heavy metals within their photosynthetically active aerial tissues. Extracellular avoidance strategies, such as exudation of rhizosphere pH modifiers or metal-chelating organic molecules, formation of a redox barrier, or mycorrhizal associations are the first defensive strategy of many plants (Dalvi and Bhalerao, 2013). Most plants are heavy metal excluders. Plants which accumulate high concentrations of heavy metals within their roots, but do not translocate these into their aboveground tissue are considered exclusion plants as well (Deng *et al*, 2018).

1.1.2 Accumulation

Non-exclusionary (uptake) plants accumulate heavy metals to concentrations above those considered toxic to most plants. These plants, collectively called metallophytes, can be used for different aspects of phytoremediation (Awa and Hadibarata, 2020). Metallophytes are divided into three subgroups: indicators, accumulators, and hyperaccumulators. Indicator plants accumulate concentrations of metals indicative of the level of contamination within the growth medium unrestrictedly. In a heavy metal contaminated area, this results in toxicity and plant death (Deng *et al*, 2018). Indicators can be used to determine the level of metal contamination within soil (Awa and Hadibarata, 2020). Accumulator plants extract concentrations of heavy metals from water or soil which exceed those within the growth medium and neighbouring plants, and store them within their tissue without toxicity occurring (Deng *et al*, 2018). Hyperaccumulator plants are accumulator plants with the unique ability to accumulate of one or a few heavy metal to extreme concentrations (at least 1% of plant dry mass), while excluding other metals (Jaffre *et al*., 1976; Deng *et al*., 2018).

1.2 Strategies of Accumulator Plants Under Heavy Metal Stress

Heavy metal tolerance is a second defensive strategy of plants, focusing on intracellular detoxification over extracellular metal exclusion (Dalvi and Bhalerao, 2013). Metal tolerance is reported to occur by two strategies.

On uptake of heavy metals, accumulator plants try first to exclude transport of toxic metals across the plasma membrane. This is achieved primarily through immobilization or modification of ions (Dalvi and Bhalerao, 2013). Following transport of metals into the plant body, metal ions are detoxified by a range of strategies based on reduced ion toxicity through inactivation or conversion to less toxic forms (Dalvi and Bhalerao, 2013). Ions are bound to strong ligands, mainly peptides – particularly phytochelatins and metallothioneins– as well as

metalloproteins and thiols, to be transported safely from cytosol to photosynthetically inactive vacuoles (Leitenmaier and Küpper, 2013). Phytochelatins are the most widely studied of these, particularly in relation to Cd tolerance of plants (Andresen *et al*, 2013; Cobbett, 2000; Hall, 2002). In vacuoles, metal ions are complexed mainly to organic acids, immobilizing these ions within sites which do not affect plant growth and development. Synthesis of stress-related molecules, such as the amino acid (AA) Pro, also occurs to aid in heavy metal detoxification (Dalvi and Bhalerao, 2013).

1.3 Hyperaccumulation

The term 'hyperaccumulation' was first encountered in a 1976 paper (Jaffre *et al*, 1976), where it was used to describe the extraordinary concentration of Ni accumulated by a New Caledonian tree *Sebertia acuminata*, later re-classified as *Pycnandra acuminata* (Reeves *et al*, 2018). This phenomenon is extremely rare, with approximately 0.2 % of angiosperms shown to exhibit hyperaccumulation (Baker *et al*, 2000). In general, for a plant to be considered a hyperaccumulator, the concentration of the accumulated metal within dry above-ground matter must exceed a suitable concentration, ranging from 100 mg kg^{-1} for Cd, to 10 000 mg kg^{-1} for Mn (Reeves *et al*, 2017). Ni hyperaccumulators accumulate at least 1 000 mg kg^{-1} of Ni within their shoots (van der Ent *et al*, 2012).

To date, there are at least 746 hyperaccumulator species globally, the majority (532) of which accumulate Ni (Corzo Remigio *et al*, 2020). The remaining species comprise hyperaccumulators of essential heavy metals Cu, Co, Zn, As, Mn, Pb and Cd; non-essential heavy metals selenium (Se) and thallium (Tl); and two rare earth elements (Reeves *et al*, 2017). The significance of the evolution of metal hypertolerance is not well known. The most strongly favoured hypothesis is that hypertolerance is a defence strategy developed for protection from herbivores, parasites and pathogens (Boyd, 2007; Raskin *et al*, 1994).

1.3.1 Uptake, transport and storage of heavy metals by hyperaccumulators

Essential mechanisms employed by hyperaccumulators include enhanced metal uptake into the root system, increased root-to-shoot translocation through xylem loading and transport, and increased detoxification of heavy metals by sequestration and compartmentalisation within leaf tissue (Bhatia *et al*, 2005; Deng *et al*, 2018).

Metal uptake occurs through interaction of plant roots with the rhizosphere. With accumulators, microbial communities associated with plant roots can have an effect on the bioavailability of heavy metals. Root exudates, such as phytosiderophores, organic acids and protons, and microorganisms within the rhizosphere can aid in the mobility and availability of heavy metals for uptake by processes like chelation (Verbruggen *et al*, 2009). The freed metal species are

absorbed into the root system, and then transported to plant shoots by xylem (Naila *et al*, 2019).

Metal tolerance mechanisms within hyperaccumulator plants are also similar to those employed by accumulator species. They include precipitation, changing of metal speciation by redox mechanism, and complexation to organo-ligands (Bhatia *et al*, 2005; Leitenmaier and Küpper, 2013).

1.4 Plant Ligands

Heavy metal accumulation within plants is associated with an increased capacity for metal ion detoxification. This is attributed largely to the binding of free aqueous metal ions to low molecular weight ligands, peptides and proteins, which render metal ions immobile and thus, non-toxic (Callahan, 2007). These ligands drive transport and sequestration of heavy metals outside of plant cells where important enzyme activity (such as photosynthesis) occurs (Leitenmaier and Küpper, 2013). Ligands have been grouped into three major classes: 1) oxygen donor ligands, including low molecular weight organic acids such as carboxylates, 2) nitrogen donor ligands, largely AAs and, 3) sulfur donor ligands such as metallothioneins and phytochelatins (Bhatia *et al*, 2005).

Heavy metal chelation is highly pH-dependent. AAs are regarded as favourable metal chelators in the roots system of plants largely due to the alkalinity of root cytoplasm (Harris *et al*, 2012), while carboxylic acids such as citrate and malate are favoured in the more acidic storage sites of plants (Bhatia *et al*, 2005). For the present study, oxygen donor ligands and nitrogen donor ligands are monitored within the hyperaccumulator *B. coddii*, and the accumulator sunflower. Some common ligands within plants are shown in Figure 1.1, as well as ChA, a unique metal ligand associated with Ni accumulation for *B. coddii*.

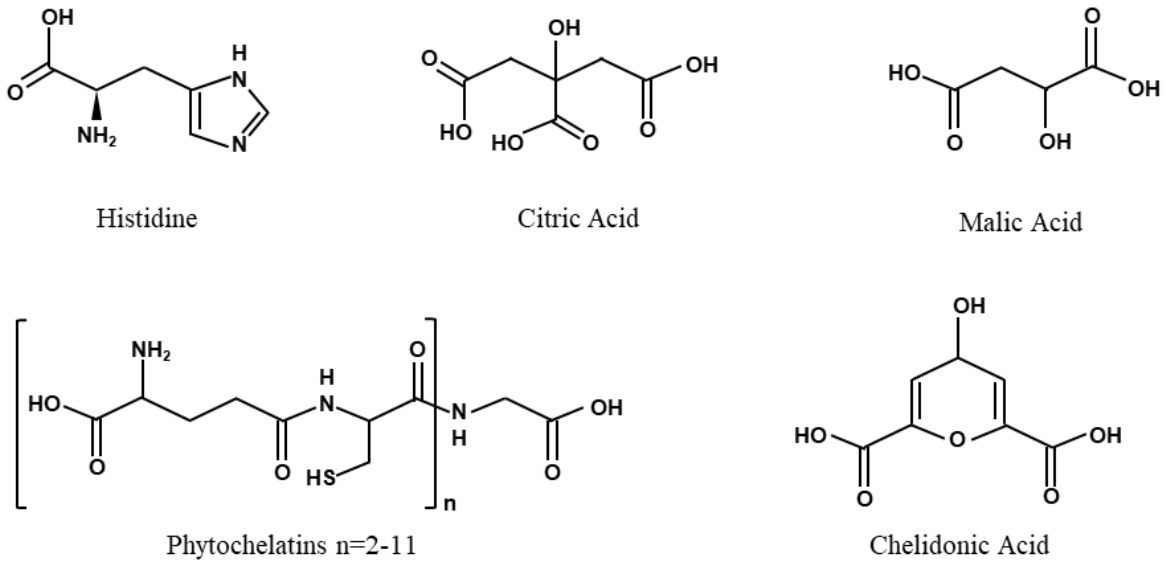

Figure 1.1 Common heavy metal ligands associated with metal uptake

1.4.1 Oxygen donor ligands

Organic acids (OAs) are important plant metabolites which are formed mainly through the tricarboxylic acid (Krebs) cycle, and to a lesser extent through C_4 photosynthesis and Crassulacean Acid Metabolism (CAM) processes, and the glyoxylate cycle (Osmolovskaya *et al*, 2018). These molecules are important in several biochemical pathways, including amino acid synthesis and energy production at the cellular level, and overall plant adaptation to the environment (Lopez-Bucio *et al*, 2000).

Many (hyper)accumulator plants contain large quantities of low molecular weight OAs such as malic, citric and oxalic acid within their roots. Due to their low association constants with metals in alkaline conditions these molecules are not expected to be strong enough metal-binders in this cytosolic environment (Rascio and Navari-Izzo, 2011). Rather, OAs are excreted by roots such that they may decrease rhizospheric pH, thus solubilizing important plant nutrients and consequently increasing the bioavailability of metal cations (Lopez-Bucio *et al*, 2000; Sheoran *et al*, 2010). In accumulator plants, this allows for the faster xylem loading and root-to-shoot translocation of metals, an essential mechanism utilised by hyperaccumulating plants (Gao *et al*, 2012).

1.4.1.1 Citric acid

Citric acid (Figure 1.1) is a plant metabolite, which is important for photosynthesis and cellular respiration (Tusei, 2019). Citric acid is among the most common OA ligands associated with hyperaccumulation (Schaumloffel *et al*, 2003). Deprotonation of this tricarboxylic acid produces the negatively charged ligand, citrate, with three chelation sites for positively charged ions, such as metals (Tusei, 2019). The presence of this tricarboxylic acid within leaf

tissue has been shown to be exacerbated by an increased uptake of cations, nutrient and metallic alike (Lee *et al*, 1978). Citric acid is shown to chelate with Ni in the New Caledonian hyperaccumulator *Sebertia acuminata* and 16 other Ni accumulating species of the New Caledonia region *(Sagner et al, 1998;* Lee *et al*, 1978). Citrate has also been shown to play a role in xylem transport of heavy metals Fe and Cd (Osmolovskaya *et al*, 2018).

1.4.1.2 Malic acid

Malic acid (Figure 1.1) is an important intermediate biosynthesised during the Krebs energy cycle (Shitan and Yazaki, 2013). In plants, this OA acts as a reserve anion in vacuoles, a precursor to oxaloacetate from which a range of amino acids are biosynthesised, and a counter ion for K and Ca cations (Ghazijani *et al*, 2018). Malate was suggested as a chelating agent of Ni within roots of *Sebertia acuminata*, and during transport through shoots into leaf vacuoles where the metal is stored (Bhatia *et al*, 2005). Malate, along with citrate, were reported to play a major role in Ni tolerance for the hyperaccumulators *Senecio coronatus* and *B. coddii* (Montarges-Pelletier *et al*, 2011). Malic acid and citric acid were also found to accumulate within the roots and shoots of sunflower plants spiked with Al, Cd and Zn (Saber *et al*, 1999).

In recent years, studies of the effect of exogenously applied organic acids on heavy metal accumulation and tolerance have been undertaken (Osmolovskaya *et al*, 2018). Chelating agents such as citrate, malate, oxalate and EDTA have been utilized in phytoremediation research as exogenous amendments to enhance metal uptake (Gao *et al*, 2012). The results of these studies have been largely ambiguous, implying a factor-dependent use for these amendments. At low concentrations (generally below 5 mmol kg^{-1}), organic acids have been found to have little to no effect on metal uptake into plants, and at higher concentrations (above 125 mmol kg^{-1}) these amendments were found to negatively affect plant growth, evidenced by reduced plant biomass (Evangelou *et al*, 2007).

Citric acid has been extensively studied as a potential exogenous soil amendment for phytoremediation. Schwab, Zhu, and Banks (2008) found that this organic acid had a greater effect on Cd and Zn mobility than both malate and oxalate. The addition of citric acid to the soil matrix of was found to enhance water-soluble metal concentrations to a lesser degree than the more efficient metal chelant, EDTA. The advantages of citric acid over EDTA – lower phytotoxicity and natural biodegradation within the environment the primary of these – make citric acid the more viable organic amendment for phytoremediation (Gao *et al*, 2012). Enhanced uptake with citric acid amendment has been shown for the following metals: Cd and Pb (Gao *et al*, 2012), U (Huang *et al*, 1998), Mg (Yang *et al*, 2018), and Ni (do Nascimento *et al*, 2020).

1.4.1.3 Ascorbic, Oxalic and Chelidonic Acid

Oxalic acid synthesis within plants is related to the regulation of ionic balances with plants as well as protection of plants from insects and animals. This organic acid is a key root exudate linked to Al tolerance for buckwheat and tea plants, and has been reported to aid in hyperaccumulation of Cd, Ni, Zn and other heavy metals (Prasad and Shivay, 2017).

Ascorbic acid (vitamin C) is an essential organic acid for both plants and animals, which acts as a radical scavenger in oxidative stress, reduces Fe^{3+} and Cu^{+}, and is involved in a number of plant defence mechanisms (Smirnoff, 2018). Elevated concentrations of ascorbic acid were reported for sunflowers grown in metal-rich tannery sludge, *Brassica juncea* (mustard) plants spiked with Zn, and *Bacopa monnieri* (water hyssop) plants spiked with Hg (Singh *et al*, 2004).

ChA (Figure 1.1) is a naturally occurring secondary metabolite found in several plants (Singh *et al*, 2016). It is found mainly in vacuoles of plants, and is thought to play a part in alkaloid transport from cytosol into vacuoles (Naicker, 2014). ChA has been determined, previously, to be a growth inhibitor in plants (Leopold *et al*, 1952). ChA is shown to chelate to Ni within the hyperaccumulator *B. coddii* (Naicker *et al*, 2016). No other hyperaccumulator to date exhibits this behaviour.

1.4.2 Nitrogen donor ligands

Biosynthesis of almost all AA metal metabolites occurs by metabolism of three molecules of the glucose and Krebs cycles to three amino acids: 3-P-glycerate to serine, α-ketoglutarate to glutamic acid (the glutamate family pathway), and oxaloacetate to aspartic acid (the aspartate family pathway) (Figure 1.2). Metabolism of these three amino acids produces all other N-containing metal metabolites, except alanine (formed by the pyruvate family pathway).

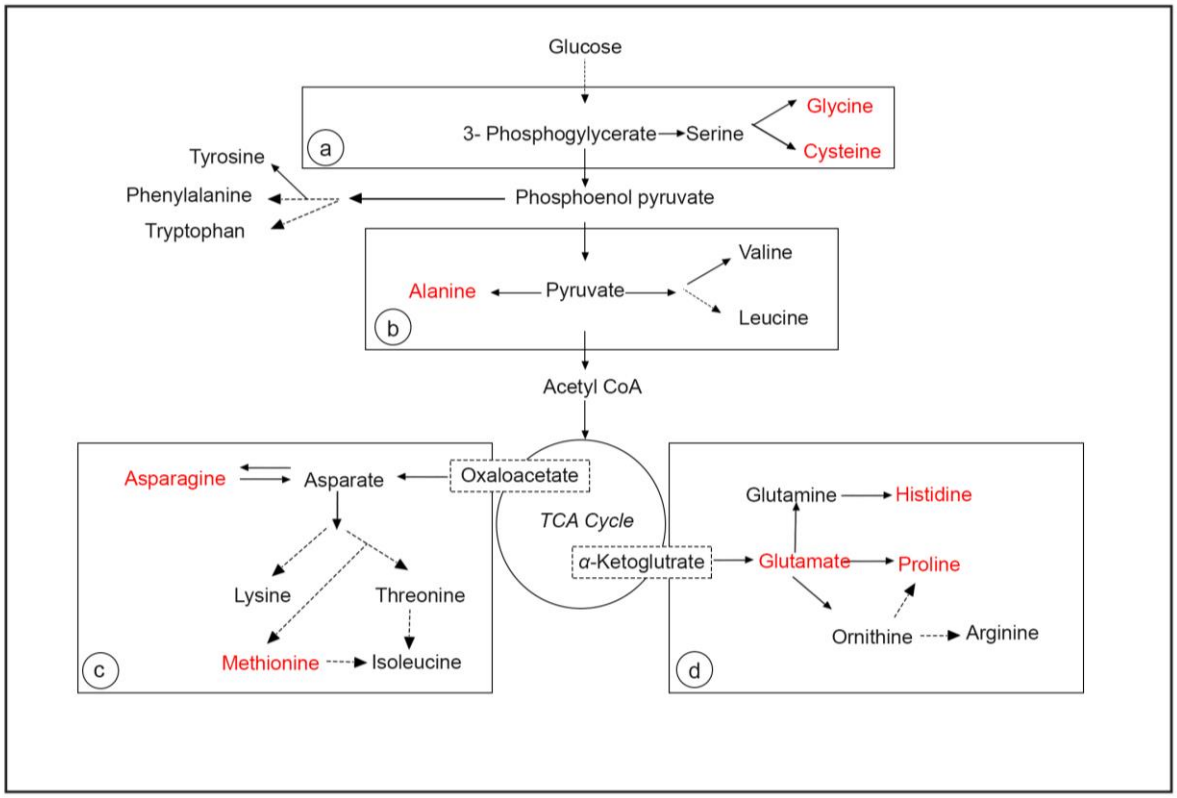

Figure 1.2 The pathways by which amino acids involved in metal metabolism are synthesised within plants: a) the 3-P-glycerate pathway, b) pyruvate pathway, c) aspartate family pathway, and d) glutamate family pathway. Red amino acids are all involved in metal metabolism. Adapted from: Planchet and Limami, 2015

The general trend for amino acids accumulation within plant was found to be stems>leaves>roots, indicating their role within plants as essential transport molecules and plant nutrient storage sites (Kumar *et al*, 2017). The average concentration of AAs (relative to Lys) yielded the result: Leu > Asp > Glu > Arg > Gly > Ile > Ser > Pro > Lys > Thr > Val > His > Phe > Tyr > Cys > Met > Trp. This result reflects concentrations of amino acids largely within seeds and fruits of plants (Kumar *et al*, 2019).

When exposed to heavy metal stress, plants often synthesise, up to millimolar concentrations, nitrogen-containing metabolites such as amino acids, peptides (glutathione and phytochelatins), and a selection of amines, including nicotianamine. For some metabolites, such as His and Pro, this synthesis is part of the primary reaction of the plants to excess heavy metal uptake (Sharma and Dietz, 2006). Application of exogenous amino acids to the plant growth medium was found to modulate membrane permeability, and thus effect ion uptake. addition of exogenous Pro to *Vigna radiata* cultures increased K^+ content; *Phaseolus* seedlings spiked with His, Pro, Met, Gln and Gly were reported to have an increased Ca load,

while addition of Ala, Asp, Glu and Trp inhibited Ca uptake in the same plants (Rai, 2002). The most widely studied amino acids for heavy metal tolerance are His and Pro.

1.4.2.1 Histidine

His (Figure 1.1) has been found to have an important impact on the mechanism of hyperaccumulation, especially for the Ni-accumulating plants of the *Brassicaceae* species (Leitenmaier and Küpper, 2013). On exposure to Ni, a proportional increase in the production of free His within the xylem sap of the Ni-hyperaccumulators *Alyssum lesbiacum*, *A. murale*, and *A. bertelonii* was observed. A similar effect was not observed for the non-hyperaccumulating species *A. montanum* (Kramer *et al*, 1996). Exposure of *A. montanum* to His however, showed an increase in the ability of the plant to transport Ni out of the roots and into the shoots, as well tolerate the metal (Kerkeb and Kramer, 2003). A similar trend was seen for the *Pisum sativum L.* (Kameda *et al*, 2002) and *Brassica juncea* L. (Kerkeb and Kramer, 2003) plants. This provides an indication that transport of Ni from root to shoot is enhanced by the availability of free His through a co-release strategy, while uptake of either agent is independent of the other (Sharma and Dietz, 2006). Exposure of the Ni hyperaccumulators *Streptanthus polygaloides*, also of the Brassicaceae family, and *B. coddii* of the Asteraceae family, to increased Ni also resulted in accumulation of His within plant xylem sap (Smith *et al*, 1999). Histidine was found, also, to complex Zn in the roots of the Zn-hyperaccumulator *Thlaspi caerulescens (Salt et al, 1999)*.

1.4.2.2 Proline

Proline is a proteinogenic amino acid which is synthesised predominantly from glutamate, and is essential for primary metabolism (Szabados and Savoure, 2010). It functions as a compatible and metabolic osmolyte, radical scavenger, electron sink, macromolecule stabilizer, and a component of the cell wall (Sharma and Dietz, 2006). Pro has a protective function in higher plants, playing roles in adaptation, recovery and signalling under stress conditions (Emamverdian *et al*, 2015). Biosynthesis of the amino acid has been found to increase under environmental stresses such as dehydration, high salinity, high or low temperatures, high radiation, oxidative stress, and heavy metal stress (Szabados and Savoure, 2010). Accumulation of Pro for plants under abiotic stress is species-specific and can be 100 times greater than the plant control (Verbruggen and Hermans, 2008).

Mechanisms by which Pro aids tolerance to heavy metal stress include: through protection of nitrogen reductases (Szabados and Savoure, 2010), by metal chelation and protein stabilisation (Emamverdian *et al*, 2015), aiding with reduction of osmotic stress (Verbruggen *et al*, 2009) and metal-induced water deficiency (Schat *et al*, 1997). Accumulation of Pro as a result of heavy metal stress appears to be dependent on a variety of factors, including plant species and preferred metal-storage sites, and experimental variables such as metal

concentration and duration of exposure (Emamverdian *et al*, 2015). Under metal stress, Pro accumulation was observed almost exclusively in plant roots for oilseed crops *B. napus* (canola) (Gohari *et al*, 2012) and *B. juncea* (mustard) (John *et al*, 2009), as well as for feed crop wheat (Lesko and Simon-Sarkadi, 2002; Li *et al*, 2013). In the presence of heavy metal stress, an increase of Pro content was observed in roots (Singh *et al*, 2004) and shoots (Zengin and Kirbag, 2007) of sunflowers.

1.5 Nickel: Essentiality, Uptake and Hyperaccumulation

Ni is reported to be naturally present in soils and surface waters at concentrations below 100 mg kg^{-1} and 0.005 mg L^{-1}, respectively (Yusuf *et al*, 2011). Global addition of Ni to the environment by anthropogenic activity is 1.2 times that of the input of natural sources annually (Bhalerao *et al*, 2015). The metallurgical, electroplating, chemical, and food industries are the highest contributing anthropogenic sources of Ni. Further sources include household and municipal wastes, vehicle emissions, electricity generation processes, production and use of inorganic fertilisers and fungicides, and exposure during mining activities (Nagajyoti *et al*, 2010; Yusuf *et al*, 2011). Concentrations of Ni within the (densely populated) Witwatersrand Gold Mining Basin of South Africa were found to be 1.2 times higher than the maximum allowable limit of the country (Kamunda *et al*, 2016).

1.5.1 The physiological essentiality of nickel

Ni is an essential micronutrient for plants, utilised in a range of physiological processes, starting from germination, and throughout the life cycle of the species (Yusuf *et al*, 2011). It is most abundantly utilised in higher plants, as an essential component of the enzyme urease, required to hydrolyse urea. Typically, Ni concentration in plants ranges between 1 mg kg^{-1} and 10 mg kg^{-1}. Ni deficiency has been reported to negatively affect the growth, nitrogen (N) metabolism, and Fe uptake of plants (Bhalerao *et al*, 2015). Symptoms of toxicity such as leaf chlorosis, inhibited root growth, accumulation of starch in the chloroplast, and swelling of the endoplasmic reticulum causing necrosis associated with excess Ni, are more commonly reported (Ker and Charest, 2010; Shaw *et al*, 2004).

1.5.2 Uptake and storage mechanisms of nickel

Currently, little is known of the mechanism of uptake of Ni into plant tissue. It has been shown, indirectly, that Ni is absorbed into the root system as Ni, from the soil solution phase, where it exists as the hydrated cation, $Ni(H_2O)^{2+}$ (Bhalerao *et al*, 2015). Thus, Ni uptake is directly related to pH of the soil solution, and availability of the Ni cation. In hyperaccumulators, Ni uptake is mainly through active transport and passive diffusion, as it is for non-accumulator plants (Yusuf *et al*, 2011). It appears, from studies of uptake kinetics of accumulator and non-

accumulator species alike, that Ni uptake may be through the low-affinity transport systems of other divalent micronutrient elements including Fe, Cu and Zn (Deng *et al*, 2018).

