NICKEL ACCUMULATION AND TOLERANCE IN *BERKHEYA CODDII* AND ITS APPLICATION IN PHYTOREMEDIATION

Kerry Slatter

NICKEL ACCUMULATION AND TOLERANCE IN *BERKHEYA CODDII* AND ITS APPLICATION IN PHYTOREMEDIATION

Kerry Slatter
BSc (Hons) (University of Cape Town)

Submitted in fulfilment of the
academic requirements for the degree of
Master of Science
In the
Department of Biochemistry
University of Natal

Pietermaritzburg July 1998

PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg from August 1994 to December 1995 and at Amplats Research Laboratories from January 1996 to July 1998, under the supervision of Dr TR Anderson. These studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

Kerry Slatter

July 1998

ABSTRACT

As pollution becomes an ever-increasing threat to the global environment pressure is being placed upon industry to "clean-up" its act, both in terms of reducing the possibility of new pollution and cleaning up already contaminated areas. It was with this in mind that Amplats embarked on a phytoremediation project to decontaminate nickel-polluted soils at one of their mine sites in Rustenburg, using the nickel hyperaccumulating plant, *Berkheya coddii*, which is endemic to the serpentine areas near Barberton, Mpumalanga.

Besides the applied aspects pertaining to the development of the phytoremediation process we were also interested in more academic aspects concerning the transport and storage of nickel within the plant tissues. In order that the progress of nickel could be followed through the plant, a radio-tracer of ^{63}nickel was placed in the soil and its movement within the plant followed by analysing the plant material, at set intervals, using a liquid scintillation counter. From these studies it was found that the nickel appeared to be transported from the roots to the leaves of the plant via the xylem. It appeared that the nickel was not confined to the leaf to which it was initially transported and so movement of nickel within the phloem also appears to occur in *B. coddii*. As nickel is generally toxic to most plants, hyperaccumulators contain elements that nullify the toxic effect of nickel. In the case of *Berkheya coddii* it is thought that the accumulated nickel is bound to malate to form a harmless nickel complex. With this in mind an assay for L-malic acid was developed in order that any effect on L-malic acid, caused by growing *Berkheya coddii* on soils containing various concentrations of nickel, could be determined. This method also enabled comparisons of L-malic acid concentrations to be made between hyperaccumulators and non-hyperaccumulators of various plant species. From the L-malic acid comparisons it was found that the nickel concentration within soils affected the levels of L-malic acid within *B. coddii* and that the levels of L-malic acid within *B. coddii* were greater than that of a closely related non-hyperaccumulator, suggesting that L-malic acid is indeed involved in the hyperaccumulation mechanism within *B. coddii*.

B. coddii was chosen as the tool in nickel phytoremediation at Rustenburg Base Metal Refineries as it was found to accumulate up to 2.5% nickel in the dry biomass, it grows rapidly and has a large above-ground biomass with a well developed root system, and it is perennial and so does not need to be planted each season. Earlier work had shown that the nickel levels

in the roots were comparatively low (up to 0.3% nickel in the dry material) and thus, for ease of harvesting and to ensure the continued vegetative growth of the plant on the planted sites, it was decided that the leaves and stems of the plants would be harvested at the end of each growing season. The plant was also found to accumulate low levels (0.006 – 0.3 %) of precious metals, including platinum, palladium and rhodium, within its above ground biomass, making it attractive for the remediation of certain soils that contain low levels of these metals.

Before *B. coddii* could be introduced to the Rustenburg area a comparison of the climatic and soil conditions of Barberton, the area to which *B. coddii* is endemic, and Rustenburg needed to be made to ensure that the plant would be able to survive the new conditions. These comparisons showed that Rustenburg receives on average, 484 mm less rain per year than Barberton, indicating that irrigation was required when the Rustenburg sites were planted out with *B. coddii*, in order to reduce water stress. Rustenburg was also found to be, on average, 4.6°C warmer than Barberton, but as *B. coddii* growth responds to wet/ dry seasons, as opposed to hot/ cold seasons, it was not felt that this temperature difference would have a negative effect on the growth of the plants. The soil comparisons showed the contaminated Rustenburg sites to be serpentine-like in nature, with respect to Barberton, again giving confidence that the plant would adapt to the conditions occurring at the contaminated sites. However, to ensure optimal growth, nutrient experiments were also performed on *B. coddii* to ascertain the ideal macronutrient concentrations required, without inhibiting nickel uptake. These trials indicated that the individual addition of 250 mg/l ammonium nitrate, 600 mg/l calcium phosphate, 2 000 mg/l calcium chloride, 600 mg/l potassium chloride and 250 mg/l magnesium sulphate enhanced plant growth and nickel uptake, suggesting that, for phytoremediation purposes, these nutrients should be added to the medium in which the plants are growing.

The growth-cycle of naturally occurring *B. coddii* plants in Barberton was also studied in order that seedlings could be germinated, in greenhouses, at the correct time of year so that the plants could be sown as the naturally occurring plants were germinating. From this information the seeds of the plants could be collected at the correct time of year and the above ground biomass harvested when the nickel concentrations were at their highest. It was found that the plants began to germinate as the first rains fell, which was generally at the beginning of

September, and plant maturity was reached at about five months, after which flowers were produced. Seeds were produced from the flowers and these matured and were wind-dispersed one month to six weeks after full bloom, usually during February. The plants then started to die back and dry out and dormancy was reached about nine months after germination, generally in about mid- to late- May. It was found that the nickel concentration was at its highest about one month after the plants had begun to dry out and thus it was decided that the above ground biomass would usually be harvested at the end of April each season, in order to achieve maximum nickel recovery.

Finally, in order that the plant's potential for use in phytoremediation could be fully assessed, field trials at the contaminated sites in Rustenburg were performed. Germination procedures were developed for the mass production of *B. coddii* and it was found that, although fully formed plants could be propagated in tissue culture, it was cheaper and faster to germinate the seeds in speedling trays, containing a zeolite germination mix, in greenhouses. It was also found that the seeds had a low germination rate, due to dehydration of the embryos and thus, in order to obtain the number of plants required, four to five times the amount of seeds needed to be sown. The two-month-old seedlings were transferred to potting bags, containing a mixture of potting soil and RBMR soil, and grown up in the greenhouse for a further three months. This growth period allowed *B. coddii* to adapt to the RBMR soil and also ensured that the plants were relatively healthy when transplanted into three prepared sites at RBMR. The plants were allowed to grow for the entire season after which the above ground biomass, comprising the leaves and stems, was harvested, dried and then ashed in an ashing vessel designed by the author, with the help of Mr K Ehlers. The ashed material was acid-leached with aqua regia in order that the base metals (mainly nickel) and precious metals could be removed from the silicates and carbonised material. The acid solution was then neutralised, causing the base metals (mainly nickel) and precious metals to be precipitated. This precipitate was then smelted with a flux in order that nickel buttons could be formed.