Upon uptake, metal ions are quickly complexed to organo-ligands, reducing toxicity that could be caused by cation exchange with xylem cell walls (Yusuf *et al*, 2011). Due to the alkaline nature of the matrix of roots cytoplasm, chelation to amino acids is expected to be favoured. This is shown to be true of the hyperaccumulator *A. lesbiacum*, for which root-His concentrations were found to be higher than a non-accumulator comparison plant, *B. juncea (Kerkeb and Kramer, 2003)*. For non-hyperaccumulator plants, excess Ni accumulates within roots. Ni is transported out of the roots and through the shoots of plants by xylem transport. Storage of nickel is typically within leaves for hyperaccumulator plants. The metal is found to be complexed to low molecular weight OAs, such as citrate, malate and, in the unique case of *B. coddii*, ChA. Ni mobility within plants is high, with re-translocation occurring via the phloem (Ahmad *et al*, 2011).

1.6 *Berkheya Coddii* R.

B. coddii is one of many members of the Asteraceae family and is a known Ni hyperaccumulator (Reeves *et al*., 2018). This perennial, summer-green plant has a reported annual biomass production of 22 t ha^{-1} (Robinson *et al*., 2003). Its high root:shoot ratio, and long-lived root biomass lend positively to the use of this plant for phytoremediation purposes (Slatter, 1998).

Varying Ni leaf concentrations have been found within *B. coddii*. The average concentration of Ni found appears to be between approximately 13 000 mg kg^{-1} (Naicker, 2014; Nematundani *et al*., 2006; Pillay, 2006) and 17 000 mg kg^{-1} (Howes, 1991; Howes *et al*, 1998; Mesjasz-Przybylowicz *et al*., 2004). The highest recorded leaf Ni concentration found is 76 100 mg kg^{-1}, from plants taken from the Songimvelo game reserve, Mpumalanga (Mesjasz-Przybylowicz *et al*., 2004). *B. coddii* has the largest plant biomass, and shortest growth cycle of all known Ni hyperaccumulators (McGrath *et al*, 2001).

Storage of excess Ni is found to be within the leaves of the plant, largely complexed to an organic acid (Slatter, 1998), identified as ChA (Naicker *et al*, 2016). An increase in ascorbic acid concentration, as well as the amino acid Pro have been observed and postulated as a stress response to excess nickel uptake within the stem and young leaves of the plant (Naicker, 2014). *B. coddii* also has the ability to accumulate metals other than Ni. With addition of 1.0 g kg^{-1} sodium thiocyanate to the growth substrate, *B. coddii* plants were found to accumulate gold (Au) within roots (49.0 g kg^{-1}) (Lamb *et al*, 2001). The plant also has the potential to accumulate Pt and Pd within leaves, and Co when soil Ni is reduced (Nemutandani *et al*, 2006; Rue *et al*, 2020).

1.7 Sunflowers (*Helianthus Annuus* L.)

For non-hyperaccumulating species, phytoextraction of heavy metals can be financially viable if: 1) the species utilised has a high biomass yield, and 2) the species provides an additional income stream, such as fragrance, fiber, food, feed or energy production (Nehnevajova *et al*, 2005). The plant species *Helianthus annuus* L., commercially known as 'sunflower', fulfils both of these criteria. It is a fast-growing plant, with high biomass, and an oilseed crop (Belhaj *et al*, 2016). A member of the Asteraceae family and native to North America, this annual herbaceous species is grown on every continent, primarily as an oilseed crop with a high protein meal by-product (Heiser, 1981; Chavez *et al*, 2011). The plant, which thrives in temperate and subtropical climates, typically grows during the summer and into early autumn, and can grow to a height of 3 m or more (Heiser, 1981).

Sunflowers have been shown to accumulate multiple metals into their tissue simultaneously. Metals (Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Th, U and Zn) have been accumulated within roots and shoots of sunflowers grown in mining areas (Alsabbagh and Abuqudaira, 2017; Kotschau *et al*, 2014), sewerage sludge (Belhaj *et al*, 2016), and tannery sludge (Singh *et al*, 2004). Heavy metal accumulation results in increased biosynthesis of chelating ligands as well as activation of defence mechanisms. Al and Zn accumulation within plant roots and shoots produced an increase in biosynthesis of malic and citric acid within plant tissues (Saber *et al*, 1999). For plants grown in sewerage and tannery sludge, increasing concentrations of glutathione, Pro and ascorbic acid were reported (Belhaj *et al*, 2016; Singh *et al*, 2004).

Research into modification of metal uptake capacity for plants by accelerating plant growth, increasing chelating ability, genetic modification and chemical transformation is ongoing (Naila *et al*, 2019). Chemical mutagenesis was successfully used on sunflower plants to improve metal accumulation and biomass yield (Nehnevajova *et al*, 2009). Addition of EDTA to metal contaminated soil selectively enhanced uptake of Cu and Ni while Cd, Pb, and Zn uptake remained consistent (Cornu *et al*, 2017). Sunflowers grown from seed under constant Ni stress accumulated 2 000 – 3 000 % more Ni within leaves and achenes than control plants (Ahmad *et al*, 2011). Root proliferation and shoot growth, concentrations of macro-nutrients (N, P, K and Ca) and micro-nutrients (Cu, Fe, Mn and Zn) were all negatively impacted by Ni accumulation. An additional finding of the study was that uptake and transport of Ni occurs by the same transport system as other micro-nutrients, Cu and Zn (Ahmad *et al*, 2011).

1.8 Aims and Objectives

Understanding the mechanisms by which metals are accumulated within plant species is vital for the propulsion of phytoremediation technology. The aim of this project was to identify and compare the key facilitators of Ni uptake in *B. coddii*, a South African Ni-hyperaccumulator, and *Helianthus Annuus L* (sunflower) a metal accumulator of the same family (Asteraceae).

The objectives of this project are thus:

- Quantification and analysis of the effect that additional bioavailable Ni has on uptake of the metal, and the natural response mechanism induced within each species;
- Observation of the effects that an organic acid amendment (ChA) and an amino acid amendment (His) has on Ni uptake and accumulation, as well as biosynthesis of organic and amino acids, for each species;
- Evaluation of the mechanistic differences between a Ni hyperaccumulator and an accumulator plant on addition of the soil enrichments.

1.9 Hypotheses

Analysis of the effect of soil enrichments on *B. coddii* and sunflowers was performed, such that the aim of the thesis could be investigated. Per the objectives of this thesis, the following hypotheses are drawn:

- Addition of Ni to the hyperaccumulator *B. coddii* will not adversely affect the development of the plant (Pillay, 2006; Naicker, 2014), unless the threshold accumulation capacity has been reached. Signs of necrosis will be observed for sunflowers, linked to the metal enrichment, and synthesis of stress-related amino acids will be increased.
- Addition of ChA will enhance the ability of *B. coddii* to accumulate Ni, while sunflowers will be adversely affected due to the effect of the organic acid on soil pH. Addition of His will increase accumulation capacity of both *B. coddii* plants, and sunflowers. Synthesis of key organic and amino acids will be observed within shoots for *B. coddii* and roots for sunflowers, related to the effects of the soil enrichments.
- The soil enrichments will result in enhanced synthesis of stress-related organic and amino acids within roots of both plants, as well as shoots of *B. coddii*.

CHAPTER 2

Experimental Methods and Materials

2.1 Introduction

This chapter outlines the materials, sampling and sample preparation techniques and instrumentation used in this project.

2.2 Materials and Instrumentation Parameters

2.2.1 Consumable Materials

All chemicals used in this experiment were of analytical or chromatography grade reagents purchased from Sigma-Aldrich, Spectrochem or Microsep (Pty) Ltd.

2.2.2 Instrumentation

The instrumentation used to obtain the objectives of this project are listed and analysis parameters for each technique is given in this section.

2.2.2.1 Sample Preparation Instrumentation

Stick Blender

A Prestige® PHB-905 Hand Blender was used to homogenise the plant samples prior to digestion.

Power: 230 V

Microwave Digestion

An Anton Paar Multiwave Go Microwave Digestion System with rotor 12HVT50, containing 12 reaction vessels.

Pressure Vessels: PTFE-TFM; 50 mL volume

Maximum Temperature: 250 °C

Maximum Pressure: 20 bar

Rotary Evaporator

The Rotavapor® R-100 rotary evaporator was used to vacuum seal vessels for digestion, and then later to dry acid-digested plant samples for the amino acid sample preparation procedure.

2.2.2.2 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry was used to analyse metal content in plant tissues. Experiments were conducted on an Agilent 7700x ICP-MS instrument, high-frequency hyperbolic quadrupole and electron multiplier detector.

Table 2.1 Operating Parameters of the ICP-MS

Gas Mode	No Gas
Forward Power (W)	1150 W
Nebulizer Type	MicroMist
Nebulizer pump Speed (rps)	0.5
Isotope measured	^{60}Ni
Isotope Limit of Detection (µg L^{-1})	0.1606
Replicates	3
Sweeps/Replicate	100

2.2.2.3 High Performance Liquid Chromatography (HPLC)

Instrument: Agilent HPLC 1260 Infinity II

Table 2.2 Instrument Parameters for HPLC-UV

Solvent Delivery Unit	VL G7111B Quaternary Pump Flow rate: 0.000 – 5.000 mL/min
Autosampler	G7129A Injection Volume: 0.00 – 100.00 µL
Diode Array Detector	WR G7115A Wavelength range: 190 nm – 950 nm Lamps: Deuterium
Data analysis software	OpenLAB CDS ChemStation Edition Rev. C.01.09 [144]

Table 2.3 HPLC-UV Operating Parameters for Organic Acid Analysis

Mobile Phase	0.04 M KH_2PO_4 adjusted to pH 2.7 with ortho-H_3PO_4
Flow Rate	1.0 mL/min
Column	Reverse Phase C_{18}, Phenomenex® Luna (2), 2.7 µm particles, 250 x 4.6 mm i.d.
Injection volume	5 µL
Gradient Type	Isocratic
Detector Wavelength(s)	220 nm for all organic acids, except ascorbic acid (254 nm)
Run Time	18 minutes

Table 2.4 HPLC-UV Operating Parameters for Amino Acid Analysis

Mobile Phase	Waters® Eluent A: ammonium formate, acetonitrile and formic acid Waters® Eluent B: Acetonitrile and formic acid Eluent C: MilliQ Water
Flow Rate	1.0 mL/min
Column	Reverse Phase C_{18}, AccQ-Tag™ 1.7 µm particles, 150 x 2.1 mm
Injection volume	5 µL
Gradient Type	Gradient Elution
Detector Wavelength(s)	254 nm
Run Time	80 minutes

2.3 Experimental Sites and Facilities

2.3.1 B. coddii

B. coddii seeds and serpentine soil were collected in Barberton, Mpumalanga in February and October, 2019, respectively. The plants were germinated and grown at the University of the Witwatersrand (Johannesburg), School of Chemistry.

2.3.2 Sunflowers

Sunflower seeds were purchased (Marltons Pet Care (Pty) Ltd: Sunflower Seed for Birds). The mixed species seeds were germinated and grown in organic compost in Johannesburg, South Africa. The plants were grown outdoors, in an area which receives all-day sunlight.

2.4 Germination, Growth and Spiking of Plants

2.4.1 *Berkheya coddii* R.

Table 2.5 B. coddii plant sets and their corresponding analyte spiking solutions

Plant Set	Chemical Used	Chemical Concentration [mg kg^{-1}]	Analyte	Analyte concentration per spike [mM]	Total Analyte concentration [mM]
Control	None	0	None	0	0
1	Chelidonic acid	1000	Chelidonic acid	5.43	27.16
2	Histidine	1000	Histidine	6.45	32.22
3	Nickel sulfate hexahydrate	10 000	Ni	170.4	852.9
4	Nickel sulfate hexahydrate and chelidonic acid	1000 each	Ni and ChA	17.04 Ni 5.43 ChA	85.29 Ni 27.16 ChA

Seeds were germinated on damp cotton wool in sealed containers for 3 weeks. For most plants, germination occurred within 7 - 11 days. Seedlings were then placed into biodegradable starter pots containing serpentine soil and grown for a further 4 weeks. Following this, the individual seedlings were transferred into larger polythene bags. After a week, plants were placed into five batches of 10 plants and separated into 4 enrichment batches and one control batch. Plants were then spiked with 20 mL of the appropriate aqueous solution (Table 2.5) every four days for five weeks. Two plants were culled from each batch every week and analysed for Ni content by ICP-MS and for organic and amino acids by HPLC-UV.

2.4.2 Sunflowers

Table 2.6 Sunflower plant sets and their corresponding analyte spiking solutions

Plant Set	Chemical used	Chemical Concentration [mg kg^{-1}]	Analyte	Analyte concentration per spike [mM]	Total Analyte concentration [mM]
Control	None	0	None	0	0
1	Chelidonic acid	500	Chelidonic acid	2.72	13.6
2	Histidine	1 000	Histidine	6.445	32.22
3	Nickel sulfate hexahydrate	1 000	Nickel	17.04	85.19

Sunflower seeds where germinated and grown in 1 L plant bags containing organic compost soil. After four weeks of growth, plants were grouped into four batches of 10 plants. Plants were spiked with 25 mL of solution for five weeks, and duplicate whole plant samples culled once a week (Table 2.6). The plants were watered daily save for the days when spiking occurred.

2.5 Plant Sample Preparation

Plant samples were prepared by a procedure adapted from Naicker (2014). Whole plants were culled and separated into aboveground (leaves and stems) and below ground (roots) sections. For sunflowers, aboveground tissues were further separated into leaves, stems and, where available, flowers. Sample sections were thoroughly washed with tap water to remove dirt and residue, then air dried overnight. Samples were ground with a stick blender, prior to extraction or digestion.

2.6 Organic Acid Analysis by HPLC-UV

2.6.1 Standard preparation for qualitative analysis of organic acids by HPLC-UV

To identify the organic acids present in plant samples, 200 mg kg^{-1} single standards of a range of organic acids which are associated with heavy metal accumulation were made. Salts of each acid were dissolved in Millipore water and analysed by HPLC-UV.

2.6.2 Standard preparation for quantitative analysis of organic acids by HPLC-UV

Concentrations of organic acids within plant samples were calculated by an external standard method.

Table 2.7 Organic acid standard calibration concentration ranges

Organic acid Standard	Concentration range (mg kg^{-1})
Oxalic acid	0 – 500
Tartaric acid	0 – 1000
Malic acid	0 – 1000
Ascorbic acid	0 – 500
Chelidonic acid	0 – 100
Citric acid	0 - 2000
Fumaric acid	0 – 100

A concentration range (0 – 2000 mg kg^{-1}) of standards were prepared for analysis of each peak (Table 2.7). From this, a calibration curve was created for each organic acid, based on the peak area obtained from HPLC-UV. The concentration range of each analyte was chosen dependent on the associated peak area of the analyte.

2.6.3 Sample preparation and analysis

Prepared sample matter (section 2.5) was weighed into sample vials (~0.01 g for *B. coddii* samples and ~0.1 g for sunflower samples). Millipore water was added to each. Sample vials were vortexed for one minute to extract organic acids, then centrifuged at 60 rpm for 3 minutes before the supernatant was filtered through a 0.45 µM nylon filter and analysed by HPLC-UV.

2.7 Amino Acid Analysis by HPLC-UV

2.7.1 External Standard Preparation for Quantitative Analysis of Amino acids

A range (0 – 0.04 mM) of amino acid standards were prepared and a calibration curve of each created for quantitative analysis of analytes by HPLC-UV.

Table 2.8 Amino Acids Contained in Waters® Amino Acid Standard Hydrolysate

Metabolic pathway	Amino Acid	Abbreviation
3-P-Glycerate	L-Cysteine	Cys
	Glycine	Gly
	L-Serine	Ser
Pyruvate	L-Alanine	Ala
	L-Leucine	Leu
	L-Valine	Val
P-enolpyruvate (shikimate)	L-Phenylalanine	Phe
	L-Tyrosine	Tyr
α-ketoglutarate	L-Arginine	Arg
	L-Glutamic Acid	Glu
	L-Histidine	His
	L-Proline	Pro
oxaloacetate	L-Aspartic Acid	Asp
	L-Isoleucine	Ile
	L-Lysine HCl	Lys
	L-Methionine	Met
	L-Threonine	Thr

The amino acid hydrolysate standard containing 17 amino acids and ammonia, provided by Waters®, was diluted in Millipore water to create a calibration standard (Table 2.8). The calibration standard was then derivatized by the Waters AccQ-Tag Method using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), resulting in stable amino acid derivatives. Briefly, excess AQC is added to the amino acid sample and the mixture is heated to 55 °C. On heating, the N-hydroxysuccinimidyl (NHS) group of AQC is cleaved from the carbamate group and replaced by the amino acid. Remaining AQC hydrolyses to produce 6-aminoquinoline (AMQ), which has a strong UV peak at 254 nm. The resultant product contains a stable amino acid derivative, and the by-products NHS, AMQ and carbon dioxide (CO_2). Derivatives and the by-product AMQ were analysed by HPLC-UV at 254 nm (Millipore Corporation, 1993).

2.7.2 Sample preparation and derivatisation

Samples were hydrolysed by the method used by Davidson (2003). Prepared sample matter (~100 µg) was weighed into 1 mL clear glass ampoules, and 1 mL of 6 M HCl added. Samples were vortexed thoroughly, centrifuged, then sealed under vacuum. The sample vessels were heated to 110 °C for 18 hours, and then excess acid dried off in a rotary evaporator. Dried samples were reconstituted in 20 mM HCl, derivatized using AQC, and amino acids analysed by HPLC-UV.

2.8 Ni Uptake Analysis by ICP-MS

2.8.1 Method Validation

Certified reference materials (CRMs) have a certified associated analyte concentration, with a given confidence interval as well as a known accompanying uncertainty (Ulberth, 2006). These materials are used to ensure accuracy of the entire method developed for sample analysis, from sample preparation to instrument parameters, and analysis procedure developed. CRM (DC73349), Bush branches and leaves was used to validate the ICP-MS method used to analyse plant samples (Appendix A). The CRM was treated similarly to the plant samples.

2.8.2 Calibration Solution Preparation

A range of metal standards were prepared (0 – 5 000 µg kg^{-1} each of ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, and ^{66}Zn) from a 10 mg kg^{-1} stock multi-element standard solution purchased from Spectrochem. This was used to quantify heavy metals using ICP-MS. A 20 µg kg^{-1} Rh internal standard, purchased from Spectrochem, was added to each standard.

2.8.3 Sample Preparation – Nickel analysis

Table 2.9 Microwave Digestion Program for Plant Sample Digestion

Step	Time (min)	Temperature Ramp (°C)
1	2	RT - 145
2	5	145
3	3	145 - 190
4	20	190

Dried, ground plant samples and CRM samples (0.1 – 0.25 g) were weighed into clean, dry Teflon vials. Nitric acid (5 mL) was added to each sample, vials were sealed and left for a pre-digestion period of 1 hour. Following this, samples underwent a 30-minute microwave digestion program (Table 2.9). Cooled samples were then filtered (0.45 µm filter paper), and diluted 20-times with Millipore water. A 20 µg kg^{-1} Rh internal standard was added to each sample.

2.9 Sample Analysis Instrumentation

Two instrumentation techniques were used for analysis of the samples:

- Mass spectrometry

ICP-MS was used to determine the metal content of plant samples

- Liquid chromatography

HPLC with ultraviolet (UV) detection, was used to identify and quantify organic and amino acids in the plants.

2.9.1 ICP-MS

Acid digested plant matter was analysed for Ni concentrations by ICP-MS. ICP spectrometry techniques have become the preferred choice for inorganic element analysis due to their ability to accommodate multi-element analysis (Hansen *et al*, 2013). Mass spectrometry detection, in recent years, has gained popularity for plant metal analysis due to its advantageous characteristics: accommodation of micro-scaled sample digestion, sub-ppt detection limits, a wide linear calibration range, and the aforementioned multi-element analysis capability (Zuluaga *et al*, 2011).

ICP-MS is the most utilised of the major spectroscopy techniques (FAA, ETA, ICP-OES and ICP-OES) because of its unique characteristic advantages. It has the lowest detection limits of all major spectroscopy techniques, making it the ideal choice for analysis of sub-ppt range inorganic analytes. It can also accommodate higher concentration ranges (mg kg-1-level), making it a more robust sample analysis tool than ICP-OES (Hansen *et al*, 2013). Additionally, ICP-MS has the unique characteristic of allowing for the analysis of specific isotopes of ions (Profrock and Prange, 2012).

2.9.2 HPLC-UV

Plant extracts were analysed by HPLC-UV for the identification and quantification of amino

HPLC is an adaptation of chromatography in which a higher flow rate and pressure are used to separate liquid sample components with the mobile phase. This allows for the use of smaller stationary phase beads with a greater surface area to volume ratio, resulting in increased interaction of mobile phase components with the stationary phase and faster elution of samples with greater resolution and repeatability of separation than traditional gravity column chromatography (Pillay, 2006).

CHAPTER 3

Results and Discussion: *Berkheya coddii* R.

3.1 Introduction

B. coddii plants were grown in serpentine soil enriched with ChA, His, Ni, or a combined Ni-ChA solution (hereafter called the co-enrichment). Plant responses to each soil enrichment were monitored and compared to responses of plants grown in non-enriched serpentine soil (control). Ni uptake and accumulation was monitored to elucidate the effects that each soil enrichment had on the hyperaccumulating capabilities of the plants (Appendix Table C1). It is presumed that additional bioavailable Ni will have the largest effect on this. The Ni soil amendment made in the present study is, to date, the largest known concentration of this metal applied to *B. coddii* over a five-week study. The responses of the plant, with regard to biosynthesis and metabolism of organic and amino acids were also observed for each soil amendment. Organic molecules are essential to the growth and development of plants, as well as the abiotic stress response thereof.

All 17 of the amino acid standards analysed (Table 2.8), were detected within *B. coddii*. However, only 12 amino acids were both identified and quantified consistently throughout the experiment within roots, and 13 within shoots (Appendix Table C2). Concentrations of arginine, cysteine, methionine, and threonine were inconsistently quantified within all plant tissues, and valine within roots. The focus of this experimental study was centred on five amino acids: Pro and His, which have both been directly related to the Ni-accumulating capacity of hyperaccumulator plants; and aspartate, glutamate and serine, from which biosynthesis of almost all amino acids related to metal metabolism occurs (Sharma and Dietz, 2006).

Ni hyperaccumulation has been linked to four main chelating ligands for *B. coddii*: malic acid, citric acid, His and ChA (Montargès-Pelletier *et al*, 2011; Naicker *et al*, 2016; Robinson *et al*, 2003; Slatter, 1998). The molar ratios of these four organic ligands to Ni within plant shoots were investigated in order to further understand the mechanism by which Ni is rendered immobile within *B. coddii* plants.

3.2 Effects of Soil Enrichment on the Growth and Development of *B. coddii*

Plant biomasses of each batch of plants over the 5-week experimental study were monitored (Appendix Table C1). The growth and development of *B. coddii* plants was affected significantly by environmental factors, such as lower daily temperatures and daylight hours and rainfall effects, since plants were grown outside of their typical growth cycle (September to February) (Slatter, 1998). For all plants, roots and shoots were very small (< 1.5 g) and

underdeveloped. All plants presented with at least 2 pairs of true leaves prior to commencement of soil amendments.

It is clear that the soil enrichments applied have a significant effect on growth and development of *B. coddii* plants over the five-week experimental period (Figure 3.1).

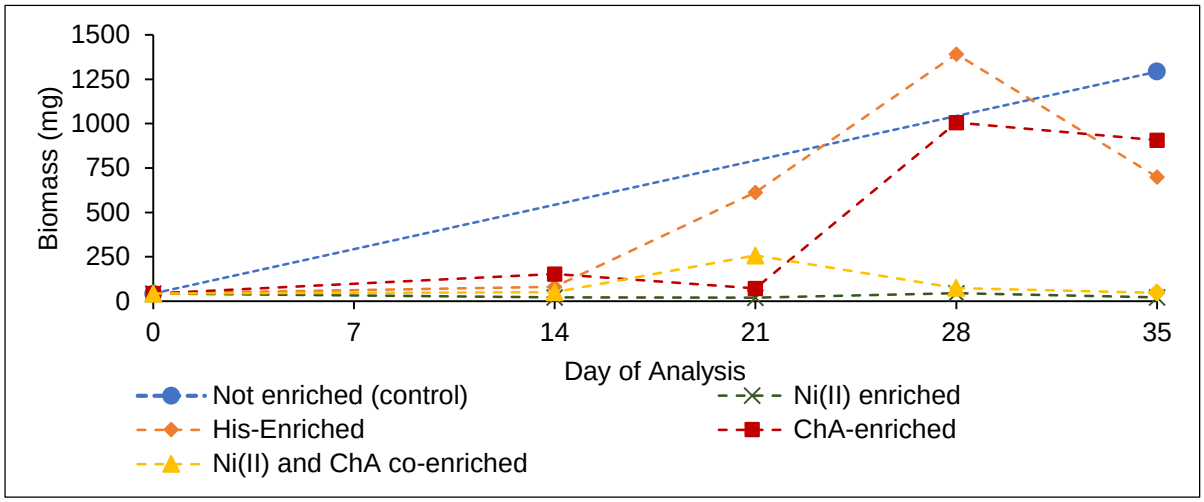

Figure 3.1 Total dry biomasses of B. coddii plants

Application of bioavailable Ni to the soil appears to have the largest negative effect on plant growth and development, observed for the Ni (II) enrichment as well as the Ni (II) and ChA co-enrichment. For both of these soil enrichments, root biomass becomes progressively smaller over the experimental period, indicating that root development is severely hindered within plants on addition of bioavailable Ni. Growth and development of shoots was also severely hindered by addition of Ni, with physical signs of necrosis observed (Figure 3.2a). Plants grown in Ni and ChA co-enriched soil fared better than plants grown in Ni-enriched soil. This is due to the ten-fold concentrated bioavailable Ni for the Ni-enrichment. Previous analyses of *B. coddii* plants grown in Ni-enriched soil did not indicate an adverse effect on growth and development with additional bioavailable Ni (Naicker *et al*, 2016; Pillay, 2006; Robinson *et al*, 1997). The response observed in the present study is likely a result of Ni transport and storage systems within plants being less developed, since their biomasses are smaller (Paul *et al*, 2020).

For all four batches of plants, a sudden growth spurt was observed after day 21 of the experimental analysis. Root biomass was comparable to the control for both the ChA and His soil enrichments (Appendix Table C1). Plants grown in His-enriched soil had larger, healthier leaves than any other soil-enriched plant batches (Figure 3.2b). This indicates that the His soil enrichment does not have a negative impact on plant growth and development, as observed for Ni soil enrichments. This observation is in keeping with a His soil enrichment for other hyperaccumulator plants of the Brassicaceae family (Kozhevnikova *et al*, 2014; Seregin *et al*,

2019). ChA soil enrichment resulted in dull, wilted leaves. This implies that a persistent essential nutrient deficiency occurs on addition of ChA to the soil medium, likely due to acidification of the soil. The availability of macronutrients N, Ca, P, K, S and Mg are all negatively impacted by soil acidification (Yadav *et al*, 2020).

Figure 3.2 B. coddii plants grown for 3 weeks in 1) Ni-enriched soil, 2) Histidine-enriched soil, and 3) ChA-enriched soil

3.3 No Soil Enrichment (controls)

B. coddii seedlings were grown in their native serpentine soil for two months outside of their natural growing cycle. A group of plants were watered with tap water, and two plants culled once a week for five weeks. Data obtained pertaining to natural plant responses observed are presented.

3.3.1 Nickel uptake and accumulation

Table 3.1 Concentration of Ni within B. coddii plants

Analysis Day	Ni Concentration/ mg kg^{-1}	
	Roots	Shoots
0	930	1 060
35	614	3 284

Ni concentrations within roots and shoots of *B. coddii* are in the range 772 ± 223 mg kg^{-1} and 2 172 ± 1 572 mg kg^{-1}, respectively (Table 3.1). Within plant roots, there is a decrease of approximately 30% in the Ni concentration from the start of the experiment (930 mg kg^{-1}) to the end (614 mg kg^{-1}). This is likely due to the uptake of Ni by the shoots and indicates that the capacity for Ni accumulation within plant roots is limited. The decrease is also associated with increased translocation pathways as the plant grows (Groeber *et al*, 2015). In contrast,

the concentration of Ni in the shoots increases gradually from 1 060 to 3 284 mg kg^{-1} over the five weeks following trends noted by other studies (Naicker *et al*, 2016; Pillay, 2006; Robinson *et al*, 2003).

3.3.2 Amino acid biosynthesis

Table 3.2 Amino Acid concentrations within untreated B. coddii plants grown in serpentine soil (RSD <5%)

	Concentration/ mg kg^{-1}			
	Roots		Shoots	
Day of Analysis	0	35	0	35
His	1300	30069	8666	11525
Pro	21081	27712	7452	1113
Asp	14057	29261	5404	6136
Ser	11941	10957	5242	4499
Glu	13868	34918	10757	7265
Gly	8581	1026	4127	3926
Ala	7157	14203	3673	2574
Tyr	4255	772	2020	5344
Lys	5233	13286	4695	3717
Ile	5333	14400	6662	3458
Leu	8881	22396	6926	5637
Phe	4655	16886	3530	3171
Val	n.d.	n.d.	4028	20

n.d. - not determined

Amino acids are naturally biosynthesised within plants. They perform a variety of roles pertaining to plant growth and development, and protection from biotic and abiotic stresses. The amino acids biosynthesised within *B. coddii* seedlings analysed are analysed below (Table 3.2). The concentrations of all amino acids within roots and shoots of *B. coddii* plants are significantly higher for the plants analysed presently than for similarly aged *B. coddii* plants analysed previously (Naicker, 2014). This is likely due to the suboptimal conditions in which the plants were grown, which led to slowed growth and development and thus, smaller plants. Amino acid metabolism produces substantial amounts of energy, which may aid plants to survive non-ideal circumstances (Hildebrandt *et al*, 2015).

3.3.2.1 Shoots

Initially, Pro is the third most abundant amino acid (Table 3.2), after glutamic acid and His, in plant shoots (7 452 mg kg^{-1}). The concentration of Pro in metal-tolerant plants is found to be constitutively higher than for non-accumulator plants (Sharma and Dietz, 2006). The presence of Pro within plant shoots is drastically reduced (a seven-fold reduction, 1 113 mg kg^{-1}) over the five weeks. This result is anomalous, given that plant leaves are underdeveloped in the present study due to abiotic stress, a condition for which Pro accumulation is synonymous. This trend may be observed as a result of preferential glutamate and His biosynthesis, to aid

in N assimilation, a major limiting factor in plant growth and productivity, and Ni transport and accumulation (Sengupta-Gopalan and Ortega, 2015).

His was accumulated in aboveground tissues (8 666 mg kg^{-1} – 11 525 mg kg^{-1}) and was the most concentrated of the monitored amino acids on day 35. His is related to heavy metal chelation and transport of heavy metals from roots into shoots (Leitenmaier and Küpper, 2013). The concentration of His within Ni hyperaccumulating plants in particular, is constitutively higher than for non-hyperaccumulator plants, for its apparent function as a Ni ligand promoting xylem loading of the metal (Ingle, 2015). This is likely the function of this amino acid in the present study.

The concentration of serine is similar on day 0 and day 35 of the experiment, 11 941 mg kg^{-1} and 10 957 mg kg^{-1}, respectively. This amino acid is linked primarily to photorespiration within plants (Hildebrandt *et al*, 2015). Thus, it is indicated that photorespiration processes of plants are stable throughout the five weeks. Glutamic and aspartic acid concentrations within plants increase significantly from day 0 to day 35 of the experiment, each more than doubling over the five weeks. Both of these amino acids play a central role in N metabolism for higher plants, as well as roles in plant growth and resistance to disease, respectively (Duff, 2015).

3.3.2.2 Roots

At the start of the experiment, Pro (21 081 mg kg^{-1}) was the most concentrated amino acid within plant roots (Table 3.2). This concentration is approximately 7 times the average concentration (10 – 50 mM) commonly reported for non-hyperaccumulating plants (Sharma and Dietz, 2006). This concentration is likely due to Pro transfer from seeds, similarly to the model plant *Arabidopsis thaliana*, Zn-tolerant *Deschampsia cespitosa*, and Cu-tolerant *Armeria maritima* (Farago and A, 1979; Smirnoff and Stewart, 1987; Verbruggen and Hermans, 2008). Pro concentration within roots on increases over the five-week experiment to 27 712 mg kg^{-1} on day 35, as plant roots grow and develop.

The concentration of His within roots was lowest of all detected amino acids at the start of the experiment (1 300 mg kg^{-1}, Table 3.2). This concentration is significantly higher than the typical concentration of His with plants (<400 mg kg^{-1}) (Richau *et al*, 2009). This is expected, as hyperaccumulator species have been found to typically have a higher abundance of root His than non-hyperaccumulator species (Ingle, 2015). His accumulation in plant roots over the five-week experiment was the most significant of all of the amino acids, with day 35 His (30 069 mg kg^{-1}) 23 times more concentrated than day 0. This indicates a more significant role of this amino acid within plant roots than merely its association with growth and development, likely related to transport of Ni as transport pathways for this metal develop.

Concentrations of the three amino acid precursors Asp, Glu, and Ser within plant roots are similar on day 0 (11 941 – 14 057 mg kg^{-1}). It is observed that, after the 35-day experimental period, Ser concentration does not change significantly (8% reduced). This indicates that

serine and its metal metabolite derivatives, cysteine and glycine, do not participate in metal accumulation within roots of *B. coddii*. On day 35 of analysis, Glu and Asp concentrations are over double their initial concentrations. This is likely due exclusively to the seven-fold increase of root biomass recorded, indicating no correlation of these amino acids to possible root Ni accumulation.

3.3.3 Organic acid biosynthesis

Table 3.3 Organic acids within B. coddii plants grown in non-enriched serpentine soil (RSD <5%)

	Concentration/ mg kg^{-1}			
	Roots		Shoots	
Day of Analysis	0	35	0	35
malic acid	5516	8303	5980	19463
tartaric acid	625	7159	894	1832
chelidonic acid	316	900	16235	16032
citric acid	3151	6533	28025	21348

Accumulation of OAs within *B. coddii* plants grown in non-amended serpentine soil is, with the exception of tartaric acid, concentrated within plant shoots (Table 3.3).

3.3.3.1 Shoots

Over the course of the 5-week experiment, the concentration of malic acid within control plant shoots increases by over three-fold, while citric acid exhibits a 23% decline in concentration. Tartaric acid concentration within leaves increases 2.5-fold over the course of the experimental study. ChA concentration remains stable.

Citric, malic and tartaric acid are all synthesised and metabolised within the energy-producing Krebs cycle of plants (Lopez-Bucio *et al*, 2000). Malate is one of the main energy-producing pathways of plants (Zhang and Fernie, 2018). It is also linked to pH regulation and has an effect on nutrient uptake and stomatal function of plants (Fernie and Martinoia, 2009). Malic acid accumulation within shoots is thus related to the unique growth challenges observed in the present experiment. In photorespiratory conditions citrate is converted in a partial, “non-cyclic” Krebs cycle to α-ketoglutarate and its derivatives, including glutamic acid, by a “citrate valve” (Igamberdiev and Eprintsev, 2016). The accumulation of glutamate pathway amino acids glutamate, His and Pro in plant shoots likely contribute to the reduced concentration of citric acid observed as it is actively, irreversibly metabolised to these molecules, essential for growth and development and the abiotic stress response of plants. Tartaric acid is linked to the support of stress responses (Burbidge *et al*, 2021), and may play such a role in the present study.

3.3.3.2 Roots

The concentration of all four OAs increases within plant roots as a function of time. Tartaric acid has the largest concentration gradient of the organic acids evaluated, increasing eleven-fold from day 0 (625 mg kg^{-1}) to day 35 (7 159 mg kg^{-1}). Malic acid is the most concentrated OA within roots throughout the 5-week experimental period, and is also the most stable within plant roots, exhibiting a concentration increase of 33%, while the other OA concentrations (citric acid and ChA) increase by more than 50% over the experiment.

For (hyper)accumulator plants, OAs have an essential role as root exudates, capable of increasing the bioavailability of heavy metals within the rhizosphere through chelation of metal ions and acidification of the soil medium (Bao *et al*, 2011). The Cd-hyperaccumulating and non-hyperaccumulating ecotype of *Sedum alfredii* were found to exude oxalate and malate into the rhizosphere, and tartaric acid exudation was unique to the hyperaccumulating ecotype *(Tao et al, 2020)*. Concentrations of OA root exudates (acetate, citrate, malate, oxalate and tartrate) for another Cd hyperaccumulator, *solanum nigrum* L., were found to be constitutively higher than for the non-hyperaccumulator ecotype (Bao *et al*, 2011). Organic acid root exudates for Ni hyperaccumulator *T. goesingense* are proposed to result in the increased dissolved organic carbon (DOC) observed around the roots of the plants, compared to non-accumulator species native to the same area (Wenzel *et al*, 2003).

3.3.4 Nickel chelation analysis

Metal coordination within plants is different for leaves of varying ages (Schaumloffel *et al*, 2003). This is assumed to be true at varying stages of development as well, since organic molecules within plant shoots are altered depending on their age.

Previous studies performed on *B. coddii* plants, grown in their native environment, have suggested that malic acid is the major chelator of Ni within plant leaves (Anderson *et al*, 1995; Slatter, 1998). X-ray absorption analyses found that both malic acid and citric acid were sufficient to act as the main chelating ligands of Ni in leaves of *B. coddii*, and His had no role *(Montargès-Pelletier et al, 2011)*. Pot trials were later performed on *B. coddii* plants grown in serpentine soil, which revealed that the ratio of Ni to malic acid in *B. coddii* leaves (1:0.013) was not sufficient for this organic acid to be the main chelator of the metal (Pillay, 2006). An alternate organic acid was found to be the main chelator of Ni within plant leaves, later characterised as the pyranone, ChA (Naicker *et al*, 2016).

Table 3.4 Molar Ratio of Ni to reported Ni-chelants chelidonic acid (ChA), malic acid (MA), citric acid (CA) and histidine (His) within shoots of control plants.

Analysis Day	Concentration /mg kg^{-1}					Molar Ratio
	Ni	MA	CA	ChA	His	Ni : MA : CA: ChA : His
0	1 060	5 980	28 025	16 235	8 666	1 : 2.5 : 8.1 : 4.9 : 3.1
35	3 284	19 463	21 348	16 032	11 525	1 : 2.6 : 2.0 : 1.6 : 1.3

Analysis of the ratio of Ni to organic acids and His for the present study are presented (Table 3.4). Typically, two molecules of ChA complex Ni. The molar ratio of Ni to ChA on day 0 (1:4.9) indicates that sufficient ChA is present within shoots to chelate Ni within plant shoots. This is not the case, however, for analyses performed on day 35 of the analysis. Ni accumulation is shown to be not proportional to biosynthesis of ChA within shoots. Thus, ChA is not to be the only chelating agent within plant shoots. This is likely a result of the requirement for larger pools of other organic acids, which are directly related to plant growth and development, such as malate and citrate. On day 0, the molar ratios of malic acid, citric acid and His are sufficient for all three of these molecules to chelate to Ni. It is likely that, in the present study, Ni chelation is the result of a combined effort of all of these organic molecules in different organs of plant shoots.

3.4 Nickel-Enriched *B. coddii*

Ni, as 10 000 mg kg^{-1} $NiSO_4.6H_2O$ solution, was added to the soil of *B. coddii* plants over a five-week period (852 mM total). This is the largest concentration of bioavailable Ni exogenously applied to *B. coddii* plants to date over a shorter period of time.

3.4.1 Nickel uptake and accumulation

The uptake of Ni from the soil occurs in a cyclic pattern for all of the soil amendments made (Appendix Table C1). This may suggest that the metal accumulates first within roots of *B. coddii* then, at some maximum concentration, is transported into plant leaves.

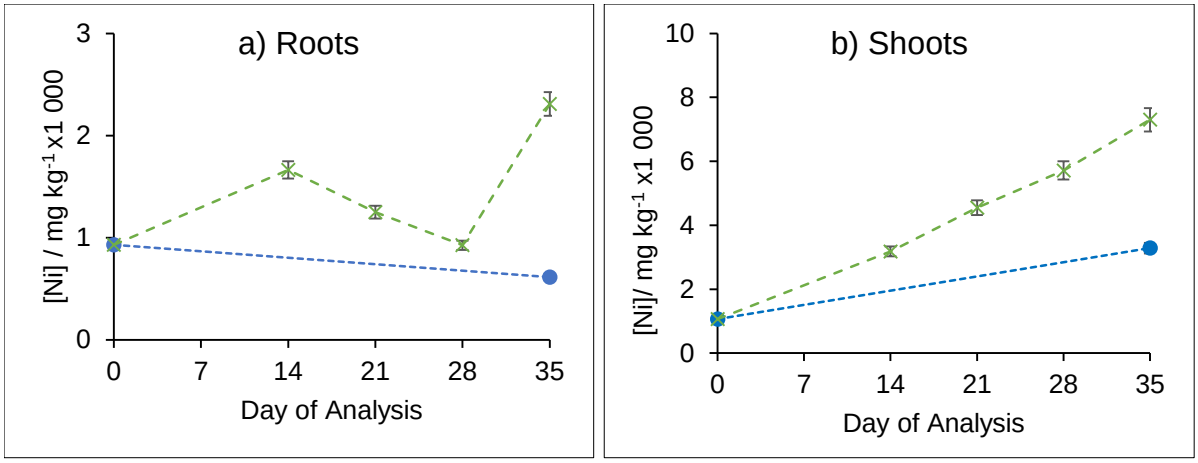

Figure 3.3 Ni concentration within a) roots and b) shoots (leaves and stems) of B. coddii R. grown in Nickel(II)-enriched serpentine soil (green) and non-enriched soil (blue)

3.4.1.1 Roots

The average concentration of Ni (1 416 ± 583 mg kg^{-1}) is double the concentration of control plants (772 ± 223 mg kg^{-1}) (Figure 3.3a). It is inferred then, that additional bioavailable Ni, as a result of the Ni soil enrichment, allows the plant to take up more of the metal in one cycle, and translocate the metal from roots to shoots at higher batch concentrations, despite the diminished root biomass resulting from the amendment (section 3.2).

3.4.1.2 Shoots

Table 3.5 Ni accumulation within B. coddii for previous studies performed

Plant section	Age of Plants / weeks	Length Study / weeks	Ni added [a]/ mM	Ni in plant/ mg kg^{-1}	Reference
Leaves	22	17	0.5[b]	8 000 – 9 000	Robinson *et al*, 2003
Leaves	40	40	100	37 050	Pillay, 2006
Leaves	12	4	280	1 447	Naicker *et al*, 2016
Shoots	12	5	852	7 300	This study

a = Highest concentration of Ni was chosen for each reference; b = Plants grown in hydroponic solution

Ni accumulation in shoots increases linearly, from 1 060 mg kg^{-1} to 7 300 mg kg^{-1} (Figure 3.3b). This trend was observed previously (Table 3.5), for plants spiked with more dilute concentrations of Ni (Naicker *et al*, 2016; Pillay, 2006; Robinson *et al*, 2003). The concentration of Ni observed is significantly higher than for similarly aged plants and similar

to that of plants grown for 10 weeks longer, but exposed to a lower concentration of Ni (Naicker *et al*, 2016; Robinson *et al*, 2003). Ni (II) accumulation is enhanced with additional bioavailable Ni.

3.4.2 Effects on amino acid biosynthesis

Amino acid accumulation within *B. coddii* is cyclic, as AAs are biosynthesised then utilised within plants.

Table 3.6 Amino acids within B. coddii plants grown in Ni-enriched soil (RSD <5%).

	Belowground/ $x10^{-3}$ mg kg^{-1}					Aboveground/ $x10^{-3}$ mg kg^{-1}					
Spike	Control		Ni (II)-enriched			Control		Ni (II)-Enriched			
Day	7	35	14	21	35	7	35	14	21	28	35
Asp	14	29	2.6	5.1	0.94	5.4	2.9	1.3	4.6	3.0	36
Ser	12	11	0.81	12	0.78	5.2	4.5	1.5	5.2	1.6	30
Glu	14	35	3.0	8.6	1.9	11	7.3	1.6	6.9	3.3	45
His	1.3	30	17	0.30	4.0	8.7	12	0.73	14	0.41	16
Pro	21	28	1.6	24	1.8	7.5	0.074	3.1	6.6	9.2	40
Ala	7.2	14	0.91	4.0	0.50	3.7	2.6	3.2	ND	1.3	29
Gly	8.6	1.0	1.1	9.2	1.2	4.1	3.9	2.1	7.4	1.6	40
Tyr	4.3	0.77	0.61	6.7	0.96	2.0	5.3	1.5	0.74	0.66	30
Lys	5.2	13	8.2	0.88	1.4	4.7	3.7	5.1	5.8	1.3	20
Ile	5.3	14	1.0	4.3	0.8	6.7	3.5	11	7.8	1.0	26
Leu	8.9	22	1.8	7.1	1.4	6.9	5.6	12	2.3	1.5	46
Phe	4.7	17	1.6	7.4	2.6	3.5	3.2	8.4	5.4	0.45	35
Val	-	-	-	-	-	4.0	0.020	2.4	ND	1.4	33

ND – not determined

3.4.2.1 Roots

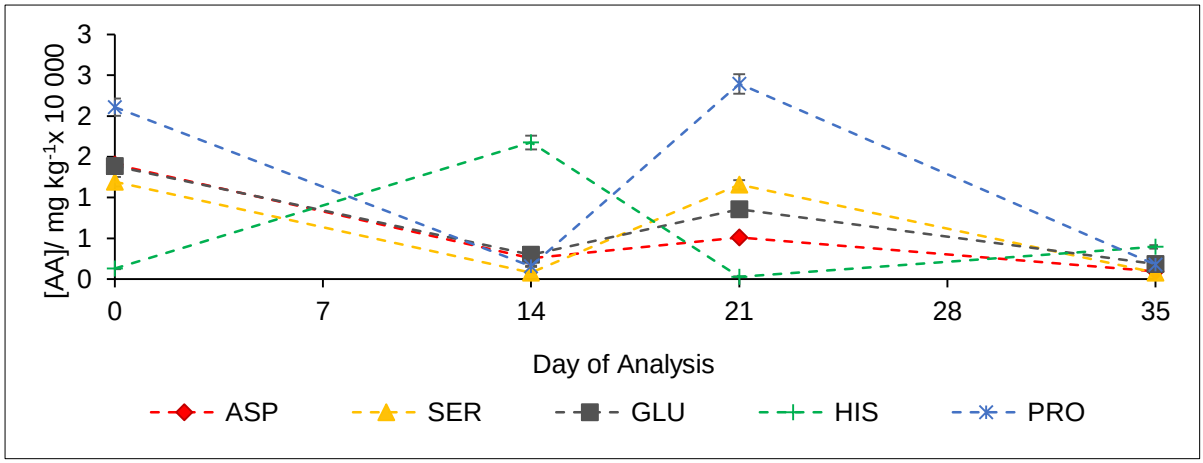

Figure 3.4 Amino acids biosynthesised within roots of B. coddii grown in Ni(II)-enriched soil

Overall, amino acid concentrations are depleted compared to control plants (Table 3.6). This is likely due to amino acid translocation to shoots in response to the negative effect of Ni on

plant biomass (Sharma and Dietz, 2006). Biosynthesis of His appears to occur in an opposing trend to that of other amino acids (Figure 3.4). This is likely due to the high energy required for His biosynthesis, which may affect synthesis of other amino acids, particularly Pro and the precursor to both, glutamate (Ingle, 2011). On day 35 of the experiment, the concentration of amino acids (502 – 3 958 mg kg^{-1}) is lower than expected, considering the cyclic trend observed. This corresponds to a spike in shoot amino acid accumulation, indicating that amino acids are translocated from roots to shoots.

3.4.2.2 Shoots

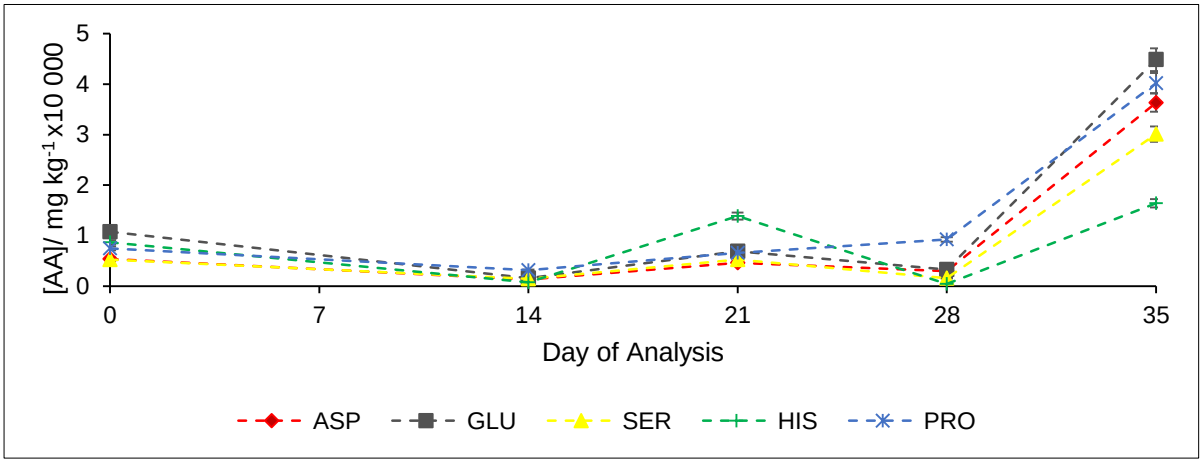

Figure 3.5 Amino acids biosynthesised within shoots of B. coddii grown in Ni(II)-enriched soil

Accumulation of AAs is observed overall within plant shoots (16 442 – 46 370 mg kg^{-1} Ni), compared to control (74 – 11 525 mg kg^{-1}). Thus, Ni-enrichment of the soil prompts biosynthesis of AAs within plant shoots, and translocation from roots to shoots. This is likely an abiotic stress response, which indicates that toxic accumulation of Ni occurs (Sharma and Dietz, 2006). A ten-fold spike in amino acid concentration is observed on day 35, for all amino acids (Figure 3.5). This accumulation appears to be dependent on organic acid accumulation within plant shoots (section 3.4.3.2).

3.4.3 Effects on organic acid biosynthesis

3.4.3.1 Roots

Accumulation of organic acids malate, citrate and tartrate occurs in a cyclic trend, while ChA concentration remains fairly stable (Figure 3.6).