Thus, from all the phytoremediation trials it was found that this process is highly successful in employing *B. coddii* for the clean-up of nickel-contaminated sites. This constitutes the first time that such a complete phytoremediation process has ever been successfully developed with *B. coddii* as the phytoremediation tool. It also appears to be the first time that

phytoremediation has been performed "commercially" to produce a saleable metal product. The success of this project has stimulated Amplats to continue with, and expand it, to include more studies on phytoremediation as well as in the biomining of certain areas containing very low levels of precious metals which, with conventional techniques, were previously not worth mining.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people and organisations for their indispensable assistance with this thesis:

Dr Trevor Anderson, my supervisor at UNP, without whom this thesis would not have fully represented all the work that we have done on phytoremediation, and who put up with all sorts of despair throughout the months of writing.

Mr Andrew Howes, a PhD student at UNP who is currently fully employed by Amplats at RBMR, for all his invaluable advice and support throughout the duration of this continuing project. Andrew was responsible for ensuring that the plants grew at RBMR and, while there was a lot of collaboration of ideas between us, it should be noted that most of the practical work done at RBMR was supervised by him.

All the people in the Analytical Chemistry Department at Amplats Research Centre (ARC) who always helped me so willingly and gave me such good advice especially Fransie Vosloo, Zenja Cruikshank, Delia Gallagher and Ronnie Lennard.

Mr Krause Ehlers, ARC, who was always so enthusiastic and who gave me so much advice and help with the ashing and smelting of the plant material.

Mr Deryck de Vaux, ARC, for giving me the support and time I needed to complete the thesis.

Mr Sandy Lambert, Mr Martin Wright and Mr Euan Kinlock, from Amplats, for their vision in seeing the merit of the project and for supporting both it and me, financially.

All the people at ARC who were able to answer all my queries about precious metals.

Mr Pierre Reinecke, ARC, for his enthusiasm which ensured the start of the commercial section of the project.

Prof. Mike Dutton, University of Natal, who contributed a lot to the conceptualisation of this project.

Mr Neville Plint for his friendship and a lot of hard work and good advice on writing up the thesis.

Ms Ann Haslau for keeping me sane whilst I was at UNP.

My folks for their understanding, patience, and support during a rather trying period.

ACKNOWLEDGEMENTS

Mr Pierre du Preez for putting up with all my frustrations and for always being there when I needed a shoulder to cry on.

Julia and Lynn for allowing me all the time I needed to complete this, but for always being there when it wasn't going well.

Amplats for its continued support of the phytoremediation and biomining projects.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAS	atomic absorption spectroscopy
AGB	above-ground biomass
AR	aqua regia
ARC	Amplats Research Centre
Au	gold
BGB	below-ground biomass
Bq	Bequerel
Ca	calcium
$Ca(H_2PO_4)_2$	calcium phosphate
$CaCl_2.2H_2O$	calcium chloride
conc.	concentrated
Cu	copper
dist.	distilled
dm^{-3}	per decimetre cubed
dpm	disintegrations per minute
Fe	iron
FIC	final insoluble concentrate
GOT	glutamate-oxaloacetate transaminase
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HNO_3	nitric acid
HPLC	high pressure liquid chromatography
ICPMS	inductively coupled plasma mass spectroscopy

ICPS	inductively coupled plasma spectroscopy
K	potassium
KCl	potassium chloride
keV	kilo electron volts
kW	kilowatt
l	litre
L-MDH	L-malate dehydrogenase
M	molar
MCi	milli Curies
Met	metallics
Mg	magnesium
mg	milligram
$MgSO_4.7H_2O$	magnesium sulphate
min	minute(s)
mm	millimetre
mol	mole
N	nitrogen
NAD	nicotinamide-adenine dinucleotide
NH_4NO_3	ammonium nitrate
Ni	nickel
nm	nanometers
NPK	nitrogen, phosphorus and potassium
P	phosphorus
Pd	palladium
PGM	platinum group metals

ppm	parts per million
Pt	platinum
RBMR	Rustenburg Base Metal Refineries
Rh	rhodium
RWB	Rand Water Board
sec	second(s)
soln.	solution
t	tons
TCRI	Tobacco and Cotton Research Institute, Rustenburg
tiss.	tissue
UNP	University of Natal, Pietermaritzburg
vol.	volume

GLOSSARY OF TERMS

antagonist	A chemical substance that interferes with the physiological action of another.
anthropogenic	Origin of pollution from man.
axillary	Growing from the angle between the stem and the leaf.
biomining	The use of accumulator plants to remove valuable metals from a deposit.
capped	To cover an area with a certain material thereby "hiding" the material beneath.
chipping	Breaking off into small pieces.
coke	The solid residue of impure carbon obtained from bituminous coal and other carbonaceous materials after removal of volatile material by destructive distillation – used in making steel.
concentrate	The final material produced from the chemical treatment of raw ore, during which most of the impurities are removed leaving a precious- base-metal rich material.
disjunct	Separated.
ecotype	An organism whose physical structure has recorded the local environment over time.
edaphic	Of or relating to soil influenced by soil rather than by climate.
endemic	Confined to a certain region. In the case of serpentine endemics the plants are confined to serpentine and other metalliferous soils.
entrained	Pulled along with.
explant	Plant formed from parent leaf and/ or stem in tissue culture.
flux	A substance that induces, aids or otherwise actively participates in fusing and prevents the formation of oxides.
forb	A broad-leafed herb other than grass.