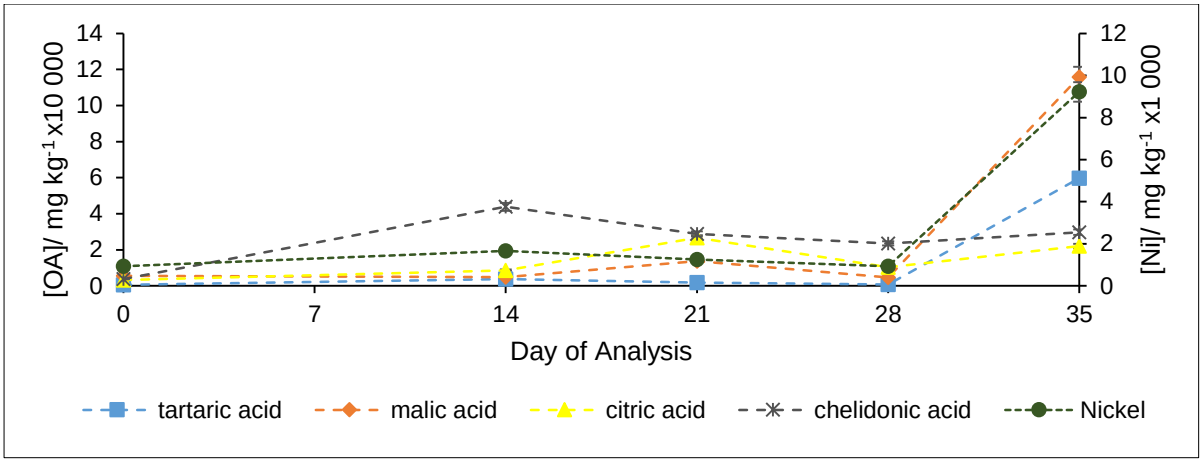

Figure 3.6 Organic acid concentrations within roots of B. coddii plants grown in Ni(II)-enriched soil

Malic acid and tartaric acid concentrations are both lower within spiked plants prior to day 35, compared to control plants. A spike in the concentrations of both of these organic acids is observed on day 35, which aligns with a corresponding Ni accumulation (Figure 3.6). These OAs are linked to the root exudate system of hyperaccumulators, and thus to increased metal accumulation within plants (Bao *et al*, 2011; Tao *et al*, 2020). Analysis of the molar ratios of these organic acids to Ni, 17:1 for malic acid and 8:1 for tartaric acid, indicates that both of these organic acids are present in excess and may be coordinated to the metal present within roots.

The average concentration of ChA within Ni-enriched plant roots (2 696 ± 744 mg kg^{-1}) is twice that of ChA within control plants (608 ± 413 mg kg^{-1}). It is also significantly larger than the concentration observed for the other soil enrichments made (Appendix Table C3). This indicates that ChA may also play some role in transport of the metal from plant roots to shoots.

3.4.3.2 Shoots

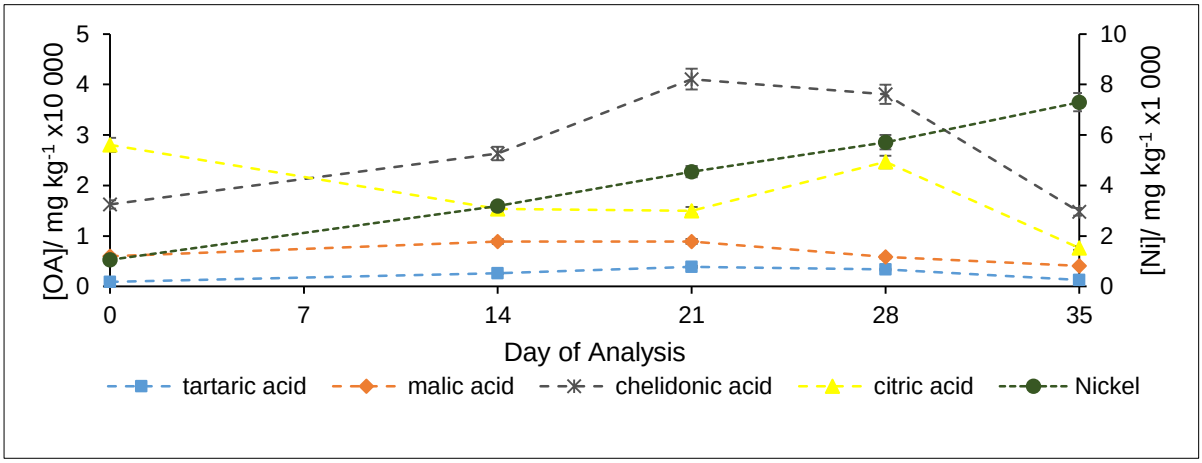

Figure 3.7 Organic acid concentrations within shoots of B. coddii plants grown in Ni(II)-enriched serpentine soil

Synthesis and metabolism of the OAs malic acid, citric acid, and tartaric acid within plant shoots occurs in a cyclic pattern (Figure 3.7). This is independent of the linear Ni accumulation pattern observed. These organic acids are thus not likely to be responsible for Ni sequestration within shoots. The average concentration of malic acid (6 742 ± 2 116 mg kg^{-1}) is half that within control plants (12 721 ± 9 534 mg kg^{-1}), indicating that this organic acid is metabolised or transported within plant shoots.

3.4.4 Nickel Chelation Analysis

Table 3.7 Molar ratio of Ni to Ni-binding organic molecules within shoots of B. coddii plants grown in Ni-enriched soil

	Day of Analysis	Biomass (mg)	Molar Ratio to Ni			
			Malic acid	Chelidonic acid	Citric Acid	Histidine
Nickel enrichment	0	9	2.5	4.9	8.1	3.1
	14	13	1.2	2.6	1.5	0.1
	21	12	0.9	2.9	1.0	1.2
	28	42	0.4	2.1	1.3	0.0
	35	19	0.2	0.6	0.3	0.9

ChA has been shown, in previous studies, to be the main chelating ligand of Ni within *B. coddii* (Naicker *et al*, 2016; Pillay, 2006). Accumulation of ChA is observed for the first four weeks of analysis, corresponding to Ni accumulation within plants (Figure 3.7,Table 3.7). The molar ratio is consistently above the requisite 2:1 up to this point in the experiment. Thus, ChA is likely to bind the metal. On day 35, however, the molar ratio (0.6) is too low for ChA to bind

Ni. This is the case for all three of the Ni-binding organic acids monitored (Table 3.7). A corresponding reduction of plant biomass by half, and a spike in amino acid accumulation was observed on this day, indicating a severe stress response of plants. It is evident that by day 35 of the experiment, severe necrosis had occurred, and the plants were likely to die. It is assumed then, that the maximum Ni accumulation has occurred within *B. coddii* plants by day 35 of the analysis and, following this, complete plant death would be observed.

3.5 Histidine-Enriched *B. coddii*

A total of 32 mM His was added to the soil matrix of *B. coddii* plants over the 5-week study (Table 2.5). It is noted that research efforts related to the effects of exogenous His to plants occur overwhelmingly on hydroponically grown samples, within temperature and humidity-controlled environments, while this project examined this on samples grown in soil, in a natural environment.

3.5.1 Nickel uptake and accumulation

Results pertaining to the addition of His to plants differ widely within plants. Exogenous application of His to the hyperaccumulator *Thlaspi caerulescens* led to an increase of xylem loading of the metal, counteracting Ni accumulation within roots and reducing Ni sequestration here (Richau *et al*, 2009). This result is in contrast with the finding for hyperaccumulator *A. lesbiacum*, for which exogenous His had no effect on xylem loading of Ni (Kramer *et al*, 1996). Ni xylem loading with addition of exogenous His was later reported for two of eight hyperaccumulators of the *Alyssum* genus, *A. pintodasilvae* and *A. obovatum*, while the other six plants did not experience the accumulation effect. This result led to the conclusion that responses to exogenous His are plant-specific, with differences observed even amongst closely related plants within the same family (Seregin *et al*, 2019). The response of *B. coddii* to exogenous His is unique to this plant.

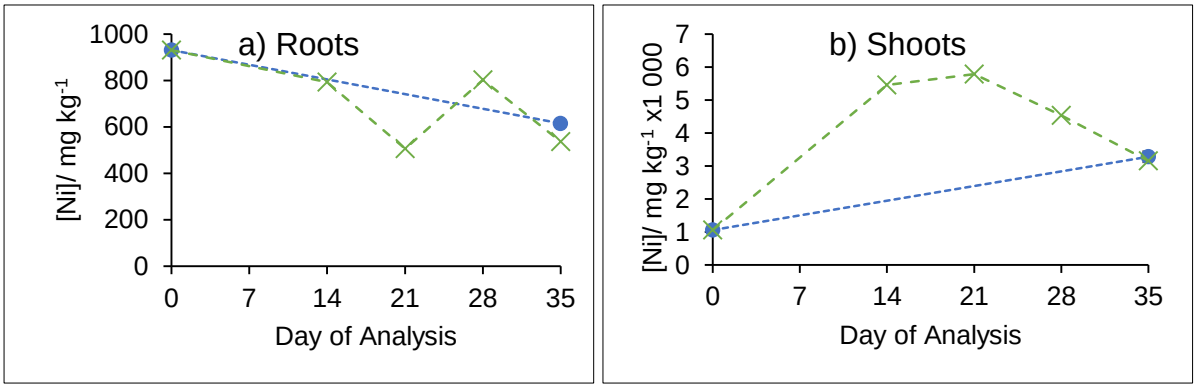

Figure 3.8 Nickel uptake and accumulation within a) roots and b) shoots of B. coddii plants grown in histidine-enriched serpentine soil (green), and non-amended soil (blue)

3.5.1.1 Roots

The concentration of Ni within roots for His-enriched plants (714 ± 184 mg kg^{-1}) is similar to the control concentration (772 ± 223 mg kg^{-1}) suggesting This suggests that addition of His to the soil matrix has no significant effect on Ni (II) uptake or sequestration thereof within plant roots.

3.5.1.2 Shoots

Contrary to roots, accumulation of Ni is observed in the shoots (4 001 ± 1 935 mg kg^{-1}) in concentrations significantly above control plants (2 218 ± 1 638 mg kg^{-1}). This indicates that Ni transport from roots into shoots is facilitated by the addition of His to the soil. The mechanism of this is likely related to the promotion of xylem loading of Ni caused by an increased root His pool, similar to *T. caerulescens* (Richau *et al*, 2009). Further investigation into the exact mechanism which *B. coddii* uses for xylem loading of Ni is required to confirm this preliminary supposition.

Ni accumulation is highest for the first 3 weeks of spiking, compared to all other soil amendments (Figure 3.8b). Thereafter, the concentration of Ni is reduced until day 35, when Ni concentration of shoots (3 157 mg kg^{-1}) is once again within a similar range as for control plants. This corresponds with the spike in plant biomass observed (Figure 3.1), when Ni transport and storage paths may be in developing further.

3.5.2 Effects on amino acid biosynthesis

3.5.2.1 Roots

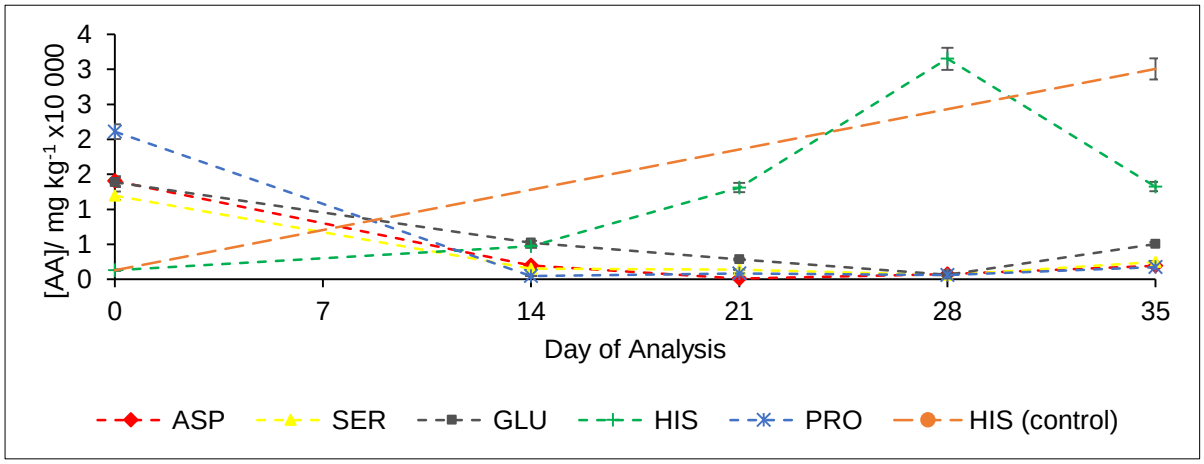

Figure 3.9 Concentrations of monitored amino acids within roots of B. coddii plants grown in histidine-amended serpentine soil (control histidine concentration gradient is added)

Accumulation of His is comparable to the control for the first 3 weeks of analysis (Figure 3.9). It accumulates within roots thereafter, from 13 105 mg kg^{-1} on day 21, to 31 522 mg kg^{-1} on day 28. This concentration is quickly reduced back to 13 235 mg kg^{-1}, indicating that a cyclic

trend is observed. Interestingly, concentrations of other amino acids are suppressed compared to the control. (Appendix Table C2). This finding indicates that the high metabolic cost of His biosynthesis within plant roots has a negative effect on biosynthesis of other amino acids within *B. coddii*. His biosynthesis has a dependence on the amino acids glutamate, from which it is synthesised, and aspartate, which acts as a N donor (Ingle, 2011).

3.5.2.2 Shoots

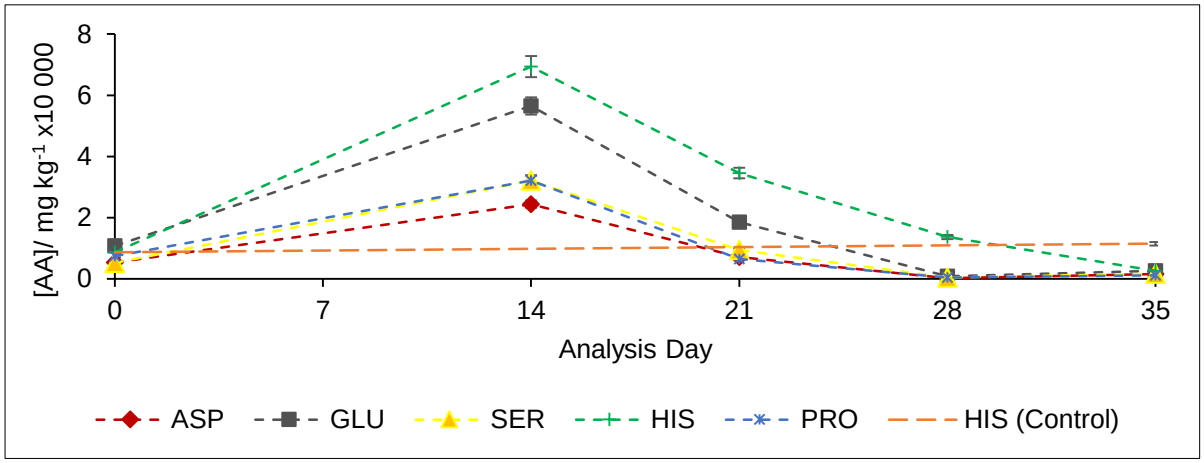

Figure 3.10 Concentrations of monitored amino acids within shoots of B. coddii plants grown in histidine-amended serpentine soil (control histidine concentration gradient is added)

A significant increase of His (8 666 mg kg^{-1} – 69 366 mg kg^{-1}) is observed in shoots up to day 14 (Figure 3.10), indicating rapid translocation into shoots. A mass translocation of amino acids from roots to shoots of plants occurs on day 14 of analysis. Thereafter, concentrations of the AAs within plant shoots drop to values which are comparable to the control. Amino acids are most likely metabolised to aid in the growth and development of plant shoots, as a corresponding biomass increases occurs after this time(Baqir *et al*, 2019).

3.5.3 Effects on organic acid accumulation

His accumulation results in a unique biosynthesis and accumulation pattern for organic acids within *B. coddii* plants.

3.5.3.1 Roots

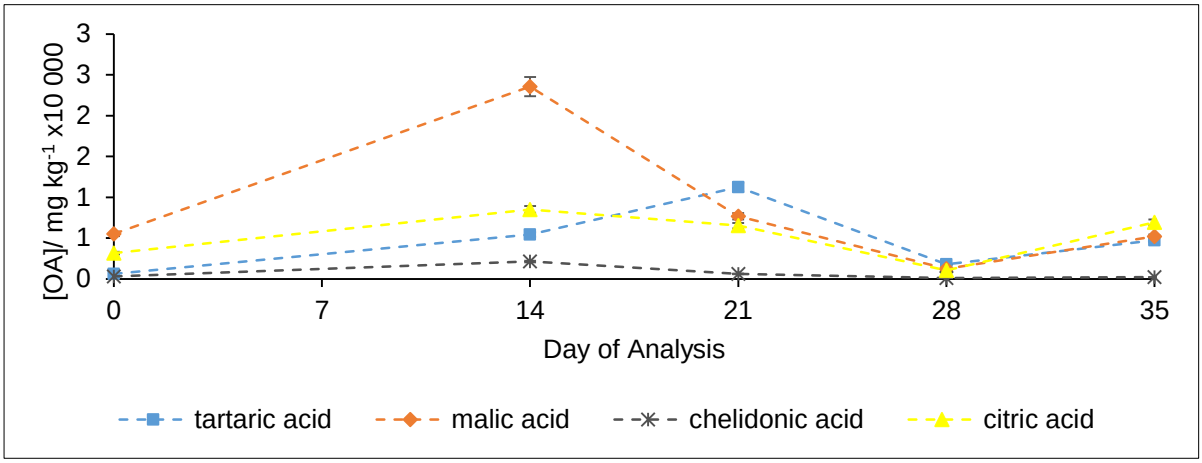

Figure 3.11 Concentrations of OAs within roots of B. coddii plants spiked with histidine

On day 14 of the experiment, a spike in the concentration of malic acid (from 5 516 mg kg^{-1} to 23 549 mg kg^{-1}) and ChA (from 625 mg kg^{-1} to 2 139 mg kg^{-1}) is observed within roots of *B. coddii* These organic acids are likely biosynthesised in response to a pH change within the root environment as a result of the soil enrichment. Outside of these two anomalous peaks, concentrations of OAs within roots of His enriched plants are comparable with control samples (Figure 3.11). Thus, the accumulation cycles of OAs are minimally affected by this soil amendment.

3.5.3.2 Shoots

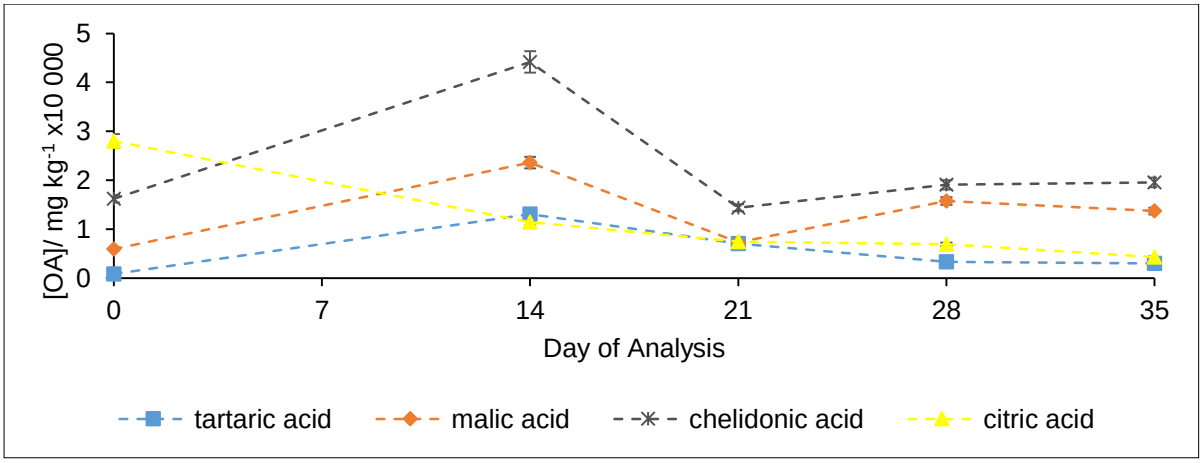

Figure 3.12 Concentrations of OAs within shoots of B. coddii plants spiked with histidine

Citric acid within plant shoots decreases steadily over the experimental study (Figure 3.12), indicating a disruption to the Krebs cycle at this point. This is likely due to the accumulation of His in-plant shoots, a product of the Krebs cycle, particularly the “citrate valve” mechanism, from which His precursor glutamate is synthesised (Igamberdiev and Eprintsev, 2016). Malic

acid concentration spikes on day 14 of analysis, likely in response to the suppression of citric acid production, since malic acid is a precursor for citric acid production by the Krebs cycle. ChA is the most concentrated of the OAs within plant shoots throughout the experiment (Figure 3.12). ChA biosynthesis is increased in *B. coddii* on accumulation of Ni, as observed in previous studies (Naicker, 2014; Robinson *et al*, 2003; Pillay, 2006). Thus, accumulation of ChA within plant shoots appears to be in response to the Ni accumulation observed when plants are spiked with His (section 3.5.1.2).

3.5.4 Nickel Chelation

Table 3.8 Molar ratio of Ni to Ni-binding organic molecules within shotos of B. coddii plants grown in histidine enriched soil

	Day of Analysis	Biomass (mg)	Molar Ratio to Ni			
			Malic acid	Chelidonic acid	Citric Acid	Histidine
Histidine enrichment	0	9	2.5	4.9	8.1	3.1
	14	61	1.9	2.6	0.6	4.8
	21	590	0.6	0.8	0.4	2.3
	28	1112	1.5	1.3	0.5	1.1
	35	438	1.9	2.0	0.4	0.3

Analysis of the molar ratios of Ni-binding organic molecules indicates that a Ni chelator is, for this soil enrichment, indiscernible (Table 3.8). It is likely that the role of His as Ni-transport molecule results in an accumulation of the metal within plant stems, affecting the molar ratio within shoots. This would, in turn, affect the ability of one to divulge which of the potential Ni-binding ligands is involved in storage of the metal.

3.6 Chelidonic Acid-Enriched *B. coddii*

3.6.1 Nickel uptake and accumulation

The concentration of Ni within roots and shoots for plants grown in ChA-amended soil is 737 ± 487 mg kg^{-1}, and 2 278 ± 1 336 mg kg^{-1}, respectively. Both concentrations are not significantly different from the concentration of the metal within the control (772 ± 223 mg kg^{-1} in roots, 2 172 ± 1 572 mg kg^{-1} mg kg^{-1}), indicating that Ni accumulation within *B. coddii* is not overtly affected by this soil amendment.

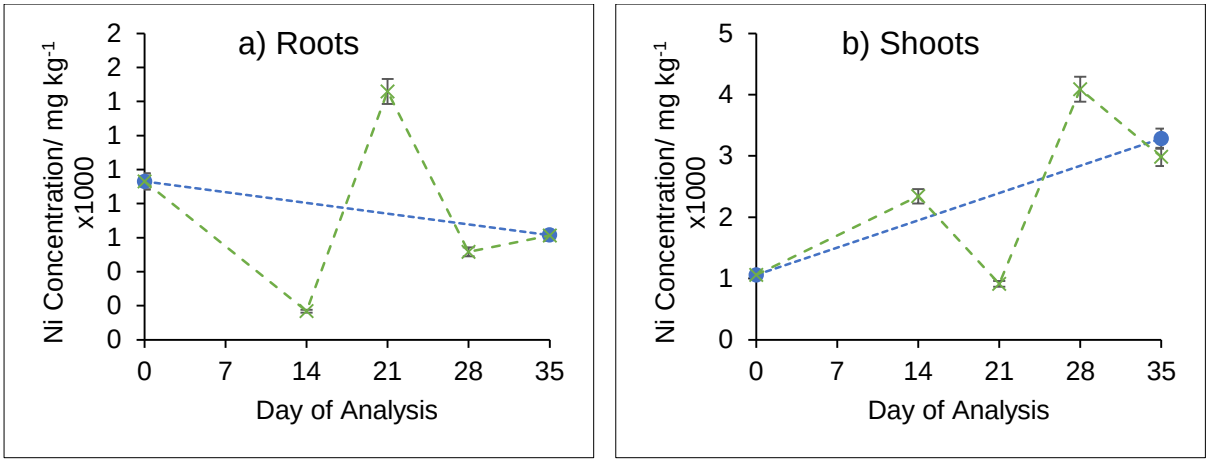

Figure 3.13 Nickel concentrations in a) roots and b) shoots of B. coddii plants grown in chelidonic acid enriched serpentine soil (green), compared to control samples (blue)

3.6.1.1 Roots

Robinson *et al* (1997) found that addition of a similarly sized OA, citric acid (192.12 g mol^{-1}), to the soil matrix of *B. coddii* resulted in a decrease in Ni accumulation within plant matter, attributed to rhizospheric competition with Ni-binding agents released naturally by plants. Both the stable aromatic ring in ChA and the additional hydroxyl bonding site in citric acid (Figure 1.1) are both indications that Ni will bind more strongly to the latter. It is likely, then, that Ni-binding root exudates form stronger bonds with the metal than ChA, rendering the effect of this OA inconsequential to *B. coddii* for Ni uptake and accumulation within aerial regions at this concentration.

3.6.1.2 Shoots

Accumulation of Ni is observed to occur by a unique cyclic trend, indicating that an open transport path may be elicited by this soil enrichment. This does not appear to have any effect on accumulation capacity.

3.6.2 Effects on amino acid biosynthesis

3.6.2.1 Roots

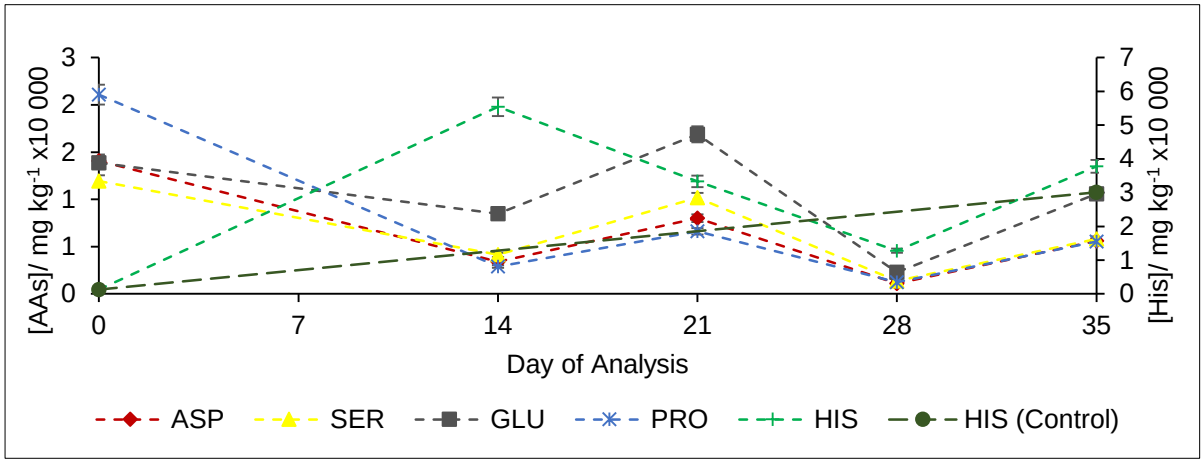

Figure 3.14 Concentrations of monitored amino acids within roots of B. coddii plants grown in chelidonic acid-amended serpentine soil

Amino acid accumulation within plant roots is, generally, suppressed by the addition of ChA to the soil medium, as concentrations are below control concentration from day 14 to day 35 for almost all AAs, with the exception of His, glycine and tyrosine (Appendix Table C2).

Tyrosine, synthesised along with phenylalanine by the shikimate pathway, is a proteinogenic aromatic amino acid instrumental to the production of secondary metabolites involved in plant responses to both biotic and abiotic stresses, such as drought, pests, and diseases (Hudson, 2015). Glycine, which is produced from a serine intermediate, accumulates within belowground samples rather than being depleted as it is in control plants (Appendix Table C2). Glycine is the smallest amino acid and, as such, creates the smallest AA chelates, which are transported readily through plant tissue. In plant roots, glycine accumulation stimulates opening of Ca ion channels (DeVille, 2018). Thus, accumulation of glycine within roots indicates a nutrient deficiency within the plant, likely Ca, as the soil is acidified and this and other essential nutrients leached out (Haynes and Swift, 1986).

His concentration spikes over the first two weeks of analysis, from 1 300 mg kg^{-1} to 55 411 mg kg^{-1} (Figure 3.14). It is inferred that this occurs in response to the nutrient imbalance within plant roots as a result of soil acidification associated with the ChA soil amendment, and the increase of Ni storage capacity within plant roots a consequence of this.

3.6.2.2 Shoots

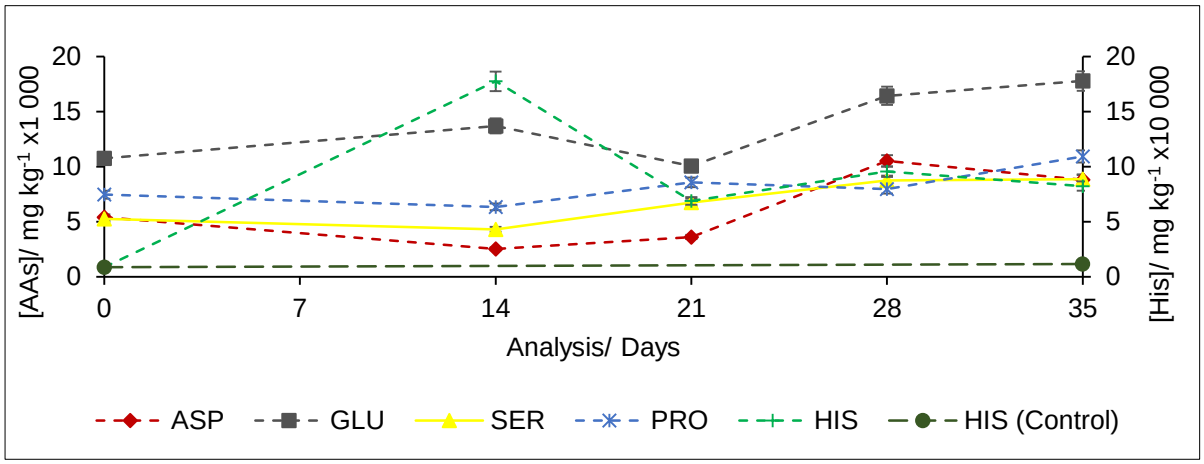

Figure 3.15 Concentrations of monitored amino acids within shoots of B. coddii plants grown in chelidonic acid-amended serpentine soil

All amino acids accumulate to concentrations two to four times higher than the control samples (Figure 3.15). Accumulation of monitored amino acids aspartate, glutamate, Pro and serine is relatively consistent over the five weeks. This is likely in response to the nutrient deficiency observed, as amino acids increase absorption and use efficiency of essential minerals (Baqir *et al*, 2019). The concentration of His is ten-fold that of any other amino acid. A concentration spike occurs on day 14, indicating an extreme response of plants to the ChA-enrichment. His concentration regulates thereafter, as is observed in plant roots. This further indicates that ChA addition to plants produces an abiotic stress response within plant tissue.

3.6.3 Effects on organic acid biosynthesis

3.6.3.1 Roots

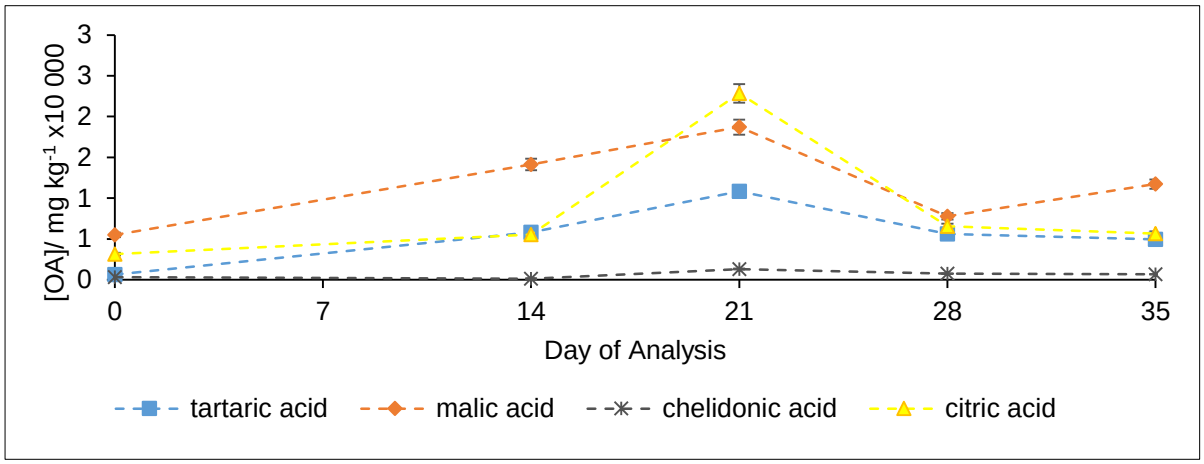

Figure 3.16 Organic acids within roots of B. coddii plants grown in chelidonic acid enriched serpentine soil

Malic, citric and tartaric acids accumulate over the first three weeks of analysis (Figure 3.16). The main function of these acids within roots is as exudates for the solubilisation of important plant nutrients (Lopez-Bucio *et al*, 2000). Thus, accumulation of these organic acids within plant roots is likely related to a nutrient deficiency within *B. coddii* plants, e.g. Ca, as observed with the accumulation of the amino acid glycine. Concentrations of these organic acids become comparable to control plant concentrations from day 28, coinciding with the normalisation of plant biomass within roots. The concentration of ChA (644 mg kg^{-1}) is comparable to that of the control indicating that addition of ChA to the soil does not affect concentrations of ChA in the roots. Further, when bioavailable, ChA is not specifically taken up by *B. coddii*.

3.6.3.2 Shoots

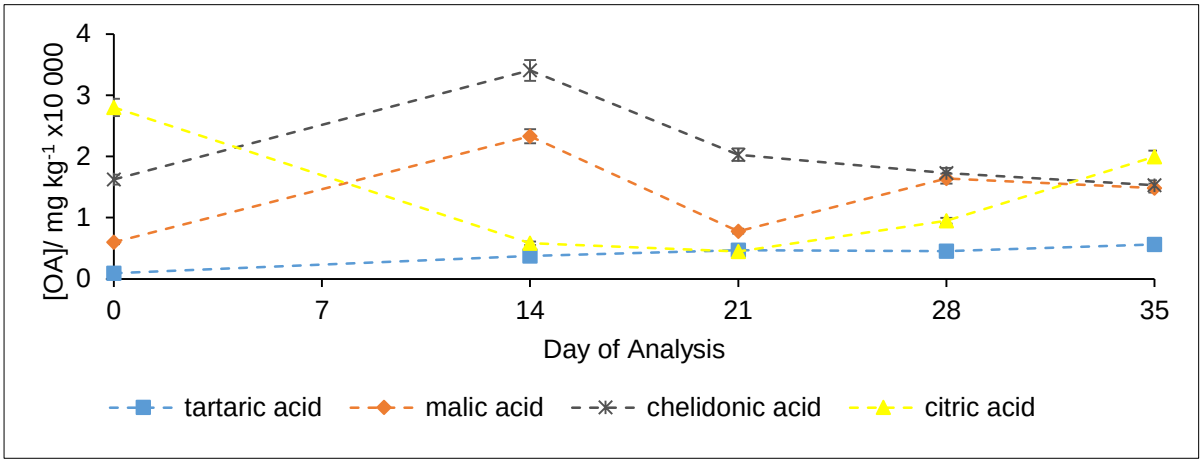

Figure 3.17 Organic acids within shoots of B. coddii plants grown in chelidonic acid enriched serpentine soil

ChA concentration peaks on day 14 (34 057 mg kg^{-1}) of the analysis (Figure 3.17). This is likely a stress response of the plant, as increased root exudates will solubilise all nutrients within the soil, including metals. The concentration of ChA within shoots decreases over the last three weeks of analysis (20 282 mg kg^{-1} - 15 320 mg kg^{-1}), becoming comparable to the average concentration within control plants (16 134 mg kg^{-1}). An opposite response is observed with regard to citric acid. Up to day 21 of analysis, citric acid is supressed within plant shoots, indicating a disruption to the accumulation of this organic acid within plant shoots and the Krebs cycle, as a result of the spike in His concentration (Igamberdiev and Eprintsev, 2016). The concentration of this OA increases again over the last two weeks of analysis, coinciding with the normalisation of shoot biomass and His biosynthesis within *B. coddii* plants. Malic acid biosynthesis and metabolism is same for ChA enriched plants as for His enriched plants, spiking on day 14 and then normalising thereafter.

3.6.4 Nickel Chelation

Table 3.9 Molar ratio of Ni to Ni-binding organic molecules within shoots of B. coddii plants grown in chelidonic acid enriched soil

	Day of Analysis	Biomass (mg)	Molar Ratio to Ni			
			Malic acid	Chelidonic acid	Citric Acid	Histidine
Chelidonic acid enrichment	0	9	2.5	4.9	8.1	3.1
	14	107	4.4	4.6	0.8	28.7
	21	48	3.7	7.1	1.5	28.6
	28	751	1.8	1.3	0.7	8.8
	35	585	2.2	1.6	2.0	10.4

Ni accumulation was not observed overall, but a unique cyclic accumulation trend occurred (section 3.6.1). This is reflected in the molar ratios of OAs to Ni (Table 3.9). As with the control samples, Ni chelation is likely divided between organic ligands as development of plants is prioritized, while His is biosynthesised in large concentrations to combat the proposed nutrient deficiency.

3.7 Nickel and Chelidonic Acid Co-Enriched *B. coddii*

The co-enrichment applied consisted of simultaneous addition of 1 000 mg kg^{-1} each of ChA and Ni - 27 mM and 85 mM total, respectively - to the soil matrix.

3.7.1 Nickel uptake and accumulation

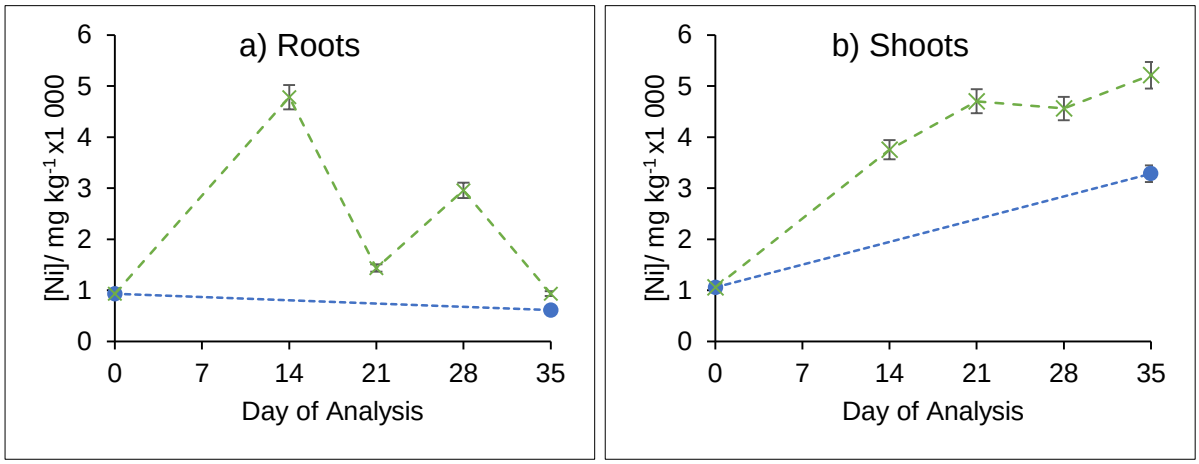

Figure 3.18 Nickel uptake and accumulation within a) roots and b) shoots of B. coddii plants grown in Ni(II) and chelidonic acid co-enriched soil (green) and control plants (blue)

3.7.1.1 Roots

The highest single concentration (4 780 mg kg^{-1}, on day 14), as well as the largest average concentration and standard deviation (2 207 mg kg^{-1} ± 1 661 mg kg^{-1}), were recorded for this co-enrichment. Thus, the cycle of Ni uptake into roots and subsequent transport to shoots is more pronounced for this soil enrichment than for any other. The ChA_2Ni complex has a strong covalent interaction between Ni and the oxygen contained within the aromatic ring of the ligand (Belian *et al*, 2014). Thus, the significantly higher concentration of Ni observed in roots during the two uptake cycles observed is likely affected by breaking of the ChA_2Ni complex by stronger ligands, such as OA root exudates malate and citrate (Verbruggen *et al*, 2009). This would result in a higher concentration of labile Ni available for uptake at root metal uptake sites. The second Ni uptake cycle into plant roots has a smaller peak than the first observed cycle (Figure 3.18). This difference is due to the significantly smaller biomass of plant roots on days 28 and 35, compared to the first three weeks of analysis (Appendix Table C1). This

reduced biomass is likely also an effect of increased labile Ni close to plant roots, as it is similar to the effect of addition of 10 000 mg kg-1 Ni observed above.

3.7.1.2 Shoots

For the first 3 weeks of the uptake study, Ni accumulation is comparable to accumulation when the plant is spiked with 10 times the concentration of Ni, and is significantly larger than for control plants. This accelerated transport from roots to shoots is likely a direct response to the high concentration of Ni taken into the plant roots, as ChA addition to plant roots was shown to have no significant effect on Ni accumulation. Over the last two weeks of the study the concentration of Ni within aboveground tissue appears to stabilise, coinciding with a smaller root-Ni uptake, linked to depletion of root biomass. It is likely that continued exposure to the $NiChA_2$ complex would eventually begin to die as plant root biomass diminishes.

3.7.2 Effects on amino acid biosynthesis

3.7.2.1 Roots

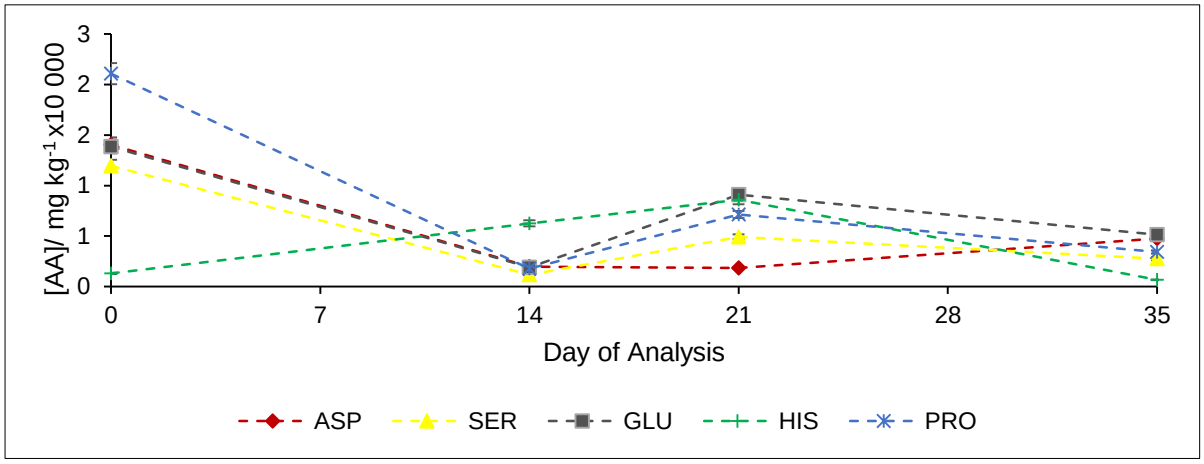

Figure 3.19 Amino acids within roots of B. coddi plants grown in Ni and chelidonic acid co-enriched soil

Similarly, to the Ni soil enrichment, an overall reduction in amino acid concentration is observed, compared to control plants (Figure 3.19, Appendix Table C2). This is likely due to amino acid translocation from roots to shoots in response to the abiotic stressor, Ni.

3.7.2.2 Shoots

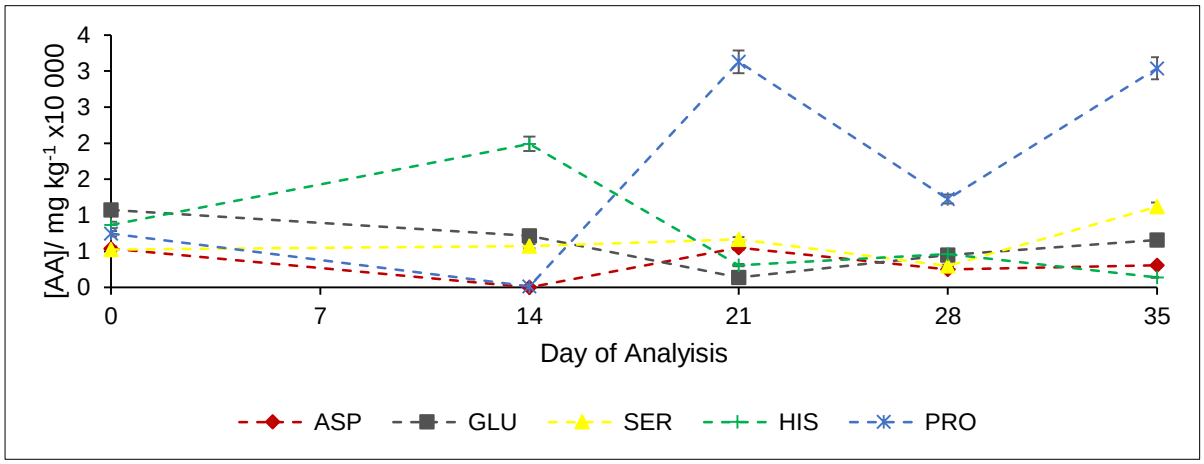

Figure 3.20 Amino acids within shoots of B. coddii plants grown in Ni(II) and chelidonic acid enriched soil

A clear stress response is observed, evidenced by the extreme accumulation of Pro within samples after 14 days (Figure 3.20). Atypical accumulation of Pro was observed for plants spiked with both the ChA-enrichment and for the Ni-enrichment, accredited above to a nutrient deficiency caused by soil acidification and leaching, and metal accumulation, respectively. Thus, the stress response of plants spiked with the Ni-ChA complex cannot definitively be attributed to either one of these ions. It is likely, rather, a combined response to the negative effects on plant development at the small biomasses of plants for the current study.

3.7.3 Effects on organic acid biosynthesis

3.7.3.1 Roots

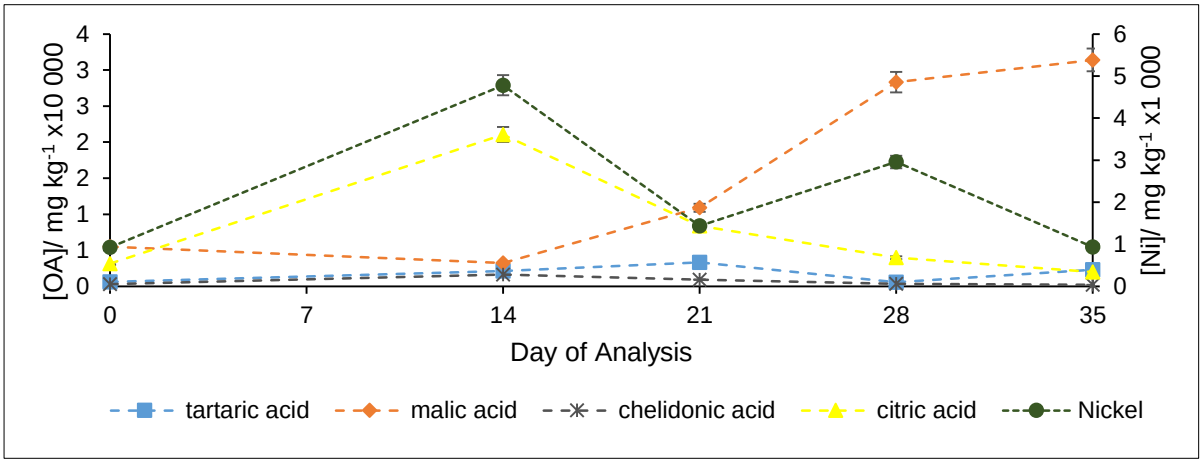

Figure 3.21 Organic acids within roots of B. coddii plants grown in Ni(II) and chelidonic acid co-enriched soil

Accumulation of ChA has been shown to remain un-affected by the presence of additional ChA within the soil matrix, for the ChA enrichment (Figure 3.21). A similar result is observed for the Ni and ChA co-enrichment, with average ChA concentration (689 mg kg^{-1}) for co-enriched plants similar to control samples (608 mg kg^{-1}). The concentration of malic acid increases steadily over the course of the experiment, indicating that, similar to plants spiked with ChA alone, a stress response is activated by this soil amendment. Malic acid is accumulated within plant roots to maintain cytoplasmic pH in a process known as “malate pH stat” (Bolan and Hedley, 2003). Citric acid concentration spikes on day 14 of analysis, but drops in the weeks thereafter. This is likely a direct result of malic acid accumulation, which disrupts the Krebs cycle and, thus, the formation of citric acid.

3.7.3.2 Shoots

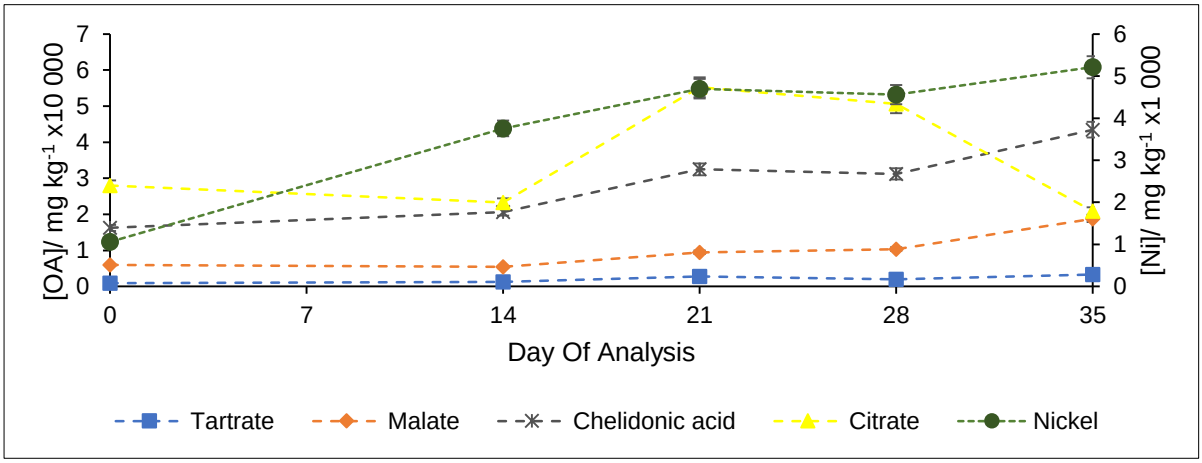

Figure 3.22 Organic acids within roots of B. coddii plants grown in Ni(II) and chelidonic acid co-enriched soil

Biosynthesis of malic acid, tartaric acid and ChA is relatively linear and increasing (Figure 3.22). On day 35, the concentrations of both malic acid (18 971 mg kg^{-1}) and tartaric acid (3 284 mg kg^{-1}) are similar to control plants (19 463 mg kg^{-1} and 1 832 mg kg^{-1} each). Thus, biosynthesis of these organic acids is related to plant growth and development, and not to Ni accumulation or to the observed stress response of plants.