glaucescent	Covered with a greyish, bluish or whitish waxy coating or bloom that is easily rubbed off.
heterogeneous	Varied.
induction	The generation of heat in a furnace allowing the fusing of substances, using a flux, at high temperatures.
leachate	A product or solution formed by leaching.
matte	Solid material containing the valuable metals, produced chemically or by smelting a concentrate.
mesophytic	Land plant growing in an environment having a moderate amount of moisture.
metal button	The moulded metal formed by casting the molten matte into a crucible and then letting it cool. The slag rises to the top of the conical crucible, while the metallic metal forms a button at the base of the crucible. When cool and solid the slag and metal button are removed from the crucible and the slag is chipped off the button leaving a clean metal button behind.
muffle	A kiln in which material can be fired without being exposed to direct flame.
mullock	Waste material from a mine.
organogenesis	A controllable process of plant formation involving the formation of shoots and roots in tissue culture (Warren, 1991).
paucity	Smallness of number or quantity.
phanerophyte	Plant which produces seeds.
phytoremediation	The removal of toxic elements from a polluted site or the stabilisation of a site using plants able to tolerate the unfavourable conditions.
plagiotropism	The tendency to grow at an oblique or horizontal angle.
podzolisation	Soil having certain minerals leached from the surface layers into a lower stratum.
pubescence	A covering of short hairs.

refractory	A substance that does not respond to conventional treatment.
scarifier	Agricultural machine with prongs for loosening without turning soil.
sclerophyllous	Lignified.
sedge	Any of numerous grass-like plants of the family Cyperaceae, having a solid stem, leaves in three vertical rows and spikelets of inconspicuous flowers, with each flower subtended by a scale-like tract.
siderophile	Iron loving.
slag	The vitreous mass left as a residue by the smelting of metallic ore.
smelt	To melt or fuse (ores) in order to separate the metallic components.
smelter	The apparatus for smelting material.
thermolabile	Easily decomposed or subject to a loss of characteristic properties by the action of heat.
tissue culture	Sterile technique for propagating plants having the identical genetic make-up.
ultramafic	Rocks containing high concentrations of magnesium and iron.
xeromorphic	Adapted to a hot, dry environment.

CHAPTER 1

INTRODUCTION

1.1 *Introduction*

As the twenty-first century draws closer, so our attention is drawn more and more towards people's effect on the environment. Until recently, nature has tended to be seen as a never-ending source of supply of commodities such as mineral energy, food, water, and fertile and habitable land. It is only now, as the supplies begin to dwindle, that we are realising that people are, perhaps, too powerful even for nature. With this in mind, more and more research is focussing on ways to preserve and remediate our environment using natural tools, so that further pollution is minimised.

Although the laws in third-world countries on environmental protection and pollution prevention tend to be less stringent than those in the first world, it is becoming apparent to the third-world industries that their first-world consumers are concerned enough about the global environment to start putting pressure on the way third-world industries produce their commodities. For example, there is no point in buying a product to reduce pollution in the first world if, in producing it in the third-world, large amounts of pollution have been created. It is with this realisation that the mining industries of South Africa are looking to "clean up their act".

Amplats, where the author is employed as a biometallurgist, is a large platinum mining company comprising a series of platinum mines in the North-West and Northern Provinces. Recently it has taken cognisance of the fact that, while mining is a fairly destructive process, the effects of mining can be lessened with good environmental practices. Thus began a series of rehabilitation projects, one of which is the phytoremediation of nickel-contaminated soil at one of the Rustenburg mines, employing *Berkheya coddii* (*B. coddii*) – the subject of this thesis. Like any plant used in phytoremediation, *B. coddii* is able to transport and concentrate metals from the soil into the harvestable parts of its above-ground shoots (Salt *et al.*, 1995).

Heavy metals, comprising about thirty-eight elements, are those elements having metallic properties, a density greater than five and an atomic number greater than 20 (Raskin *et al.*, 1994). With respect to biological life, these elements are toxic in excessive quantities and can cause the death of most living organisms. However, certain plants and microorganisms have evolved mechanisms enabling these organisms to cope with conditions of metal contamination.

In order to perform effective phytoremediation with *B. coddii* it was important to first gain insight into the plant as well as any other metal tolerant plants used as phytoremediation tools. Thus *B. coddii* and other metal-tolerant plants were studied with respect to their growth-cycle, how they function, why they are able to take up, accumulate and tolerate heavy metals, where the heavy metals are concentrated and stored, and what their nutritional and climatic requirements are for optimal growth. This literature review addresses the current knowledge on all these aspects and also gives an overview of the many plants occurring world-wide that are able to tolerate and accumulate heavy metals and which may, therefore, be good candidates for phytoremediation projects. In addition, since the metal toxic soil described at the Rustenburg mine waste site is serpentine in nature, the mineralogy, geology, chemistry, toxicity, physical and nutrient characteristics of this soil are also reviewed.

1.2 *The Mineralogy and Geology of Serpentine Soils*

1.2.1 Chemical Composition of Serpentine

The term "serpentine", as used by biologists, is not entirely accurate as it tends to describe most ultramafic rocks i.e. those rich in ferromagnesium minerals, even though they may not contain any minerals of the serpentine group (Proctor and Woodell, 1975). The word ultramafic is derived from the fact that these rocks contain high concentrations of magnesium (*ma*) and iron (*f - ferrous*) – ultra*mafic*. These ultramafic rocks have been recognised by their floral cover for several hundred years owing to the fact that they support highly unusual flora which is usually xeromorphic and impoverished - poor in species and individuals (Brooks, 1987). In some cases the flora differs so greatly where the ultramafic rock occurs that the geology of the area can be mapped from the air due to vegetation patterns alone. It is the ultramafic rocks that generally give rise to the serpentine soils. Thus the term "serpentine" in this thesis relates to the botanist's, rather than the mineralogist's, perspective.

Ultramafic rocks vary greatly in their chemical and mineral composition, but the most commonly occurring ultramafics are the igneous rocks peridotite and dunite and their metamorphic derivative, serpentine (Peterson, 1979). Essentially these ultramafic rocks are composed of various combinations of the minerals olivine, clinopyroxene, orthopyroxene, amphibole, horneblende, talc and serpentine (Brooks, 1987; Proctor and Woodell, 1975). Various accessory minerals such as iron oxides, chromite, spinel and biotite are common (Proctor and Woodell, 1975). The variations in rock composition are reflected in the colour, which may range from shiny green to bluish or almost black (Walker, 1954). Serpentine minerals are derived from a group of layer silicates represented by the formula $Mg_6Si_4O_{10}(OH)_8$. Significant amounts of elements such as Ni, Co and Fe^{2+} frequently substitute for magnesium in serpentine (Proctor and Woodell, 1975).

The chemical process of serpentisation (Uren, 1992) is as follows:

$$6[(Mg_{1.5}Fe_{0.5}SiO_4)] + 7H_2O \rightarrow 3[Mg_3Si_2O_5(OH)_4] + Fe_3O_4 + H_2$$

In 1956 Faust *et al.* divided serpentine minerals into two classes, A and B, with regards to their minor element content (Proctor and Woodell, 1975). *Class A* serpentines are those derived from ultramafic igneous rocks by hydrothermal alteration, and have a relatively rich content of minor elements, particularly nickel, chromium, cobalt and scandium. *Class B* serpentines are derived by the alteration of magnesium-rich rocks such as various types of metamorphic dolomites and limestones, and have a much lower minor element content as well as a lower iron content. The *Class B* serpentines are much more likely to be associated with calcium-containing minerals and thereby contain higher calcium levels than do the *Class A* serpentines. This calcium appears to have an ameliorative effect on the toxicity of the heavy metals associated with serpentine as well as on the magnesium. Due to its low calcium and high nickel contents (section 4.3.2), it is thought that the serpentine on which *B. coddii* naturally occurs in Barberton, is a *Class A* serpentine.

Trace elements are contained in ultramafic rocks and it is largely their abundances that control the vegetation types, which may be supported by serpentine-derived soils (Brooks, 1987). In general it is the siderophile (iron-loving) elements like cobalt, chromium, iron and nickel that are strongly enriched in ultramafics relative to other rock-types. Plant nutrients such as

calcium, nitrogen, phosphorus and potassium have much lower relative abundances and this significantly restricts the vegetation types that can be supported by soil resulting from the weathering of this rock.

1.2.2 Weathering of Ultramafic Rocks

The weathering of ultramafic rocks by various processes produces the "serpentine soils", high in magnesium and low in calcium, which support the serpentine floras (Peterson, 1979). The proportion of weathering products (minerals formed, or substantially altered, during weathering) and weathering residues (minerals present in the parent rock which remain unchanged in the soil) in serpentine soils is variable (Proctor and Woodell, 1975), although most contain both weathering products and residual minerals in the clay fraction. All serpentine soils contain clay although the content tends to vary greatly - some soils are exceedingly rocky and stony, others have a very high clay content, while others show an intermediate condition between these two extremes.

The type of soil produced from the weathering of ultramafic rocks depends not only on the parent material but also on factors such as climate, relief, time and biological activity (Brooks, 1987; Proctor and Woodell, 1975). Thus an extremely diverse range of soils is produced from ultramafic rocks making it difficult to generalise about the composition of a "typical" serpentine soil. Most serpentine soils have the following common characteristics:

- High concentrations of siderophile elements;
- Low concentrations of plant nutrients e.g. nitrogen, phosphorus and potassium, and a low Ca/Mg ratio compared with non-serpentine soils;
- Lower clay contents than "normal" soils; and
- Thin soils with a number of rock outcroppings (Anonymous, 1997).

Generally the soils that form are reddish, brown or grey in colour at the surface, often changing to yellowish or greenish in the lower layers (Walker, 1954). The colours are due to the chemical and physical properties of serpentine, discussed briefly in the next section (1.3).

1.3 *The Physical and Chemical Properties of Serpentine Soil*

Serpentine soils tend to be fairly infertile mainly as a result of the chemical composition of the soil, although the physical nature of the soil (particularly its well-drained character) also has a negative effect on plant growth. It must be noted that although some toxic chemicals may be present in high concentrations in the soils only that amount available to the plant (plant available metals) should be taken into account when considering plant toxicity (Brooks, 1987).

1.3.1 Physical Properties of Serpentine Soils

The ecology of a soil tends to determine the type of plant able to grow on it (Antonovics *et al.*, 1971). For instance, a coarse soil will have a low water capacity and, if it is dark in colour, a high temperature, and so will support plants with deep roots and narrow leaves. A fine soil will have a higher water capacity and, if light in colour, a low temperature and so supports plants with shallow roots and a generally tufted appearance. Many serpentine soils in temperate regions are shallow and stony causing a low water capacity as well as restricted soil depth for root penetration (Proctor and Woodell, 1975). Thus plants growing in these regions will often experience drought conditions which may be exacerbated by a scantiness of vegetation causing the plants to be exposed to excessive wind and isolation. This, in turn, causes instability of the soil showing that the plant-soil interaction is "two-way". Serpentine soils that develop on level ground are not very shallow or stony, but have poor drainage, due to a lack of course material, causing, once again, poor plant growth. The serpentine soil in Barberton, which is dark in colour but deep and non-stony with a fairly high water capacity during the rainy season, supports the relatively shallow root system of *B. coddii* although, as the plant is a shrub, the vegetation is not tufted in appearance.

The soil properties of serpentine soils are also unfavourable e.g. lumpiness, compactness, viscosity and stickiness when wet, vertical cracking when dry and poor water permeability, again leading to poor plant growth. However, the physical properties alone do not account for the unfavourable vegetation conditions of serpentine soils - it is the nutrient and toxic properties of the soil that tend to have the greater influence.

1.3.2 Chemical Properties of Serpentine Soils

The lack of plant nutrients in serpentine soils plays a major role in the infertility of these soils although, at pH 6.8 (the pH of most serpentine soils), the nutrients are optimally available (Brooks, 1987). These low levels of nutrients are mirrored in the serpentine plants - they contain much lower levels of nitrogen, potassium and phosphorus than do plants growing on non-serpentine soils, although often the concentration of these nutrients is greater in the leaves of serpentine plants than in the surrounding soil. (Medina *et al.*, 1994). This indicates that plants growing on serpentine soils can accumulate nutrients.

1.3.2.1 Nitrogen, Phosphorus and Potassium

It appears that serpentine soils contain low levels of at least one of the nutrients nitrogen, phosphorus or potassium (NPK) (Proctor and Woodell, 1975) and this is thought to have a negative effect on plant growth. However, in experiments where serpentine soils have been fertilised with NPK it is often seen that there is no satisfactory improvement in plant growth, and in some cases plants have shown a marked increase in the severity of toxicity symptoms (Proctor, 1971a). Why this occurs is not yet fully understood but it is thought that the addition of nitrate anions might result in a greatly increased solubility of toxic cations, such as magnesium.