Citric acid biosynthesis is disparate to the other organic acids. The two-fold spike in concentration from day 14 to day 21 (23 271 – 55 235 mg kg^{-1}), and the subsequent metabolism of citric acid from day 28 to day 35 (50 619 – 20 927 mg kg^{-1}), both indicate a significant disruption to the Krebs cycle of plants, likely related to the nutrient deficiency observed within plant roots.

3.7.4 Nickel Chelation

Table 3.10 Organic acids within roots of B. coddii plants grown in Ni(II) and chelidonic acid co-enriched soil

Day of Analysis	Biomass (mg)	Molar Ratio to Ni (II)			
		Malic acid	Chelidonic acid	Citric Acid	Histidine
0	9	2.5	4.9	8.1	3.1
14	38	0.6	1.8	1.9	2.0
21	67	0.9	2.2	3.6	0.2
28	69	1.0	2.2	3.4	0.4
35	44	1.6	2.7	1.2	0.1

The molar ratio of malic acid to Ni is not consistently large enough for this OA to be the ligand which binds Ni. This indicates that, the amendment causes a disruption to the ligands binding

Ni. The accumulation gradient of ChA within plant shoots corresponds to Ni accumulation for this soil enrichment (Table 3.10). Thus, at this plant biomass, enough ChA is biosynthesised to accommodate the rate of Ni accumulation which occurs when plants are grown in Ni and ChA co-enriched soil.

3.8 Chapter Conclusions

Four soil enrichments were applied to the Ni hyperaccumulator *B. coddii*: Ni (II), ChA, His and Ni with ChA as a co-enrichment. The effects that each of these soil enrichments had on Ni uptake and accumulation, organic acids and amino acids within the plants were analysed.

Ni uptake from the soil occurs in a cyclic pattern for all of the soil amendments made (Appendix Table C1). This may suggest that the metal accumulates first within roots of *B. coddii* and then, at some maximum concentration, it is transported into plant leaves.

The growth and development of plants was most affected by addition of bioavailable Ni to the soil. For both soil enrichments containing Ni, root biomass was sequentially diminished, indicating necrosis.

For the Ni soil enrichment, shoot biomass was also diminished in the last week of the study, a physical indication that plants were dying. This corresponded with a spike in amino acid biosynthesis, indicating a severe stress response. Analysis of molar ratios of organic acids to the metal also indicated that insufficient concentrations of all three possible Ni-binding organic acids (malate, citrate and ChA) were contained within plant shoots. This was likely the cause of the diminished shoot biomass, as free Ni is toxic to plants above millimolar concentrations.

The His soil enrichment produced a significant increase in Ni concentration within plant shoots, while root concentrations were observed to be similar to control samples. This indicated that additional His facilitates Ni transport from roots to shoots. Analysis of the molar ratios of Ni to organic molecules presented a unique erratic trend. This indicated that the Ni-His molecules were likely maintained as such within plant stems, disallowing for a distinct Ni-binding molecule to be determined.

Plants grown in ChA enriched soil had smaller, duller leaves than His-enriched plants. This was found to be the result of a nutrient deficiency, likely Ca. Ni accumulation was similar to control samples, and malic acid the most likely Ni-binding ligand as well.

The Ni and ChA co-enrichment was found to yield results similar to those of the prior enrichments containing only one of each. The Ni accumulation capacity of plant roots was increased, due to complexation of Ni by ChA. This also contributed to the diminished root biomass observed. Plants appeared better able to contain the heavy metal within shoots as well, with ChA consistently in sufficient concentration to bind Ni here, as previously seen on addition of bioavailable Ni to soil (Naicker *et al*, 2016; Pillay, 2006).

CHAPTER 4

Results and Discussion: Sunflowers

4.1 Introduction

Sunflower plants were grown in uncontaminated soil, soil spiked with ChA, His, or Ni. The effects that each soil enrichment had on growth and development, Ni uptake and accumulation, and biosynthesis of organic molecules (amino acids and organic acids) were monitored. These responses were compared to a control sample batch.

Physical responses of plants and sample dry weights indicate the effect of each soil amendment on the growth and development of the plants, a key factor in the determination of heavy metal accumulation capacity of the plant. In some batches, plants grew to their reproductive stages, whilst others remained within their vegetative stages throughout the study (Appendix Table D1).

It is observed that the amino acids Asp, Ser and Glu are precursors to the synthesis of almost all of the heavy metal metabolite amino acids synthesised within plants (Figure 1.2). The results pertaining to amino acid responses are thus based on these three amino acids, as well as His and proline for their large contribution to heavy metal stress tolerance.

Six organic acids (ascorbic acid, citric acid, fumaric acid, malic acid, oxalic acid, and tartaric acid) linked to metal accumulation and detoxification, were monitored in the present study.. Of these organic acids, three consistently accumulate to significantly large concentrations: fumaric acid, malic acid and citric acid (Appendix Table D3).

4.2 Effect of Soil Enrichments on Growth and Development of Sunflowers

Figure 4.1 Sunflower plants a) on day 0, prior to commencement of spiking, and b) on day 25, after plants underwent their seventh (of nine) soil amendment. Plant batches are labelled as follows: 1) Ni spiked; 2) ChA spiked; 3) Histidine spiked; and 4) control batch

Plant biomasses of each batch of plants over the 5-week experimental study were monitored. There were differences in plant health for the different amendments over the 35-day period.

Table 4.1 Sunflower plant dry biomass of roots and shoots (stems and leaves)

	Analysis Day	Dry Biomass (g)			
		Control	ChA Spiked	His Spiked	Ni Spiked
Roots	7	0.27	0.49	0.43	1.54
	14	1.06	0.94	1.41	3.52
	21	2.17	1.95	4.81	3.00
	28	0.43	0.75	8.91	1.08
	35	4.62	3.98	8.76	4.76
Shoots	7	1.31	1.26	2.21	3.54
	14	5.99	4.90	4.91	3.81
	21	4.04	4.51	6.71	5.77
	28	4.81	5.84	7.58	5.40
	35	9.87	7.82	10.80	10.95

Of the amendments, the addition of ChA to the soil appeared to have the least effect on plant growth and is most comparable to the control in appearance and biomass (Figure 4.1,Table 4.1).

His amendments resulted in plants which were taller than all other plant batches, with healthier, larger leaves (Figure 4.1b). The below ground biomass is the most obvious indication of this difference, with a day 35 sample mass (8.76 g) which is almost double the other samples (Table 4.1). This enhanced growth may be linked to the natural role of His as one of the key amino acids responsible for growth and development in plants (Ingle, 2011).

Ni soil amendments resulted in leaf dehydration, observed by wilting leaves (Figure 4.1b). The resilience of sunflower plants was observed clearly, as leaves regained sturdiness within days of being spiked with Ni, throughout the experiment. Thus, a similar dry mass was observed for Ni-spiked, ChA-spiked, and control plants (Table 4.1). This indicates that a non-phytotoxic concentration of the metal was applied. It is assumed, in fact, that the plants would have proceeded to grow in these conditions to full age, as observed for sunflowers grown in soil irrigated significantly higher Ni concentrations (Stoikou *et al*, 2017).

4.3 Nickel Uptake and Accumulation

4.3.1 Control Samples

The overall concentration of Ni within sunflowers ranged from 6 to 13 mg kg^{-1} (Appendix Table D1), and is within in the range for non-accumulator plants (1 – 10 mg kg^{-1} on average) (Bhalerao *et al*, 2015).

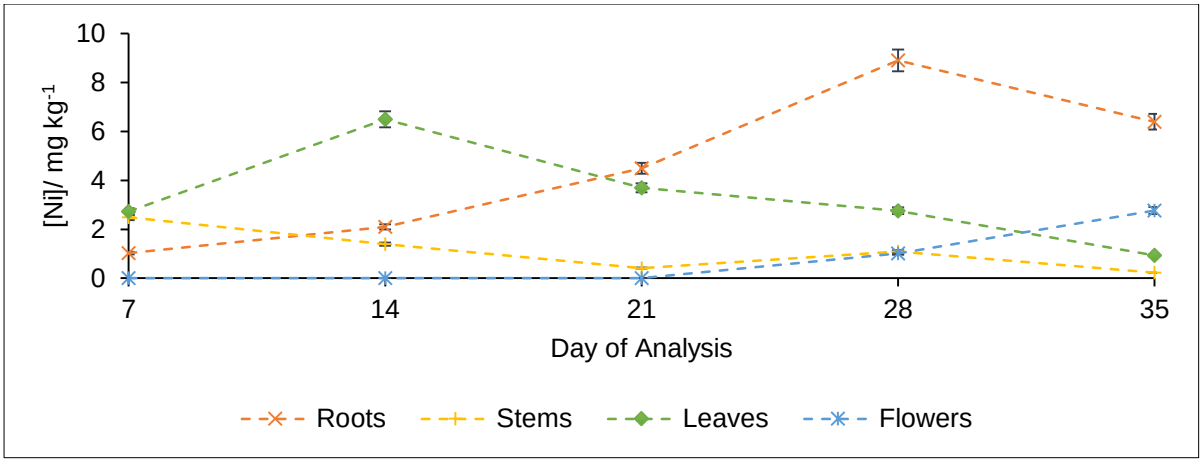

Figure 4.2 Concentrations of Ni(II) within control samples of sunflowers

Ni in plant roots, stems and leaves appears to be controlled by plant development. Translocation of the metal is related to development of blossoms, observed by the reduction in root, stem and leaf concentrations during reproductive stages (Figure 4.2). This is likely due to the requirement of the metal for the enzyme urease, a highly active form of which is present within plant seeds (Fabiano *et al*, 2015).

4.3.2 Nickel Enrichment

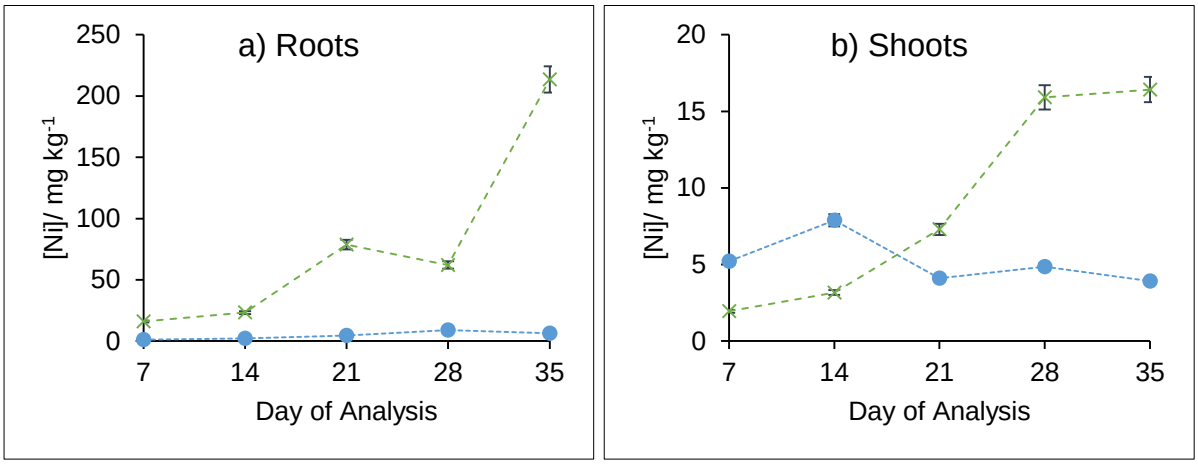

Figure 4.3 Ni within a) roots and b) shoots of Ni enriched sunflowers. Ni enriched (green); control (blue)

Additional bioavailable Ni resulted in enhanced uptake of the metal, with final concentrations significantly above the control (Figure 4.3a). Thereafter, root accumulation of Ni increased significantly over the five-week study.

Little Ni bioaccumulation is observed within shoots of plants, compared to roots, over the experimental period (Figure 4.3b). A spike in stem concentration is observed between days 14 and 21 of analysis, from 1.2 mg kg^{-1} to 6.2 mg kg^{-1}, corresponding to an increase in stem biomass (Table 4.2). This marks a significant alteration to the capacity for plants to translocate

the metal from roots to shoots. Ni is consistently more concentrated within stems thereafter, and spikes significantly within leaves, from 1.1 mg kg^{-1} on day 21 to 12.6 mg kg^{-1} on day 28 (Appendix Table D1). This is likely done to reduce toxicity within roots and provide a more natural root environment stimulating further growth and development. The translocation mechanism in plants is often a mechanism of detoxification by chelation of heavy metals within sites which are inactive (Salt *et al*, 1998). Similar mechanisms of metal control are noted in other heavy metal accumulators such as buckwheat, for which Al detoxified by chelation to oxalate in leaves and roots of buckwheat (Ma *et al*, 1997). The primary storage site of additional Ni is clearly within plant roots, a trend observed in similar previous studies (Belhaj *et al*, 2016; Eissa *et al*, 2014; Kacálková *et al*, 2014; Stoikou *et al*, 2017).

4.3.3 Histidine Enrichment

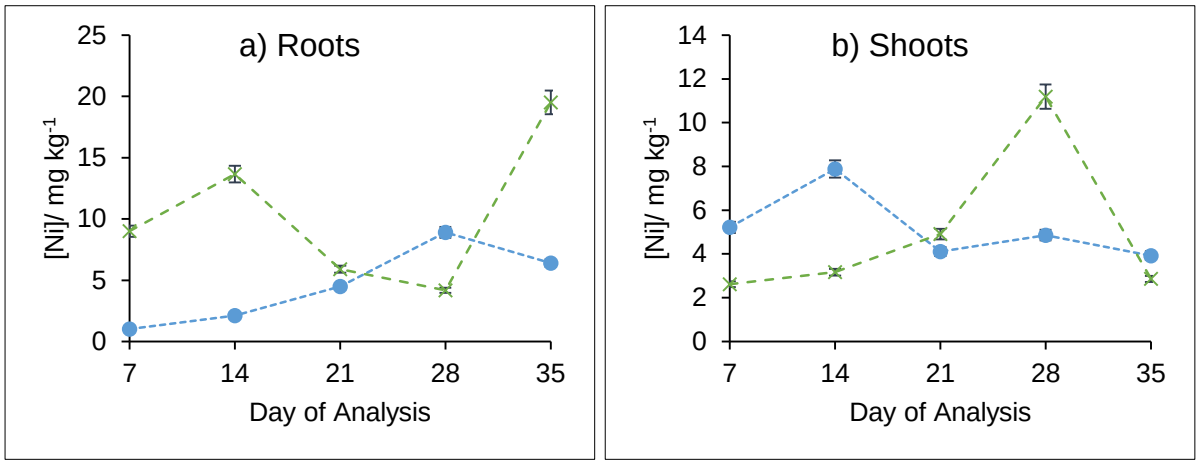

Figure 4.4 Ni accumulation within a) roots and b) shoots of histidine enriched sunflowers. His enriched (green); control (blue)

Spiking soil with His resulted in a cyclic uptake of Ni into roots (Figure 4.4a), increasing the average concentration to 10.5 mg kg^{-1}. The presence of His in the soil appears to facilitate Ni uptake into roots. Translocation of Ni into stems and leaves was not enhanced by addition of His and remained low (Appendix Table D1). The spike in Ni concentration occurring within plant leaves on day 28 (Figure 4.4b, 11.2 mg kg^{-1}) is likely related to the development of flowers, for which Ni plays an essential role in the active enzyme urease (Fabiano *et al*, 2015).

4.3.3 Chelidonic Acid Enrichment

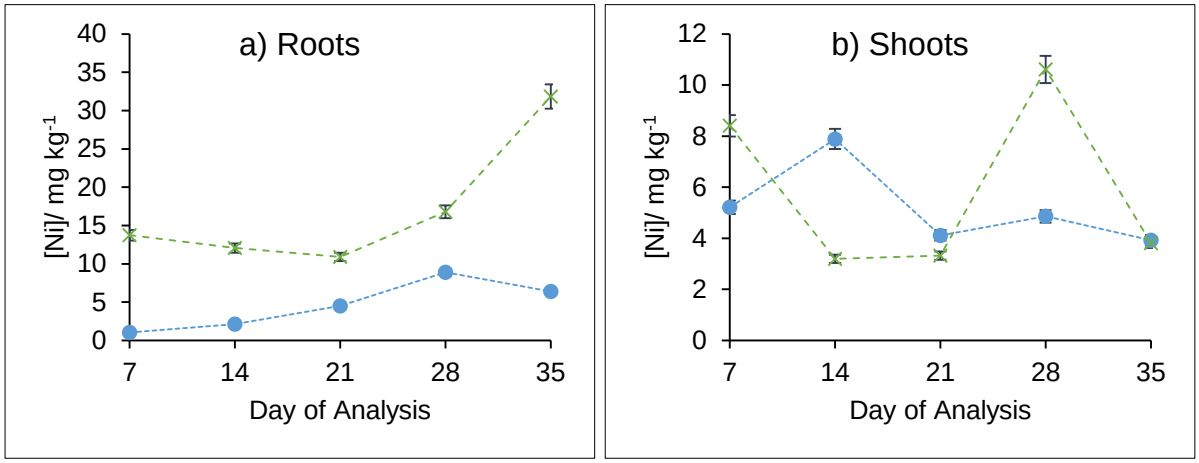

Figure 4.5 Ni accumulation within a) roots and b) shoots of ChA enriched sunflowers. ChA enriched (green); Control (blue)

A consistent increase in Ni accumulation is observed with addition of ChA to the soil, with day 35 concentration of the metal (31.8 mg kg^{-1}), significantly larger than control samples 6.4 mg kg^{-1} (Figure 4.5a). This may be attributed to increased bioavailable Ni within soil, as a result of the soil pH decrease which ChA could cause. Translocation of Ni into plant shoots is facilitated by this soil amendment (Figure 4.5b). This is related to stem growth and development after day 21 of analysis, and may indicate that root to shoot translocation of Ni is dependent on the xylem loading capacity of plants.

4.4 Amino Acid Biosynthesis and Metabolism

In general, AA concentration was found to be highest in plant stems, followed by leaves, and lowest in plant roots (Appendix Table D2). Roots and leaf vacuoles are known storage sites used by plants for metal ion detoxification (Leitenmaier and Küpper, 2013).

Table 4.2 Monitored Amino Acids within roots, stems and leaves of sunflowers.

	Root Concentrations/ mg kg^{-1}																			
	Control					Nickel Enriched					Histidine Enriched					Chelidonic Acid Enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Asp	1419	5552	3131	6850	3863	10960	9737	NA	5527	6221	4830	NA	6676	9909	29148	8449	8230	NA	9909	10173
Glu	853	5514	3159	6530	3740	10581	7334	NA	5566	6307	5553	NA	6336	11027	31686	7480	8878	NA	10880	19773
His	768	2472	506	5644	1155	3448	8871	NA	546	2052	1355	NA	2000	7991	6001	2422	2826	NA	2253	452
Pro	1319	3698	2303	3825	2382	7522	7596	NA	3664	6639	3810	NA	5546	7689	20112	6894	4859	NA	7317	9419
Ser	1345	2633	2191	3124	2599	7116	1806	NA	3757	3288	3401	NA	5253	4990	20252	3398	4916	NA	5502	10522

	Stem Concentrations/ mg kg^{-1}																			
	Control					Nickel Enriched					Histidine Enriched					Chelidonic Acid Enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Asp	19776	7258	6827	21023	NA	2347	1569	15849	4333	4864	31364	5325	7947	6201	1549	9328	6921	3649	9654	2616
Glu	5836	26254	5736	26712	NA	1319	3002	15190	3458	5600	18947	3948	7492	6647	1382	7435	9755	3364	10629	3088
His	2277	3339	1371	5549	NA	123	1885	3498	1042	1854	3926	1114	1923	164	513	756	1656	1102	2016	905
Pro	3678	10113	2985	12462	NA	1465	1786	16466	3588	12366	10985	10776	12983	6682	3360	8291	28539	6103	22810	7636
Ser	1983	10746	2166	9834	NA	415	2640	6684	1511	3259	6925	2301	2982	3316	906	2589	4815	1715	4496	1837

	Leaf Concentrations/ mg kg^{-1}																			
	Control					Nickel Enriched					Histidine Enriched					Chelidonic Acid Enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Asp	3105	4144	12852	10063	5292	3975	21921	8084	16045	5284	4799	3366	19231	4909	6145	10343	10403	NA	11887	11819
Glu	2372	3772	11315	7006	6092	4110	20829	9880	18340	6052	3486	4197	23797	5582	6093	10393	9714	NA	12865	13764
His	788	2196	2656	3023	1929	955	5681	2214	5396	1342	1635	1865	6236	1977	2130	3297	8459	NA	4014	1565
Pro	951	6627	6872	9638	4725	3657	19692	9384	10053	4879	3727	4941	22427	3149	4488	6272	5958	NA	11536	12144
Ser	966	3623	5442	4162	4737	2308	10659	4422	9658	3026	3109	2293	11887	2682	2940	4525	4371	NA	6420	6645

NA – not analysed

4.4.1 Control Samples

Concentrations of amino acids were found to be higher than concentrations for a wide range of food plants (Appendix Table D2), but lower than concentrations within sunflower meal (Kumar *et al*, 2017; Ivanova *et al*, 2013). Glu and Asp concentrations were highest, similar to other food crops (Kumar *et al*, 2017). These acidic amino acids are both important precursors to the synthesis of essential amino acids - Glu in the synthesis of His, and Asp for the synthesis of Lys, Thr, Met and Ile. Heavy metal metabolites Pro and Arg, and the peptide glutathione are also synthesised from Glu (Galili, 2011; Qiu *et al*, 2020).

Concentrations of amino acids within roots increase over the experimental study in a cyclic trend, as biosynthesis, transport and metabolism occur (Table 4.2). A similar trend is observed within plant stems, where concentrations of amino acids are highest due to their essential role in transport of N and other essential nutrients via the xylem and phloem by osmotic pressure or cyclosis (Ohyama *et al*, 2017; Price and Okumoto, 2015). In leaves, concentrations of amino acids increase in a cyclic trend up to day 21 of analysis, during vegetative growth (Table 4.2). Thereafter, concentrations of the acidic precursors, aspartate and glutamate are significantly reduced as these amino acids are transported into developing flower buds. Pro, His and Ser concentrations are not affected as significantly.

4.4.2 Nickel Enrichment

After one week of spiking, concentrations of all five amino acids within plant roots are higher for Ni-enriched plants than for control plants (Table 4.2). This corresponding amino acid response to Ni accumulation indicates a stress response within plant roots (Sharma and Dietz, 2006). His is most affected for this soil enrichment, peaking at 8 871 mg kg^{-1} on day 14 of analysis, and then subsequently dropping overall between day 28 and day 35, as the metal accumulates within plant leaves. This indicates that His is involved in the Ni transport mechanism of sunflowers.

A cyclic trend of AA accumulation is observed within leaves (Table 4.2), unlike the accumulation trend observed within control samples. Peak concentrations for Ni-enriched plants were twice those for control plants, in response to the additional metal stress. Concentrations within leaves do not spike with the influx of Ni observed on day 21 of analysis (section 4.3.2), indicating that they do not play a significant role in Ni sequestration.

4.4.3 Histidine Enrichment

The concentration of His within roots on day 7 (1 355 mg kg^{-1}) is significantly higher than for control plants (768 mg kg^{-1}), indicating that His is taken into roots from the soil. His has been found to play a role in regulation of biosynthesis of other amino acids (Stepansky and Leustek, 2006). This is seen within sunflower plants, as biosynthesis of other amino acids within root

tissue is also increased, improving capability for plants to grow and develop. The AA response mechanism within leaves was similar to control samples, peaking on day 21 of analysis with development of flowers (Table 4.2).

4.4.4 Chelidonic Acid Enrichment

The average concentration of His in roots (1 988 mg kg^{-1}) was comparable to control samples (2 109 mg kg^{-1}). This implies that accumulation of His within plant roots is related more strongly to transport of heavy metals into shoots, as shown for the Ni spike, than it is for sequestration within roots. Concentrations of Asp, Ser, Glu and Pro are at least twice that of control plants on day 7, and consistently increase thereafter (Table 4.2). Various amino acids, including Pro, Glu and Ser, have been shown to have an effect on membrane permeability of plants (Rai, 2002). Thus, additional uptake of an external molecule, such as ChA, may account for accumulation of these amino acids within plant roots. Concentrations of amino acids in leaves were, on average, 3-times larger on day 7 for plants grown in ChA enriched soil (Table 4.2), indicating that a stress response unrelated to Ni uptake and accumulation was induced throughout plant sections. A consistently increasing accumulation trend was observed thereafter for all amino acids except His, which was metabolised (Table 4.2). His biosynthesis is an energy intensive process which may be a hindrance to the stress response observed (Ingle, 2011).