Generally, phosphorus levels in serpentine soils are low, owing to the fact that serpentine has a very high affinity for soluble phosphorus (Brooks, 1987). Interactions between heavy metals and phosphate tend to exacerbate the effects of a phosphate shortage. For instance, iron, aluminium and chromium all seem to cause a reduction in the ability of plant roots to translocate phosphorus from soil to leaves (Proctor and Woodell, 1975).

1.3.2.2 Calcium and Magnesium

Some researchers feel that the low Ca/ Mg ratio in serpentine soil plays a significant role in its infertility, whilst others think that the Ca/ Mg ratio has very little effect on the serpentine vegetation (Proctor and Woodell, 1975; Brooks, 1987). There are three ways in which calcium and/or magnesium may affect the serpentine vegetation and these are discussed below.

The factor that requires first consideration is magnesium toxicity. The assimilation of mineral nitrogen appears to be adversely affected by high magnesium concentrations (low Ca/ Mg ratio), as is subsequent nitrogen metabolism and, therefore, protein production (Proctor and Woodell, 1975). Nitrate absorption into the roots also appears to be much lower when it is supplied as magnesium nitrate, as opposed to calcium nitrate, again indicating the adverse affects of high levels of magnesium. Not only is the magnesium itself toxic but it also has an antagonistic effect on other plant nutrients, such as potassium and phosphorus, rendering them unavailable to the plant (Yang *et al.*, 1985). Serpentine flora has thus developed specific tolerance mechanisms, including exclusion, to cope with high magnesium levels (Gabbrielli and Pandolfini, 1984).

Calcium deficiency is the second factor that requires consideration. If calcium is present in high enough ratios, it is able to counteract the deleterious effects of ions such as Na^+, Ni^{2+}, Mg^{2+} and H^+ on plant growth. However, the deficiency of calcium in serpentine soils results in a lowered tolerance of plants to the other elements present in the soil, and thus limits vegetation in such areas (Brooks, 1987). The surviving serpentine vegetation has adapted to the low calcium levels by tolerating these deleterious ions whose effects are no longer rendered useless by the calcium. It appears that serpentine vegetation has also adapted directly to the low calcium levels, as there is a decrease in serpentine vegetation with increasing calcium.

The third factor requiring consideration is the actual Ca/ Mg ratio, which is low in serpentine soils, although it is felt that this factor is secondary to the nickel toxicity of serpentine soils (Brooks, 1987). In general, for "normal", healthy plant growth, a Ca/ Mg ratio of greater than one is required; serpentine soil, however, usually shows a ratio of 0.12 - 0.4 (Brooks, 1987) indicating a much lower exchangeable Ca/ Mg ratio than "normal" soil and luxuriant plant growth is not expected. It is interesting to note that in a serpentine area in Scotland, where the Ca/ Mg ratio is 0.7, the vegetation is much more luxuriant than usual indicating the importance of the Ca/ Mg ratio (Brooks, 1987).

1.3.2.3 Nickel and Chromium

Of the three siderophiles, nickel, cobalt and chromium, it is the high level of nickel found in serpentine soils that is most likely to cause infertility (Brooks, 1987) and it is this element to

which *B. coddii* has successfully adapted. About 1% of the nickel in serpentine soil is generally available to the plants, as the hydrated nickel (II) ion, which far exceeds the available chromium and cobalt levels (Peterson, 1979).

Until recently nickel was not generally regarded as an essential element in plant nutrition, but it appears this hypothesis has changed. Nickel has been shown to be essential, in trace amounts, for animals, several bacteria and a strain of blue-green algae, and there is increasing evidence that suggests that nickel is also essential for all plant growth – both of accumulators and non-accumulators (Eskew *et al.*, 1984). In this regard, Brown *et al.* (1987) have shown that nickel is essential for plant growth, plant senescence, nitrogen metabolism, iron uptake and it may also play a role in phytoalexin synthesis and plant disease resistance. Furthermore, Eskew *et al.* (1984) have shown that the absence of soil nickel causes the accumulation of toxic urea in soybeans (*Glycine max* [L.] Merr.), resulting in necrosis of leaflet tips.

Although *B. coddii* has adapted to the typical nickel concentrations in serpentine soil, it is useful to be familiar with the factors that can cause nickel toxicity in case such problems arise when growing *B. coddii* at the contaminated Rustenburg sites. The factors that contribute to nickel toxicity are:

- pH – the lower the pH of a soil, the higher the availability of nickel to the plant, resulting in an increase in soil toxicity;
- Iron deficiency - if the plant availability of iron is much lower than that of nickel, the soil becomes toxic as there is no iron to compete with the nickel;
- Low levels of organic matter in serpentine soil means lower nickel chelation by organic acids, which compounds nickel toxicity; and
- Low levels of calcium, nitrogen, potassium and, to a certain extent, magnesium compound the nickel toxic effect of serpentine soils.

The chromium content in serpentine soils, although variable, tends to be in the order of 5 000 µg/g (Brooks, 1987). However, the availability of chromium to plants in serpentine soils at their mean pH of 6.8 is very low suggesting the chromium toxicity should not be a problem among serpentine flora. Indeed there is no real evidence that chromium plays a toxic role in

serpentine soils, and there is also no evidence that plants develop mechanisms to accumulate or even tolerate chromium.

1.4 *Serpentine Vegetation and Adaptations to Nutrient Stress*

In 1932 Braun-Blanquet stated, "the serpentine ridges of the Alps in their dark deathly hardness are among the most depressing lonely phenomena of nature, and the popular reference of "Tote Alp" (dead alp) is quite appropriate". Thus, in general, ultramafic soils are fairly barren and there is a pronounced difference between the type of species populating these soils and those found on non-ultramafic soils (Proctor and Woodell, 1975).

Initial work on serpentine vegetation showed that tolerant plants seemed to only belong to one or two species. It was thus thought that these plants could be placed into a class of their own. However, as work has progressed, plants from families as widely separated as Cyperaceae, Compositae (the family to which *Berkheya coddii* belongs), Orchidaceae, Gramineae, Leguminosae and Ranunculaceae have been found growing on, and tolerating, serpentine soils (Appendix 1) (Antonovics *et al.*, 1971).