4.5 Organic Acid Biosynthesis and Metabolism

Table 4.3 Monitored organic acids within sunflowers.

Concentration/ mg kg^{-1}									
Plant Section		Roots				Leaves			
Soil Enrichment	Day of Analysis	Chelidonic acid	Citric acid	Fumaric acid	Malic acid	Chelidonic acid	Citric acid	Fumaric acid	Malic acid
No enrichment (control)	7	23	9051	1057	11437	0	5597	10707	11371
	14	0	1462	1964	5583	0	2649	6471	9464
	21	0	12505	2345	7012	NA	NA	NA	NA
	28	0	7162	488	24490	0	307	509	7939
	35	0	6542	1801	9198	0	1715	1638	8950
Nickel	7	0	22397	4848	12415	0	3070	8771	9850
	14	0	39542	76	4600	0	836	4556	6367
	21	0	2912	32	4734	NA	NA	NA	NA
	28	0	24288	1871	7173	0	2844	6221	9742
	35	0	7587	2170	7969	0	688	10827	11036
Histidine	7	0	7746	114	12481	0	1778	8365	7182
	14	0	20477	1645	11546	3	447	985	3310
	21	0	20785	524	7445	0	0	252	402
	28	0	12237	2136	19964	NA	NA	NA	NA
	35	0	8334	444	24893	0	118	252	1253
Chelidonic acid	7	41	9056	380	12274	0	3049	348	10287
	14	11	2255	1556	2513	10	30286	3908	4655
	21	8	16846	1681	6787	28	881	1299	3619
	28	10	4197	2054	3660	0	3168	3226	14125
	35	0	2736	1049	20023	47	3398	1280	54931

NA – not analysed

4.5.1 Control Samples

Malic acid is the most concentrated organic acid within plant roots, as observed within other non-hyperaccumulator plants (Appendix Table D3) (Igamberdiev and Eprintsev, 2016). Malic acid concentration is also the most stable of the three organic acids over the experimental study (Table 4.3). Citric acid and fumaric acid behave similarly within plant roots, exhibiting a steep declining concentration gradient for the first four weeks of analysis, then accumulating once again, on day 35, to approximately 15% of their concentrations on day 0. Accumulation of malic acid is linked directly to reduced concentrations of citric acid and fumaric acid, since fumaric acid accumulation controls malate conversion to pyruvate, citric acid is converted to malate through oxaloacetate by the Krebs cycle (Igamberdiev and Eprintsev, 2016). Thus, a

decreased concentration of fumaric acid and citric acid is expected as malic acid remains concentrated within roots.

Organic acids within leaves accumulate in a cyclic trend (Table 4.3). Up to day 21 of analysis, the cycle of synthesis and metabolism for malic acid and citric acid is the same. When plants enter their reproductive phase, after day 21, citric acid concentration decreases while malic acid accumulates within leaves.

4.5.2 Nickel Enrichment

The average concentration of malic acid does not alter significantly (7 399 mg kg^{-1} for enriched plants, compared to 7 545 mg kg^{-1} for control). Concentrations of fumaric acid and citric acid normalise within plant roots with the addition of Ni, rather than decreasing as for control plants (Table 4.3). Analysis of the molar ratio of malic acid and fumaric acid to Ni on day 35, when the metal is most concentrated (23:1 and 26:1, respectively) are both sufficient to chelate the metal. However, organic acids are unlikely to be responsible for Ni sequestration within roots, due to the alkaline environment (Harris *et al*, 2012).

The accumulation cycle of citric acid (Table 4.3) was significantly larger for plants enriched with Ni, peaking on day 14 (39 542 mg kg^{-1}). This indicates that the Krebs cycle is enhanced within sunflower leaves. The concentration of malic acid within leaves increases almost two-fold from day 21 to day 35 (4 734 – 7 969 mg kg^{-1}) coinciding with the spike in Ni accumulation observed (Figure 4.3) and indicating a possible Ni detoxification response within sunflowers. It is inferred, therefore, that malic acid and citric acid biosynthesis aids in sequestration of Ni within leaves of sunflowers.

4.5.3 Histidine Enrichment

A suppression of organic acid concentration within plant roots is observed, especially metal-chelating malic acid (Table 4.3). This is likely due to the bioaccumulation of amino acids within roots of plants resulting from this amino acid soil amendment (section 4.4.3).

Average concentrations of monitored organic acids within leaves are comparable to control samples (Appendix Table D3). The accumulation trend of citric acid is altered during vegetative growth, with citric acid concentrations consistently higher up to day 21 of analysis than for control plants (Table 4.3). This indicates an enhanced Krebs cycle, likely related to biosynthesis of amino acids to aid in growth and development of plants.

4.5.4 Chelidonic acid enrichment

ChA is not present naturally in sunflower plants, evidenced by the absence of this organic acid within control, Ni enriched, and His enriched plants (Appendix Table D3). Thus, the concentration of this organic acid within plant roots of sunflowers is due exclusively to uptake of exogenous ChA (Table 4.3). A corresponding drastic increase in malic acid concentration

is observed, with final concentration (54 931 mg kg^{-1}) significantly higher than control samples (8 950 mg kg^{-1}). This organic acid has been linked directly to the facilitation of heavy metal uptake into plant roots, as well as tolerance of heavy metals within plants (Ghazijahani *et al*, 2018). In leaves, concentrations of organic acids were comparable to control samples, even on day 28, when additional Ni accumulation was observed (Table 4.3). This is likely a result of ChA chelation to the metal as it is transported to leaves. The concentration of ChA decreases over the experimental period, indicating a detoxification process within leaves. A spike in malic acid concentration is observed on day 35, corresponding to the insufficient ChA concentration for Ni chelation (Table 4.3). This indicates that malic acid may replace ChA as the Ni-ligand. Thus, the addition of ChA to the soil medium may facilitate heavy metal uptake for accumulators and hyperaccumulators which utilise malic acid for metal sequestration and transport.

4.6 Conclusions – Effects of Soil Amendments on Capacity for Sunflowers to Accumulate Heavy Metals

Addition of Ni to the soil matrix of sunflower plants induces uptake of this metal into plant roots, where it is largely accumulated. Transport of the heavy metal from roots of plants into shoots occurs after 21 days of spiking with Ni, rather than from the first week of spiking, indicating a stress-related transport of the metal out of roots, rather than a hyperaccumulation mechanism. Biosynthesis of amino acids related to metal tolerance of the plant occurs within roots and shoots of plants. His is known to play an important role in Ni transport and sequestration. Pro, an amino acid which is directly related to abiotic stress reduction, is synthesised more extensively within leaves of Ni-spiked sunflowers, than it is for control plants. This stress response further concludes that sunflower plants do not comfortably accumulate this heavy metal, confirming that this plant is a metal accumulator and not a hyperaccumulator.

His is an essential AA which plays an important role in a variety of plant systems, including growth and development, and regulation of the synthesis of other amino acids. Exogenous addition of His to sunflowers resulted in the acceleration of plant growth, and induced production of amino acids within plant roots. Organic acid accumulation within roots was supressed by His uptake, indicating a possible hindrance to metal uptake for accumulator plants should plants be grown in contaminated soils. The impact of this soil enrichment on Ni uptake and accumulation was far less than the other two enrichments made.

Sunflowers do not produce ChA naturally. Thus, accumulation of ChA within plant roots is directly related to the exogenous application of the organic acid. Uptake of ChA into plant roots also induces uptake of Ni into plant roots, and stimulates an accumulation of the amino acids Pro, Glu, Asp, and Ser. His concentration within plant roots is not significantly altered by uptake of ChA and Ni, indicating a stronger correlation with metal transport for this amino acid,

as opposed to sequestration. Uptake of ChA into plant roots also induces malic acid synthesis within plant roots, an organic acid associated with tolerance of heavy metals for a variety of hyperaccumulators.

CHAPTER 5

Consolidated Discussion, Conclusions and Future Work

5.1 Introduction

Quantification of Ni within plant tissues confirmed that accumulation of the metal is contained within roots for the metal tolerant species, sunflowers, and in shoots of the hyperaccumulator *B. coddii*. Ni transport was found to occur in a cyclic pattern within *B. coddii* indicating, for the first time, that an accumulation capacity is achieved prior to transport from roots into shoots for this hyperaccumulator. Addition of 842 mM Ni to the soil of *B. coddii* plants resulted in toxicity. The threshold level of Ni accumulation was reached within the plants (7 300 mg kg^{-1}). It is clear that the capacity for Ni accumulation is directly related to the stage of development of plants, as the average concentration of Ni within *B. coddii* is between 13 000 mg kg^{-1} (Naicker, 2014; Nematundani et al., 2006; Pillay, 2006) and 17 000 mg kg^{-1} (Howes, 1991; Howes et al, 1998; Mesjasz-Przybylowicz et al., 2004). Necrosis was not observed within sunflowers, but plant development was indeed also negatively affected by this soil enrichment. Organic and amino acids, the key facilitators of Ni uptake and accumulation within plants, were monitored for both plant species. The role of ChA was further explored and confirmed as significant to the hyperaccumulation process of *B. coddii*, along with malic acid. Amino acid accumulation was linked to metal and nutrient stress, and a role in transport of Ni was identified for histidine within sunflowers. ChA soil enrichment had a negative effect on growth and development in both plants, while exogenous addition of His enhanced plant growth for both species.

Table 5.1 Concentrations of nickel, organic acids and amino acids in non-accumulator plants, sunflowers (HA) and B. coddii (BC)

		Concentration (g kg^{-1}) at day 35								
		Non-accumulator	Control		Nickel Enriched		Histidine Enriched		Chelidonic Acid Enriched	
	Analyte		HA	BC	HA	BC	HA	BC	HA	BC
Roots	Ni	-	0.00639	0.614	0.214	2.31	0.0195	0.537	0.0318	0.613
	Chelidonic acid	0	0	0.900	0	2.55	0	0.218	0.0472	0.678
	Citric acid	0.78[1]	1.72	6.53	0.688	22.1	0.118	6.92	3.40	5.67
	Malic acid	1.37[1]	8.95	8.30	11.0	116	1.25	5.19	54.9	11.7
	Aspartate	0.18[2]	3.86	29.3	6.22	0.939	29.2	1.91	10.2	5.61
	Glutamate	0.33[2]	3.74	34.9	6.31	1.86	31.7	5.05	19.8	10.6
	Histidine	0.0038[2]	1.16	30.1	2.05	3.96	6.00	13.2	0.452	37.8
	Proline	-	2.38	27.7	6.64	1.79	20.1	1.75	9.42	5.52
Shoots (Leaves)	Ni	0.01[3]	0.00392	3.28	0.0164	7.30	0.00286	3.16	0.00381	2.99
	Chelidonic acid	0	0.00	16.0	0.00	43.5	0.000	19.5	0.00	15.3
	Citric acid	(4.792[4])	10.5 (6.54)	21.4	14.5 (7.59)	20.9	15.7 (8.33)	4.32	7.47 (2.74)	19.9
	Malic acid	(8.362[4])	21.0 (9.20)	19.5	21.6 (7.97)	18.8	58.2 (24.9)	13.7	29.9 (20.0)	14.8
	Aspartate	(0.067[5])	32.8 (5.29)	2.89	10.2 (5.28)	36.4	12.6 (6.15)	1.60	14.4 (11.8)	8.79
	Glutamate	(0.357[5])	35.2 (6.09)	7.27	11.7 (6.05)	44.9	11.2 (6.09)	2.66	16.9 (13.8)	17.8
	Histidine	(0.023[2])	9.06 (1.93)	11.5	3.20 (1.34)	16.4	4.81 (2.13)	2.69	2.47 (1.57)	82.3
	Proline	(0.515[5])	22.3 (4.73)	0.0735	17.3 (4.88)	40.2	14.9 (4.49)	1.18	19.8 (12.1)	10.9

Sources: 1 = (Jones, 1998) 2 = amino acids within non-accumulator *A. conyzoide (Zhu et al, 2018)* 3 = (Bhalerao *et al*, 2015) 4 = (Kumar *et al*, 2017) 5 = (Kumar *et al*, 2015)

5.2 Growth and Development of Plant Species

Concentrations of organic and amino acids within *B. coddii* and sunflowers are both higher than is commonly found in non-accumulator plants (Table 5.1). Biosynthesis and metabolism of organic molecules is dependent on multivariate factors, such as age of plants, their type of carbon fixation, and their environment (Jones, 1998). As such, concentrations of organic molecules differ within plants, even of the same species.

The differences in amino acid concentrations between the three groups clearly reflects the role of amino acids in development of plants (Table 5.1). Growth of *B. coddii* was significantly affected by the environment, and stunted root growth is the main reason that amino acid biosynthesis was significantly higher than the non-hyperaccumulator species. Development of sunflowers was normal, and further along than the comparison species, *Ageratum conyzoides* L., resulting in higher concentrations of free amino acids (Roy *et al*, 2013). Concentrations of amino acids within shoots of sunflowers are higher than within *B. coddii*. This may be related to growth and development of plant stems, where amino acids are most concentrated (Kumar *et al*, 2019) and reflect the differences in growth between sunflowers and *B. coddii* during the experiment.

Organic acid concentrations within *B. coddii* are higher than the non-hyperaccumulator species, with the exception of malic acid (Table 5.1). A key determinant of organic acid levels within roots is the cation-anion balance, a factor which is dependent on the soil environment (Jones, 1998). Malate accumulation within sunflower roots may indicate that excess cations such as K^+ were present within the soil. ChA is naturally present only in *B. coddii*, further implicating its unique role in the uptake of Ni.

5.3 Effects of Nickel Soil Enrichments

Uptake of Ni into roots was apparent for both *B. coddii* and sunflowers (Table 5.1). Storage of the metal was in shoots for the hyperaccumulator and in roots for the non-hyperaccumulator. The impact of this significant difference between the two species was reflected in biosynthesis of organic and amino acids.

Increasing the concentration of bioavailable Ni in the soil results in an increased accumulation thereof within roots of sunflowers, and a corresponding spike in the amino acid pool, indicating a stress response rather than a tolerance mechanism (Table 5.1). A positive correlation was determined between uptake of ChA and biosynthesis of malic acid within plant roots, indicating a possible use for this organic acid to increase metal uptake capacity of non-hyperaccumulator species. Amino acids, linked to the stress response of plants, accumulate within roots of sunflowers, in response to the influx of Ni (Sharma and Dietz, 2006). The opposite is true for

B. coddii plants, where amino acid concentrations are suppressed compared to control samples, as they are utilised for xylem loading and transport of Ni into shoots (Sharma and Dietz, 2006).

Ni accumulation within shoots of *B. coddii* plants was linked to accumulation of ChA prior to day 35, when necrosis was almost complete. On day 35, no monitored organic molecule was concentrated enough to chelate to Ni. This likely contributed to plant necrosis (Table 5.1). Ni detoxification is thus related to amino acids within roots, observed within the non-hyperaccumulator, and organic acids within shoots for hyperaccumulators.

5.4 Effects of Organic Soil Enrichments

5.4.1 Histidine

The pool of amino acids within roots of sunflowers was enhanced by addition of exogenous histidine, and suppressed in *B. coddii* (Table 5.1). This may be related to the concentration balance of root amino acids which is typically in the range of 1 – 10 mM, with those in soil (0.01 – 10 µM) (Jones and Darrah, 1994). Due to the high concentration of amino acids in roots of the hyperaccumulator, addition of exogenous His to the soil increases the soil-root balance, disrupting the natural transport system of amino acids. Competition between soil organisms and plant roots also affect uptake of free amino acids (Jones *et al*, 2005).

OAs within sunflowers are suppressed in roots and enhanced within shoots (Table 5.1), indicating a response mechanism of organic acid biosynthesis or metabolism, due to disruption of the Krebs cycle.

5.4.2 Chelidonic acid

ChA uptake is observed within sunflowers, but not within *B. coddii* (Table 5.1). The soil enrichment also had a more significant impact on key factors related to phytoremediation for sunflowers: Ni uptake was enhanced, and metal-chelating malic acid was biosynthesised in roots.

An overall negative impact was observed for *B. coddii* plants, resulting in reduced biomass and accumulation of amino acids within plant shoots due to the perceived nutrient deficiency triggered by the enrichment.

5.5 Conclusions

Organic and amino acids play multiple essential roles in the uptake and accumulation of heavy metals. Additional Ni resulted in necrosis for both *B. coddii* and sunflowers. The threshold accumulation capacity of *B. coddii* seedlings was observed (7 300 mg kg^{-1}) for the first time, corresponding with a suppression of Ni-binding organic acid synthesis (ChA, malate and citrate). This resulted in a severe stress response, indicated by a spike in amino acid

concentrations within plants. Storage of Ni was primarily within roots of sunflowers. Transport of the metal to aboveground tissue was linked to a stress response for sunflowers, rather than an hyperaccumulation mechanism, observed for *B. coddii*. A corresponding accumulation of proline, an amino acid synonymous with exogenous stress, was observed within leaves of sunflowers.

Research into the modification of metal uptake capacity is ongoing, with focus on acceleration of plant growth, increasing chelating ability, and genetic and chemical modification (Naila *et al*, 2019). One such modification analysed includes the addition of organic modifiers, such as EDTA, malic acid or citric acid (Cornu *et al*, 2017; Gao *et al*, 2012; Osmolovskaya *et al*, 2018). The impact of exogenous addition of organic modifiers ChA and His on organic and amino acids has never been assessed for either *B. coddii* or sunflowers. ChA was not significantly accumulated within *B. coddii*, and a nutrient-related deficiency was observed, likely due to an altered soil pH. Uptake of ChA had a unique impact on sunflowers, stimulating accumulation of amino acids and inducing synthesis of malic acid, an organic acid associated with metal tolerance for a variety of accumulators.

Addition of His increased transport of Ni for *B. coddii* plants, likely as Ni(His)2, presenting a unique erratic trend of organic acid concentrations in plant shoots. Additional His resulted in enhanced growth of sunflowers, and a corresponding spike in synthesis of amino acids in plant roots. Organic acid concentrations within roots were suppressed by this soil enrichment, indicating a possible hindrance to metal uptake for accumulator plants should plants be grown in contaminated soils.

5.6 Future Work

Increasing the length of the soil enrichment studies would provide further insight into their effects on the species with growth and development, and would be beneficial to all aspects of this experiment.

The enrichment studies have also highlighted aspects that need to be further explored. These include:

B. coddii

- The identification of all significant role players in Ni uptake using HPLC-ICP-MS/MS.
- Manipulation of uptake based on amino acid amendments.

Sunflowers

- The impact of ChA on sunflowers should be further investigated. A ChA and metal co-enrichment would improve understanding of the co-uptake strategy postulated for the two molecules.

This future work would further improve understanding and knowledge of possible applications to plants for the purpose of phytoremediation, thus increasing the appeal of this technique of soil decontamination.

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APPENDIX A

LIST OF CHEMICALS

SOIL ENRICHMENTS

Sigma-Aldrich, Nickel(II) Sulfate Hexahydrate, ACS Reagent, 98% purity, Product No. 227676

Sigma- Aldrich, Chelidonic acid, 98% purity, Product No. 382272

Sigma-Aldrich, L-Histidine, 99% purity, Product No. H8000

HPLC-UV

Organic Acids

Sigma-Aldrich, Fumaric Acid, 99% purity, Product No. 47910

Sigma-Aldrich, Citric Acid (Anhydrous), 97% purity (GC), Product No. C2404

Sigma-Aldrich, L-Ascorbic Acid, 99% purity, Product No. A5960

Sigma-Aldrich, DL-Malic Acid, 98% purity (capillary GC), Product No. M0875

Sigma-Aldrich, Oxalic Acid, 99% purity, Product No. 241172

Sigma-Aldrich, DL-Tartaric Acid, 99% purity, Product No. T400

Sigma- Aldrich, Chelidonic acid, 98% purity, Product No. 382272

Solvents

Sigma-Aldrich, Potassium phosphate monobasic, 99% purity, Product No. P0662

Sigma-Aldrich, Phosphoric acid, ACS Reagent, >85%/wt. in H_2O, Product No. 695017

AMINO ACIDS

Waters, Amino Acid Hydrolysate Standard, Part No. WAT088122

Waters, AccQ-Fluor Reagent Package, Part No. WAT052880

Solvents

Waters, AccQ-Tag Eluent A Concentrate, Part No. WAT052890

Waters, AccQ-Tag Eluent B, Part No. WAT052895

ICP-MS

Spectrochem, 10 mg kg^{-1} Multi-Element Stock Solution

Spectrochem, 10 mg kg^{-1} Rhodium Standard

Nitric Acid, ACS Reagent, 70% purity, Product No. 438073

APPENDIX B

CERTIFIED REFERENCE MATERIAL DATA SHEET

Certificate of Certified Reference Materials

NCS DC 73347 — NCS DC 73351

DC73347	Hair
DC73348	Bush Branches and Leaves
*DC73349	Bush Branches and Leaves *
DC73350	Leaves of Poplar
DC73351	Tea

Issued in 2004

Approved by China National Analysis Center for Iron and Steel

(Beijing China)

Element	analytical method
Ag	AAN ICP-MS INAA
Al	COL ICP INAA XRF
As	AF ICP-MS INAA
Au	AAN
B	COL ES ICP ICP-MS
Ba	AAN ICP ICP-MS INAA XRF
Be	AAN ICP ICP-MS
Bi	AF ICP-MS
Br	COL IC INAA XRF
Ca	AA ICP INAA XRF
Cd	AAN ICP-MS POL
Ce	AAN ICP-MS POL
Cl	COL IC INAA
Co	AA AAN ICP ICP-MS INAA
Cr	AA ICP-MS INAA
Cs	ICP-MS INAA
Cu	AA ES ICP ICP-MS XRF
Dy	ICP ICP-MS
Er	ICP ICP-MS
Eu	ICP ICP-MS INAA
F	COL ISE
Fe	AA ICP INAA XRF
Ga	AAN ICP-MS
Gd	ICP ICP-MS
Hf	INAA
Hg	AA AF
Ho	ICP ICP-MS
K	AA IC ICP INAA XRF
La	ICP ICP-MS INAA
Li	AA AAN FP ICP ICP-MS
Lu	ICP-MS INAA
Mg	AA ICP XRF
Mn	AA ICP ICP-MS INAA XRF

Element	analytical method
Mo	AA COL ICP-MS POL
N	COL VOL
Na	AA IC ICP INAA XRF
Nb	ICP-MS
Nd	ICP ICP-MS INAA
Ni	AA AAN ICP ICP-MS
P	COL ICP XRF
Pb	AA AAN ES ICP ICP-MS POL XRF
Pr	ICP ICP-MS
Rb	AAN ICP-MS INAA XRF
Re	ICP-MS
S	COL IC ICP VOL XRF
Sb	AF INAA
Sc	ICP ICP-MS INAA
Se	AF COL INAA
Si	COL GR XRF
Sm	ICP ICP-MS INAA
Sn	ES ICP-MS POL
Sr	AA ICP ICP-MS INAA XRF
Ta	INAA
Tb	ICP ICP-MS INAA
Th	ICP-MS INAA
Ti	AA COL ICP XRF
Tl	ICP-MS
Tm	ICP ICP-MS
U	ICP-MS INAA LF
V	ICP ICP-MS INAA POL XRF
W	COL POL
Y	ICP ICP-MS
Yb	ICP ICP-MS INAA
Zn	AA ICP ICP-MS INAA XRF
Zr	ICP-MS

Note:

AA: Atomic Absorption Spectrometry
AAN: Non-Flame Atomic Absorption Spectrometry
AF: Atomic Fluorescence Spectrometry
COL: Colorimetry
ES: Emission Spectrography
FP: Flame Photometry
GR: Gravimetry
IC: Ion Chromatography
ICP: Inductively Coupled Plasma emission spectrometry
ICP-MS: ICP Mass Spectrometry
INAA: Instrument Neutron Activation Analysis method
ISE: Ion Selective Electrode method
LF: Laser Fluorescence Spectrometry
POL: Polarography
VOL: Volumetry
XRF: X-Ray Fluorescence Spectrometry

Professor Wang Haizhou, Chief

China National Analysis Center for Iron and Steel

CERTIFIED VALUES(Certification 1990, Revision 2003) OF REFERENCE MATERIAL FOR VEGETABLE AND HUMAN HAIR

μg/g	NCS DC 73347	NCS DC 73348	NCS DC 73349	NCS DC 73350	NCS DC 73351
Ag	0.029±0.008	0.027±0.006	0.049±0.007	(0.013)	(0.018)
Al%		0.214±0.022	0.20±0.03	0.104±0.006	(0.30)
As	0.28±0.05	0.95±0.12	1.25±0.15	0.37±0.09	0.28±0.04
Au ng / g	(2.5)				
B	(1.3)	34±7	38±6	53±5	15±4
Ba	17±2	19±3	18±2	26±4	58±6
Be	0.063±0.020	0.056±0.014	0.051±0.004	0.021±0.005	0.034±0.006
Bi	0.34±0.02	(0.022)	0.023±0.005	0.027±0.002	0.063±0.008
Br	(0.36)	2.4±0.4	3.0±0.4	7.2±1.4	3.4±0.5
Ca%	0.29±0.03	2.22±0.13	1.68±0.11	1.81±0.13	0.43±0.04
Cd	0.11±0.03	0.14±0.06	(0.38)	0.32±0.07	0.057±0.010
Ce	0.12±0.03	2.4±0.3	2.2±0.1	0.49±0.07	1.0±0.2
Cl%		(1.13)	(1.92)	(0.23)	
Co	0.071±0.012	0.39±0.05	0.41±0.05	0.42±0.03	0.18±0.02
Cr	0.37±0.06	2.3±0.3	2.6±0.2	0.55±0.07	0.80±0.03
Cs		0.27±0.03	0.27±0.02	0.053±0.003	0.29±0.02
Cu	10.6±1.2	5.2±0.5	6.6±0.8	9.3±1.0	17.3±1.8
Dy	(0.017)		(0.13)	(0.036)	(0.074)
Eu	(0.006)	0.037±0.002	0.039±0.003	0.009±0.003	0.018±0.002
F		24±3	23±4	22±4	320±31
Fe	54±10	1020±67	1070±57	274±17	264±15
Gd			(0.19)	(0.043)	(0.093)
Hf		0.14±0.02	(0.15)	(0.026)	(0.033)
Hg	0.36±0.08			0.026±0.003	(0.013)
Ho			(0.033)		(0.019)
K%	(0.002)	0.85±0.05	0.92±0.10	1.38±0.07	1.66±0.12
La	0.049±0.011	1.23±0.10	1.25±0.06	0.26±0.02	0.60±0.04
Li	2.0±0.1	2.4±0.4	2.6±0.4	0.84±0.15	(0.36)
Lu			(0.011)		(0.007)
Mg%	0.036±0.004	0.287±0.018	0.48±0.04	0.65±0.05	0.17±0.02
Mn	6.3±0.8	58±6	61±5	45±4	1240±70
Mo	0.073±0.014	0.26±0.04	0.28±0.05	0.18±0.01	0.038±0.007
N%	14.9±0.1	1.20±0.02	1.50±0.03	2.56±0.06	3.32±0.09
Na	152±17	1.[illegible]±0.10%	1.96±0.18%	200±13	44±6
Nd		(1.1)	1.0±0.1	(0.22)	(0.44)
Ni	0.83±0.19	1.7±0.4	1.7±0.3	1.9±0.3	4.6±0.5
P	170±10	830±40	1000±40	1680±60	2840±90
Pb	8.8±1.1	7.1±1.1	47±3	1.5±0.3	4.4±0.3
Pr			(0.24)		(0.12)
Rb		4.2±0.2	4.5±0.6	7.6±0.8	74±5
S%	4.3±0.3	0.32±0.03	0.73±0.06	0.35±0.04	0.245±0.022
Sb	0.095±0.016	0.078±0.020	0.095±0.014	0.045±0.006	0.056±0.006
Sc	0.008±0.001	0.31±0.03	0.32±0.04	0.069±0.007	0.085±0.013
Se	0.60±0.04	0.184±0.013	0.12±0.02	0.14±0.02	(0.072)
Si%	0.087±0.008	0.58±0.04	0.60±0.07	0.71±0.08	(0.21)
Sm	(0.012)	0.19±0.01	0.19±0.02	0.038±0.006	0.085±0.023
Sn			(0.27)		
Sr	24±1	345±11	246±16	154±9	15.2±0.7
Tb		(0.026)	0.025±0.003		(0.011)
Th		0.37±0.02	0.36±0.04	0.070±0.010	0.061±0.009
Ti	2.7±0.6	95±18	95±20	20.4±2.2	24±4
U		(0.11)	(0.12)	(0.028)	
V		2.4±0.3	2.4±0.4	(0.64)	(0.86)
W		(0.06)	(0.06)		
Y	0.084±0.020	(0.63)	0.68±0.02	0.145±0.015	0.36±0.04
Yb		0.063±0.014	0.063±0.009	0.018±0.004	0.044±0.005
Zn	190±9	20.6±2.2	55±4	37±3	26.3±2.0

Note: Data behind "±" are standard deviation.

1. Data enclosed in brackets show these individual values are uncertified, for reference only.
2. The sample is packed in glass bottle with size less than 80 meshes.
   The package is: DC73348— 73351 35g/bottle; DC73347 10g/bottle and 20g/bottle
3. The sample should be stored in drier.
4. Minimum weight 0.5g sample should be taken for analysis.
5. The certification will expire in Dec.2010. although we reserve the right to make change as issue revisions.

APPENDIX C

Data: *Berkheya coddii* R.

Table C1 Biomass and Ni Concentrations within B. coddii over the experimental study

Soil Enrichment	Day of Analysis	Concentration Added (mM)		Biomass (mg)			Ni Concentration (mg kg^{-1})	
				Roots	Shoots	Total	Roots	Shoots
Not Enriched (Control)	0	0		35	9	44	930	1060
	35	0		260	1 033	1 293	614	3284
Ni Enriched	14	341		8	13	21	1663	3181
	21	511		7	12	19	1251	4550
	28	682		3	42	44	926	5715
	35	852		2	19	22	2309	7300
His Enriched	14	26		21	61	82	792	5463
	21	39		24	590	614	506	5787
	28	52		280	1 112	1 392	802	4535
	35	58		262	438	700	537	3157
ChA Enriched	14	11		46	107	153	168	2342
	21	16		24	48	72	1459	912
	28	22		255	751	1006	517	4090
	35	27		322	585	906	613	2985
Ni and ChA Co-enriched	14	11	34	34	13	51	4780	3755
	21	16	51	191	258	38	1436	4701
	28	22	68	6	75	67	2957	4559
	35	27	85	3	47	69	933	5211

Table C2 Amino Acids within B. coddii plants

Root Concentration/ mg kg^{-1}																			
Soil Enrichment		Control		Ni Enriched				Histidine Enriched				Chelidonic acid (ChA) Enriched				Ni and ChA co-enriched			
Pathway	AA / Day	0	35	14	21	28	35	14	21	28	35	14	21	28	35	14	21	28	35
Aspartate	Asp	14057	29261	2572	5133	na	939	1957	113	272	1907	3387	204	1105	5610	1957	678	NA	4749
	Ile	5333	14400	1014	4317	na	821	885	777	634	2100	3953	6979	1077	4710	1276	479	NA	1586
	Lys	5233	13286	8168	876	na	1366	1591	83	603	1871	bdl	5516	1141	7081	2278	420	NA	1304
	Met	bdl	bdl	bdl	232	na	bdl	bdl	bdl	bdl	bdl	114	bdl	bdl	137	bdl	bdl	NA	bdl
	Thr	ce	35307	ce	ce	na	138	ce	ce	ce	40	ce	147	ce	ce	ce	ce	NA	453
Serine	Ser	11941	10957	808	11573	na	783	1502	1379	574	2474	4131	10188	1355	5792	1157	636	NA	2744
	Cys	bdl	bdl	bdl	bdl	na	756	bdl	591	bdl	933	bdl	bdl	bdl	bdl	971	bdl	NA	bdl
	Gly	8581	1026	1063	9282	na	1150	7303	2842	5492	4782	9015	10322	2066	5256	1014	3441	NA	2997
Glutamate	Glu	13868	34918	3007	8580	na	1860	5214	2855	626	5051	8485	16874	2283	10599	1888	638	NA	5147
	Arg	ce	64978	ce	ce	na	bdl	ce	ce	ce	bdl	ce	bdl	bdl	ce	ce	ce	NA	4543
	His	1300	30069	16746	307	na	3958	4705	13105	31522	13235	55411	33336	12785	37769	6258	8577	NA	667
	Pro	21081	27712	1574	23929	na	1786	466	837	584	1745	2889	6612	1224	5524	1783	1147	NA	3425
Pyruvate	Ala	7157	14203	905	3975	na	502	703	955	615	1859	2963	7527	1012	4770	666	898	NA	2714
	Leu	8881	22396	1775	7096	na	1418	1609	1548	1050	3373	6633	11138	1687	7654	2336	913	NA	2932
	Val	7488	bdl	bdl	7230	na	bdl	bdl	bdl	691	bdl	bdl	bdl	1198	4999	bdl	bdl	NA	2397
Shikimate	Phe	4655	16886	1632	7370	na	2584	218	1478	709	3885	7121	8338	1003	5004	4044	270	NA	1481
	Tyr	4255	772	611	6712	na	964	1363	1435	276	2854	4494	12429	322	2770	1233	96	NA	994

na - sample not analysed; bdl - peak area below calibration detection limit; ce - co-eluted peaks (for ARG and THR)

Table C2 (Amino Acids within B. coddii plants) continued

Shoot Concentration/ mg kg^{-1}																			
Soil Enrichment		Control		Ni Enriched				Histidine Enriched				Chelidonic acid (ChA) Enriched				Ni and ChA co-enriched			
Pathway	AA / Day	0	35	14	21	28	35	14	21	28	35	14	21	28	35	14	21	28	35
Aspartate	Asp	5404	6136	1295	4622	2983	36348	24439	7184	278	1602	2532	3608	10527	8790	bdl	5495	2480	3070
	Ile	6662	3458	10629	7816	1028	26460	26156	6328	432	796	833	4891	6392	5257	2665	4726	1771	5977
	Lys	4695	3717	5110	5835	1278	20317	26052	5282	593	1213	1869	6038	9143	7637	3444	4077	2310	2597
	Met	bdl	bdl	2679	bdl	bdl	bdl	1028	245	bdl	167	bdl	73	387	bdl	bdl	bdl	bdl	bdl
	Thr	ce	3522	3330	ce	495	80259	64	ce	ce	13	ce	45	62	ce	755	12782	260	4372
Serine	Ser	bdl	4499	1462	bdl	1561	30103	32190	9463	418	1736	4308	6741	8735	8862	5736	6651	3001	11215
	Cys	bdl	bdl	bdl	bdl	bdl	bdl	2093	4823	bdl	bdl	bdl	bdl	540	bdl	4688	bdl	2430	bdl
	Gly	4127	3926	2064	7366	1630	40210	40737	13107	2571	1770	13927	6611	11922	11269	1402	6785	832	11256
Glutamate	Glu	10757	7265	1578	6887	3278	44868	56518	18576	845	2662	13694	10046	16441	1076	7147	1410	4485	6582
	Arg	ce	bdl	bdl	ce	279	bdl	bdl	ce	ce	bdl	ce	bdl	bdl	ce	2500	bdl	bdl	13232
	His	8666	11525	730	13889	405	16422	69366	34592	13713	2690	177523	68943	95484	82339	19915	3083	4614	1407
	Pro	7452	1113	3125	6555	9248	40207	32158	6548	423	1180	6344	8572	7947	10924	161	31289	12271	30392
Pyruvate	Ala	bdl	2574	3197	bdl	1284	28510	25635	7097	269	1357	2419	4723	6668	6758	10171	5323	2011	5409
	Leu	6926	5637	11571	2280	1505	46370	41400	10011	753	1663	1067	7692	10560	9155	4990	7907	3043	9956
	Val	bdl	20	2411	bdl	1385	33703	30274	20	346	1069	2296	5180	6559	6409	bdl	6171	bdl	7046
Shikimate	Phe	3530	3171	8440	5442	446	35198	23700	7750	514	688	9510	4568	8141	2882	2725	5723	1485	12247
	Tyr	bdl	5344	1542	735	659	30173	42836	11197	303	514	1031	2324	685	1786	444	3598	3477	11087

Table C3 Organic acids within B. coddii plants

Concentration/ mg kg^{-1}									
Soil Enrichment	Day of Analysis	Roots				Shoots			
		tartaric acid	malic acid	chelidonic acid	citric acid	tartaric acid	malic acid	chelidonic acid	citric acid
No Enrichment (Control)	0	625	5516	316	3151	894	5980	16235	28025
	35	7159	8303	900	6533	1832	19463	16032	21348
Ni Enriched	0	625	5516	316	3151	894	5980	16235	28025
	14	3737	4750	3755	8593	1258	5399	20664	23271
	21	1789	13639	2465	26678	2789	9475	32487	55235
	28	747	4728	2016	10370	1982	10362	31192	50619
	35	59734	115721	2547	22107	3284	18791	43454	20927
Histidine Enriched	0	625	5516	316	3151	894	5980	16235	28025
	14	5433	23549	2130	8511	13125	23619	44181	11469
	21	11236	7673	604	6520	7093	7374	14385	7447
	28	1789	1222	96	1043	3327	15757	19084	6920
	35	4742	5193	218	6915	3031	13727	19541	4316
Chelidonic Acid (ChA) Enriched	0	625	5516	316	3151	894	5980	16235	28025
	14	5821	14128	149	5527	3755	23314	34057	5820
	21	19866	18709	1322	22842	4670	7793	20282	4497
	28	5643	7791	755	6564	3757	16387	17278	9513
	35	4955	11723	678	5668	5640	14844	15320	19944
Ni and ChA Co-enriched	0	625	5516	316	3151	894	5980	16235	28025
	14	2139	3233	1631	21061	2636	8894	26342	15394
	21	3317	10908	942	8399	3905	8926	41053	14974
	28	589	28334	344	3976	3349	5836	38047	24682
	35	2320	31406	211	1986	1308	4075	14792	7657

APPENDIX D

Data: Sunflowers

Table D1 Biomass and Ni Concentrations within sunflowers. NA - not analysed

Soil Enrichment	Day of Analysis	Concentration Added (mM)	Biomass (mg)			Ni Concentration (mg kg^{-1})		
			Roots	Stems	Leaves (Flowers)	Roots	Stems	Leaves (Flowers)
Not Enriched (Control)	7	0	274	491	817	1.02	2.49	2.72
	14	0	1064	2449	3543	2.10	1.39	6.50
	21	0	1948	2132	1907	4.49	0.42	3.69
	28	0	2221	2827	1979 (333)	8.90	1.09	2.76 (1.02)
	35	0	3976	5990	3881 (904)	6.39	0.22	0.93 (2.77)
Ni Enriched	7	17	1536	1648	1891	15.93	0.82	1.12
	14	34	2842	1723	2086	23.32	1.25	1.92
	21	51	NA	3316	2455	NA	6.20	1.10
	28	68	1079	3495	1910	62.10	3.36	12.56
	35	85	4756	6363	4591	213.53	5.38	11.04
His Enriched	7	6	427	1054	1152	9.00	1.29	1.33
	14	13	1408	3245	1663	13.66	0.99	2.18
	21	19	4815	2554	4153 (741)	5.90	0.21	3.33 (1.37)
	28	26	8912	4031	3549	4.18	0.38	10.80
	35	32	8759	7283	3519 (883)	19.51	0.19	1.63 (1.04)
ChA Enriched	7	3	492	227	1038	13.72	5.81	2.60
	14	5	944	2364	2536	12.06	1.68	1.51
	21	8	2173	2555	1955	10.90	1.27	2.05
	28	11	4010	3404	2435	16.78	4.15	6.46
	35	14	4622	4465	3356	31.85	1.63	2.18

Table D2 Concentrations of Amino acids within sunflowers.

Root Concentration/ mg kg^{-1}																		
Soil Enrichment		Control					Ni Enriched				Histidine Enriched				Chelidonic Acid Enriched			
Pathway	AA / Day	7	14	21	28	35	7	14	28	35	7	21	28	35	7	14	28	35
Aspartate	Asp	1419	5552	3131	6850	3863	10960	9737	5527	6221	4830	6676	9909	29148	8449	8230	9909	10173
	Ile	569	2214	1437	3023	1962	5149	6760	2536	2521	2712	3670	5319	13196	2200	3667	5052	8690
	Lys	362	1499	1058	2353	1660	3878	661	1963	2631	2255	69	6439	9450	2345	3980	3256	11166
	Met	185	697	495	810	575	1602	706	751	708	795	1268	1348	5102	878	1041	1402	2436
Glutamate	Thr	853	5514	3159	6530	3740	10581	7334	5566	6307	5553	6336	11027	31686	7480	8878	10880	19773
	Ser	768	2472	506	5644	1155	3448	8871	546	2052	1355	2000	7991	6001	2422	2826	2253	452
	Cys	1319	3698	2303	3825	2382	7522	7596	3664	6639	3810	5546	7689	20112	6894	4859	7317	9419
3-*P*-glycerate	Gly	1345	2633	2191	3124	2599	7116	1806	3757	3288	3401	5253	4990	20252	3398	4916	5502	10522
	Glu	1513	3559	2365	4469	2970	8275	BDL	4071	3503	3768	7022	BDL	24179	3758	5337	8916	16219
Pyruvate	Arg	BDL	2661	1817	3565	2434	6711	6102	310	0	3005	4398	6285	19140	3387	BDL	6016	BDL
	His	1164	3725	2426	5217	3397	8554	3424	4559	4649	4325	5975	9508	23825	4008	6705	6608	14631
	Pro	1007	2626	1976	3232	2432	6834	BDL	3576	3296	3590	5161	5220	18124	3693	4953	4901	9124
Shikimate	Ala	758	3280	1334	6670	2713	6914	2299	3592	3465	2604	4051	12373	16817	2372	3866	5368	15327
	Leu	BDL	1323	1191	1000	900	3877	BDL	840	1449	1965	3422	BDL	8804	952	2330	2313	1453

NA - not analysed; BDL - below calibration detection limit

Table D2 (Amino acids within sunflowers) cont.

Stem Concentration/ mg kg^{-1}																				
Soil Enrichment		Control				Ni Enriched					Histidine Enriched					Chelidonic acid Enriched				
Pathway	AA / Day	7	14	21	28	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Aspartate	Asp	19776	7258	6827	21023	2347	1569	15849	4333	4864	31364	5325	7947	6201	1549	9328	6921	3649	9654	2616
	Ile	988	10423	1432	7187	558	1499	3508	934	2713	8141	1427	3315	2768	740	2221	3336	1501	3526	1421
	Lys	1003	7457	1404	5326	508	482	4740	966	3280	4534	1176	1020	3297	743	1835	3993	830	2838	1168
	Met	355	3276	547	2124	133	99	1510	331	679	1500	329	565	638	199	BDL	BDL	17	1038	366
Glutamate	Thr	5836	26254	5736	26712	1319	3002	15190	3458	5600	18947	3948	7492	6647	1382	7435	9755	3364	10629	3088
	Ser	2277	3339	1371	5549	123	1885	3498	1042	1854	3926	1114	1923	164	513	756	1656	1102	2016	905
	Cys	3678	10113	2985	12462	1465	1786	16466	3588	12366	10985	10776	12983	6682	3360	8291	28539	6103	22810	7636
3-*P*-glycerate	Gly	1983	10746	2166	9834	415	2640	6684	1511	3259	6925	2301	2982	3316	906	2589	4815	1715	4496	1837
	Glu	2745	9402	2709	9527	522	4451	7309	1891	3958	9332	2148	1461	3040	1039	3103	6839	1620	4991	2220
Pyruvate	Arg	BDL	BDL	2048	8696	447	1523	5151	55	3157	5925	1821	2144	2779	665	2633	8082	1209	5043	1937
	His	1468	13969	2240	9158	773	2436	5774	1592	3951	8434	1996	2614	3551	1039	3159	5904	1699	4697	2135
	Pro	2052	10181	2155	9692	551	2015	5547	1473	3236	9714	1811	2625	2656	911	2713	5208	1698	4508	1962
Shikimate	Ala	2393	4007	1463	6553	535	2420	3854	1062	3530	6872	2984	805	3143	1571	2712	3120	1756	3834	1429
	Leu	176	3447	234	2588	50	902	1657	283	1613	2274	236	179	1460	510	321	2839	204	594	974

Table D2 (Amino acids within sunflowers) cont.

Leaf Concentration/ mg kg^{-1}																				
Soil Enrichment		Control					Ni Enriched					Histidine Enriched					Chelidonic acid Enriched			
Pathway	AA / Day	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	28	35
Aspartate	ASP	3105	4144	12852	10063	5292	3975	21921	8084	16045	5284	4799	3366	19231	4909	6145	10343	10403	11887	11819
	ILE	520	2547	4618	3418	3441	2645	9273	3508	8175	2337	2564	2184	8632	2290	2333	3472	3572	6277	6103
	LYS	477	1292	4210	2601	2092	1745	8062	2983	8631	1960	1781	2235	8366	2414	2558	3548	3602	5506	5422
	THR	X	1260	1646	1392	1490	668	3154	1293	2897	710	598	36	3321	808	751	999	777	1804	2318
Glutamate	GLU	2372	3772	11315	7006	6092	4110	20829	9880	18340	6052	3486	4197	23797	5582	6093	10393	9714	12865	13764
	HIS	788	2196	2656	3023	1929	955	5681	2214	5396	1342	1635	1865	6236	1977	2130	3297	8459	4014	1565
	PRO	951	6627	6872	9638	4725	3657	19692	9384	10053	4879	3727	4941	22427	3149	4488	6272	5958	11536	12144
3-*P*-glycerate	SER	966	3623	5442	4162	4737	2308	10659	4422	9658	3026	3109	2293	11887	2682	2940	4525	4371	6420	6645
	GLY	952	4796	6369	4866	6277	3008	13407	6275	16788	3716	3813	3168	14655	4360	6268	6299	7430	8816	7474
Pyruvate	ALA	799	2540	5397	3770	3636	2205	11348	5043	10098	BDL	BDL	2254	10849	2714	252864	n.d.	BDL	6590	7562
	LEU	957	4680	7951	5923	6282	4583	16292	6468	15028	4864	5011	3758	15787	3996	4374	6846	6599	10992	11403
	VAL	1415	3546	5995	4704	4519	2701	12464	4826	10198	3341	3605	2497	12149	2601	2811	4827	4415	7230	7905
Shikimate	PHE	1709	5614	6410	6047	5951	5042	11123	4014	9369	2864	4709	3636	12683	3293	3607	5540	5837	9557	9071
	TYR	2459	4337	3702	3933	4082	2275	9037	2918	7373	2318	BDL	1707	7320	1604	1533	2821	2537	4771	5305

Table D3 Concentrations of organic acids within sunflowers.

Roots/ mg kg^{-1}																				
	No enrichment (control)					Nickel enriched					Histidine enriched					Chelidonic acid enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Ascorbic Acid	52	40	NA	50	57	22	39	NA	27	72	25	32	0	NA	18	13	68	10	53	399
Chelidonic acid	0	0	NA	0	0	0	0	NA	0	0	BDL	3	0	NA	0	0	10	28	0	47
Citric acid	5597	2649	NA	307	1715	3070	836	NA	2844	688	1778	447	BDL	NA	118	3049	30286	881	3168	3398
Malic acid	11371	9464	NA	7939	8950	9850	6367	NA	9742	11036	7182	3310	402	NA	1253	10287	4655	3619	14125	54931
Fumaric acid	10707	6471	NA	509	1638	8771	4556	NA	6221	10827	8365	985	252	NA	252	348	3908	1299	3226	1280
Oxalic acid	620	466	NA	177	238	217	426	NA	462	453	702	276	183	NA	147	ce	231	281	407	2377
Tartaric acid	1421	BDL	NA	3133	526	BDL	290	NA	625	116	2912	167	76	NA	230	45261	707	721	1714	4162

NA - not analysed; BDL - below calibration detection limit

Stems/ mg kg^{-1}																				
	No enrichment (control)					Nickel enriched					Histidine enriched					Chelidonic acid enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Ascorbic Acid	81	197	58	214	88	98	98	41	111	123	159	605	155	136	131	68	71	33	89	106
Chelidonic acid	BDL	0	0	0	0	0	0	0	BDL	BDL	0	0	0	0	0	BDL	13	47	18	BDL
Citric acid	26304	57428	5111	4944	3954	113779	12487	76488	5175	6921	19645	21139	1130	2411	5334	282211	2331	120338	89559	4736
Malic acid	22013	10569	747	5133	5865	11654	14420	678	13929	7532	1182	22	7275	11374	4125	17398	2879	1497	9613	11563
Fumaric acid	13155	8110	56577	27518	11823	7646	11471	11009	11502	13652	4795	19332	4525	14177	23748	9475	23810	8749	10048	9921
Oxalic acid	1268	1133	881	963	577	882	506	721	871	247	630	2744	1554	688	204	1424	1620	423	835	362
Tartaric acid	BDL	2790	11597	6554	1177	9495	4484	BDL	BDL	1672	BDL	1576	1454	797	602	BDL	3719	26029	11682	646

Table D3 (Concentrations of organic acids within sunflowers) cont.

	Leaves/ mg kg^{-1}																			
	No enrichment (control)					Nickel enriched					Histidine enriched					Chelidonic acid enriched				
Day of Analysis	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35	7	14	21	28	35
Ascorbic Acid	620	133	286	185	107	531	744	377	396	115	463	410	275	135	214	137	73	127	288	7
Chelidonic acid	23	0	0	0	0	0	0	0	BDL	0	0	0	0	0	0	41	11	8	10	0
Citric acid	9051	1462	12505	7162	6542	22397	39542	2912	24288	7587	7746	20477	20785	12237	8334	9056	2255	16846	4197	2736
Fumaric acid	1057	1964	2345	488	1801	4848	76	32	1871	2170	114	1645	524	2136	444	380	1556	1681	2054	1049
Malic acid	11437	5583	7012	24490	9198	12415	4600	4734	7173	7969	12481	11546	7445	19964	24893	12274	2513	6787	3660	20023
Oxalic acid	202	426	114	401	236	271	444	422	471	437	211	470	279	739	475	745	BDL	187	587	387
Tartaric acid	1725	714	168	11913	6304	3504	2147	4925	1039	7157	2160	2346	4705	12076	8942	2393	1880	5954	4449	BDL