Serpentine flora is interesting in that very few of the plant species are endemics (also known as *bodenstets, paleoendemics* or metallophytes). Instead, most of the plants are able to grow on both serpentine and non-serpentine areas and such plants are known as *bodenvag* plants, *neoendemics* or pseudometallophytes (Kruckeberg, 1954; Baker, 1987). Pseudometallophytes are those plants that have evolved on a metalliferous soil, being derived from neighbouring non-metallophytes in response to the unfavourable edaphic and environmental conditions of the substrate (Kruckeberg, 1954). The northern European serpentine plants tend to be *neoendemic* as their flora has only formed recently from the last glaciation (Wild and Bradshaw, 1977). Metallophytes have a widespread but highly disjunct distribution (Proctor and Woodell, 1975) and it is assumed that these plants colonised, at one time, a much greater range of substrates but have now been restricted to their present sites by competitive pressures or climatic changes. Most of the southern African serpentine flora is *paleoendemic*, as plant species were not eliminated in the last glaciation (Wild and Bradshaw, 1977).

Serpentine vegetation is extremely variable but generally the plant species are paucitic, dwarfed and xeromorphic (Brooks, 1987) with chloritic, narrow and glaucescent leaves, having a strong sclerenchymatic development. The root systems are enlarged to allow for maximum nutrient uptake, as the nutritional imbalance at the root level becomes more pronounced on ultramafic soils (Medina *et al.*, 1994). Plant species of serpentine soils tend to have fewer stomata, more tightly packed palisade cells and a higher vacuolar sap content than do plants growing on non-serpentine soils. *B. coddii* has fairly narrow leaves that are neither chloritic or glaucescent. The plants do, however, have the large roots systems usually associated with serpentine flora.

No forestation of serpentine flora occurs (Proctor and Woodell, 1975) and *B. coddii* is typical of the shrubby growth usually associated with these soils, although it shows none of the usual plagiotropism. The most common vegetation occurring on serpentine soils consists of the odd coniferous tree in combination with various sclerophyllous shrubs and grass-like plants including forbs. There are fewer phanerophytes on serpentine soils than on other soils, although *B. coddii* is a fairly prolific seed-producer. In some species, particularly those found in regions with a marked subtropical dry season, greater pubescence occurs and the plants are distinctively coloured (Ernst, 1972). In some cases trees or shrubs, not indigenous to serpentine soils, have been planted and, although able to grow, they tend to be very stunted and never reach full maturity. An example of this is seen in Barberton, Mpumalanga, where blue gums have been planted on a serpentine site. The trees are thirteen years old but are equivalent in size to 5-year-old trees growing on normal soils. The general lack of forestation and the dwarfed growth of plants occurring on serpentine soils, causes greater species diversity than in non-serpentine areas (Proctor and Woodell, 1975). For instance, some of the most diverse and extensive serpentine floras are found in the serpentine area of New Caledonia (Brooks *et al.*, 1990). Other important serpentine areas include Greece, Turkey, Cuba, Zimbabwe, and the state of Gioás in central Brazil.

Generally, infertile habitats are dominated by perennial, rather than annual, plant species (Chapin, 1980) and this is true of *B. coddii*, which is a perennial, deciduous plant. Like most deciduous plants, *B. coddii* stores its minerals and carbohydrates in its non-photosynthetic tissues, which are then utilised for rapid spring growth. In order to lessen leaching of minerals from its leaves by contact with water, *B. coddii* has a well-developed smooth cuticle and a

fairly erect leaf angle. It is thought that, like other deciduous plants, *B. coddii* translocates half or more of the maximum nitrogen and phosphorus content of leaves to other parts of the plant before leaf abscission (Chapin, 1980). Calcium, which is immobile in the phloem, and potassium are not translocated at all and tend to be made available to the plant in the leaching of the leaf litter.

Figure 1 shows that infertile soils generally give rise to slow growing, stress tolerant species having low capacities for photosynthesis and nutrient absorption. However, as reported by Chapin (1980) no species exhibits all of these traits and certain characteristics e.g. high root absorption capacity and slow relative growth, seldom occur together. Plants themselves also play an important role in changing the nature of the toxic habitat (Antonovics *et al.*, 1971), as the root systems stabilise the soil and the aerial parts offer some shelter to subsequent colonisers. Above all, however, they contribute organic matter and humus to the surface layers of the soil, which not only increases the nutrient status of the soil and forms complexes with the metals, but also improves the texture of the soil.

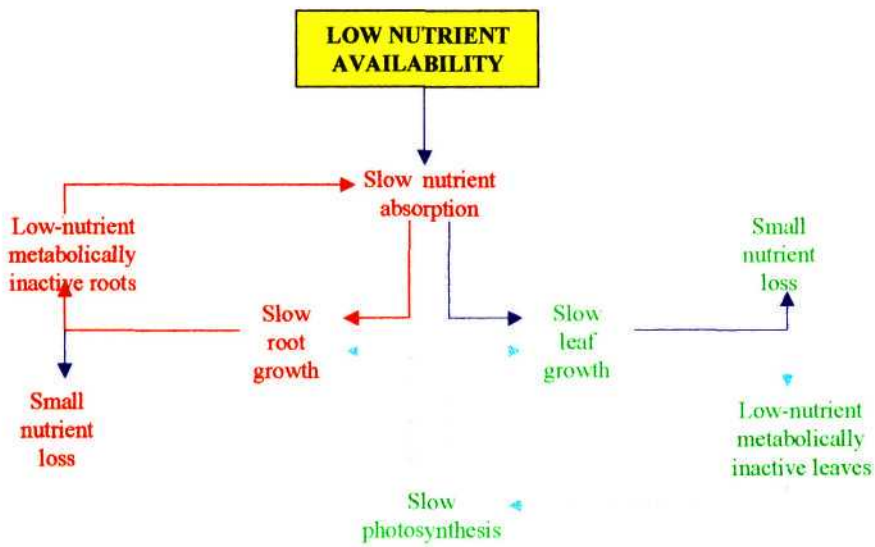


Figure 1: Nutrient-Stress Tolerant Strategy (Chapin, 1980)

1.5 *Hyperaccumulators*

Hyperaccumulators have been defined by Baker and Brooks (1989) as those plants that contain over 1 000 mg/kg of any of Co, Cu, Cr, Pb, or Ni, or over 10 000 mg/kg (1%) Mn or Zn, in

the dry matter (Appendix 1). These plants are all limited to serpentine soils where a high level of mineralisation occurs. Nickel hyperaccumulators, including *B. coddii*, which contains over 1% nickel (dry weight) (Morrey, *et al*; 1989; Proctor and Woodell, 1985; Baker and Brooks, 1989) in its leaves, are the most common hyperaccumulating plants, with over 300 having been reported (AJM Baker, *pers. comm.*). Cobalt, copper and zinc hyperaccumulators are also fairly common (Raskin *et al.*, 1994) but only a few manganese, lead, cadmium, chromium and selenium hyperaccumulators have been described.

In non-nickel accumulating plants, nickel concentration tends to be greatest in the roots and reproductive organs with levels ranging from 0.05 – 5 μg/g dry mass (Ogoleva and Cherdakova, 1981). Conversely, in nickel-hyperaccumulators, the nickel concentration is greatest in the leaves (1 000 – 35 000 μg/g dry mass) and it is always greatly elevated over the concentration that is provided by the soil (Hausinger, 1993). Nickel distribution in hyperaccumulators also tends to be greater in the growing tissues than in the less metabolically active tissues although, upon plant maturation, it is remobilised (Hausinger, 1993).

The evolutionary significance of metal hyperaccumulation remains obscure although it appears to be closely linked to metal tolerance, which enables plants to colonise otherwise toxic soils (Baker and Brooks, 1989). Plants growing in soils contaminated with various heavy metals tend to possess tolerance to all these metals, although hyperaccumulation of only the principal metal present seems to occur (Baker, 1987). Boyd and Martens (1991), have given four hypotheses as to what may have caused the hyperaccumulation of toxic metals in plants to occur:

1. As a means of metal tolerance through disposal of the metal from the plant by annual leaf loss. This causes the nickel concentrations in the rhizosphere to be reduced while those in the topsoil are increased allowing the nickel to be easily washed away by natural precipitation (Hausinger, 1993);
2. As a mechanism for drought resistance, since transpiration could be reduced or osmotic pressure increased by epidermal nickel accumulation;
3. By initial inadvertent uptake of metals, to which certain plants were able to adapt; and

4. In defence against herbivores and pathogens as workers have found that, when a hyperaccumulating plant is grown on a non-mineralised soil for a few years, the plant becomes more susceptible to fungi and insects (Gabbrielli *et al.*, 1997).

The hyperaccumulator studied in this project, and reviewed in section 1.6 below, is *Berkheya coddii* – a nickel hyperaccumulator endemic to the serpentine soils of the Barberton area, Mpumalanga, RSA. In accordance with observations on other serpentine sites the area is covered with a relatively diverse flora including grasses and woody, shrubby plant species (Wild and Bradshaw, 1977; Baker and Brooks, 1989 and Morrey *et al.*, 1989).

1.6 *Berkheya coddii:* Taxonomy, Morphology and Habitat

Berkheya coddii is endemic to the Barberton area of Mpumalanga, RSA. It belongs to the Compositae family, of the Asterales order, in the Asteridae subclass of the Dicotyledonae superorder of plants, and was first described by Roessler in 1959 (Mabberley, 1987; Roessler, 1959). He gave the following phenotypic description of the plant (paraphrased from the Latin description): "*Berkheya coddii* (Figure 2) is an upright, herbaceous perennial, about 1.5 metres in height when mature. It has one or more evenly leafed stems branching from a basal rootstock. The leaves are alternate, sessile, membranous, ovate-lanceolate (broadest below the middle) and pointed at the end. The leaves are approximately 10 - 15 cm long and 3 – 4.5 cm wide, with approximately 1 mm long teeth on the margin. The leaves are bigger at the bottom of the plant, becoming smaller just below the capitulum which itself has 8 – 10 spined and excurrent bracts. The head on top of the stem consists of a flower with an almost corymbus inflorescence shape, approximately 4 – 6 cm in diameter. It has a lobed corolla with many flowers in a disc on the thickened apex. The achenes are approximately 1.5 cm long and concealed. It also has a pappus."

Berkheya coddii is found in six small regions of southern Mpumalanga (Figure 3) where outcrops of serpentine occur along the Barberton greenstone belt (Balkwill *et al*, 1997; Anhaeusser, 1985). Like most serpentine flora it is unable to compete with other plant species and so is confined to these ultramafic areas. The underlying geology of the serpentine soil on which *B. coddii* occurs is similar to that of most serpentine soils: there is a basal dunitic unit, followed by an olivine peridotite, through harzburgite, orthopyroxenite, ortho plus clino

pyroxenite and terminating in a gabbroic unit (Wuth and Barnes, 1986; Proctor and Woodell, 1975; Brooks, 1987).

The fact that *B. coddii* is a hyperaccumulator and indigenous to Southern Africa has led to interest in it as a tool in phytoremediation of certain nickel-contaminated Rustenburg mine sites. Obviously, before *B. coddii* could be used in phytoremediation, studies on whether or not it would be able to survive the Rustenburg conditions needed to be performed. One of the areas of concern was whether it would be able to adapt to the Rustenburg climate and soil conditions (sections 4.3.1 and 4.3.2).

Figure 2: Mature Flowering *Berkheya coddii* Plant at Six Months

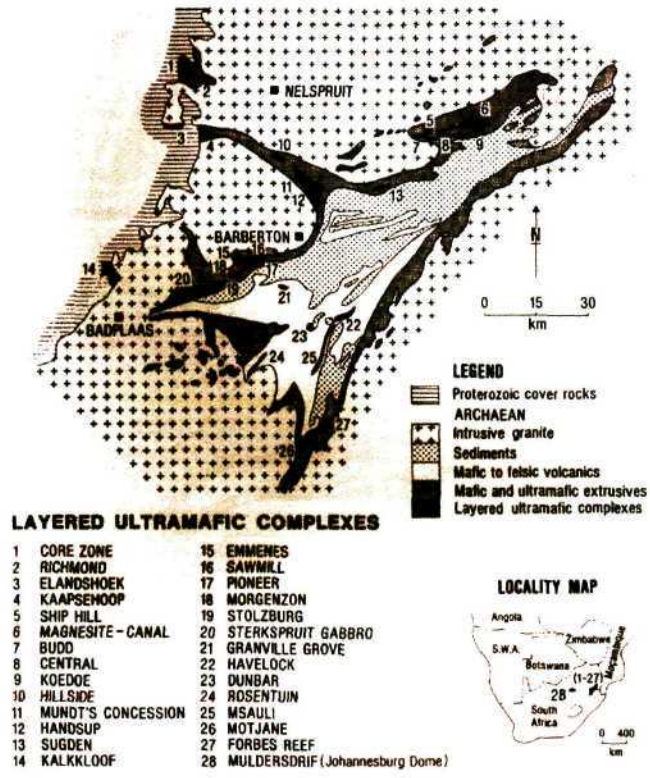


Figure 3: Locality Map Showing the General Geology of the Barberton Greenstone Belt and the Distribution of Layered Ultramafic Complexes. (Anhaeusser, 1985).

B. coddii is found at the sawmill (16) on the Queens River.

1.7 *Metal Uptake and Transport in Berkheya coddii and Other Plants*

Compared with species from more fertile soils, plants growing on infertile soils, like serpentine, usually exhibit a low nutrient absorption rate per plant (Chapin, 1980). This causes serpentine flora to absorb fewer nutrients, compared with "normal" plants, when growing under high nutrient conditions. However, when growing under low nutrient conditions, serpentine flora is able to absorb similar quantities or even more nutrients than "normal" flora growing under similar nutrient conditions. In order to maximise its nutrient uptake *B. coddii*, like other plants growing on infertile habitats, has a high root : shoot ratio, and its large root biomass is extremely long lived in order to allow maximum nutrient uptake. In addition it is thought that certain mycorrhizal fungi, associated with some serpentine flora (Gonçalves *et al.*, 1997),

improve the mineral nutrition of soils by enhancing mineral uptake by the plants. At present, it is uncertain whether *B. coddii* benefits from these mycorrhizal associations. Diazotrophic bacteria also confer greater tolerance of vegetation to serpentine soils (Mercky, *et al.*, 1997) as they allow plants to utilise dinitrogen, which causes root development to be enhanced. These bacteria appear to have become resistant to nickel as a result of nickel salts being continuously liberated by the decay of nickel-rich leaf litter, rather than as a result of the nickel ions generated by weathering of nickel-containing rocks (Schlegal *et al.*, 1991). This suggests that nickel-hyperaccumulating plants give rise to a nickel cycle causing continuous percolation of the topsoil by nickel ions.

The large root system that permits nutrients to be taken up by *B. coddii* also allows nickel to be accumulated by the plant, and the rate and concentration of this accumulation depends on the nickel ion concentration in the surrounding soil, as well as organic and inorganic particulates able to chelate the nickel (Hausinger, 1993). Generally, only hydrated nickel in the 2+ valency state as $Ni(H_2O)_6^{2+}$ in the soil, is available to the plants, although many plants are able to mobilise soil-bound metals in other valency states once an area (zone) around the roots has been depleted of elements (Uren, 1992; Salt *et al.*, 1995; Levinson, 1974); it has not yet been established whether or not *B. coddii* is able to mobilise soil-bound metals. Several chemical processes are involved in zone repletion, of which the two main processes are:

1. Mass flow of the soil solution to replace the liquid directly absorbed into the root, and
2. Diffusion of the constituents of the soil solution toward the root zone down the concentration gradient created by absorption of these elements into the root.

Zones can also be repleted using biological processes. Work done on grasses shows that plants, either directly or via mycorrhizal fungi or root-colonising bacteria, are able to solubilise metals (Crowley *et al.*, 1991) and it is thought that the mechanisms used are common to all plants, including *B. coddii*. In this solubilisation of metals, metal-chelating molecules such as phytosiderophores produced by plants, or siderophores produced from root-colonising microorganisms, are initially secreted to chelate and solubilise the metal, generally in response to iron and zinc deficiencies. Metal-bound reductases, released by the plasma membrane, then reduce the soil-bound metals (Hausinger, 1993), after which they are solubilised by the plant

roots through acidification of the soil environment with protons. The environment created by all these plants at their root tips and in the adjacent soil appears to be so corrosive that even minerals normally considered to be relatively stable in soils, such as silicates and sulphides, can be extracted (Levinson, 1974).

Once it has been removed from the soil, the soluble nickel moves towards the actively absorbing regions of the roots where it is taken up by the apoplastic or symplastic pathways (Uren, 1992; Salt *et al.*, 1995). All divalent cations appear to be taken up by a common absorption process in vascular plants (Hausinger, 1993) and there is evidence to suggest that nickel is actively absorbed across the plasmalemma using the same carrier site as copper and zinc. In *B. coddii* it also appears that the soluble nickel can be absorbed across the plasmalemma of the roots using the calcium carrier site (section 4.3.5.2).

Once the nickel has entered the root it is translocated, in a non-toxic, bound form, from the root, via the xylem sap, to all plant tissues. Nickel distribution within the plants is not uniform and varies with tissue, the age and the species of the plant (Hausinger, 1993). The nickel enters the xylem vessels by crossing the casperian strip by symplastic transport, as apoplastic transport is blocked (Hausinger, 1993; Neumann and Chamel, 1986). It appears that this symplastic transport is the rate-limiting step in metal translocation to the shoot, as the xylem cell walls have a high cation exchange capacity, causing metal cation movement to be severely retarded. In addition to translocation from the roots, via the xylem, nickel can also be remobilised from one tissue to another e.g. from an apical to a lower leaf. This movement of nickel tends to be mediated by phloem, again in a bound form (Hausinger, 1993; Neumann and Chamel, 1986). In this regard, Howes (*pers. comm.*) has reported the presence of radioactive ^{63}nickel (^{63}Ni) in aphids that have been grown on *B. coddii* treated with ^{63}Ni. As aphids feed exclusively on phloem sap, this result suggests that nickel in *B. coddii* can be remobilised from one tissue to the other.

It should be noted that no in depth study has been performed on the mechanisms of nickel uptake within *B. coddii*, but it is expected that, as the mechanisms given above hold true for metal distribution in most plants, they will also be the mechanisms by which nickel is taken up and distributed within *B. coddii*. It is hoped that more work will be performed on *B. coddii* to

elucidate the mechanism by which nickel is rendered plant-available in the soil, as well as determining the method by which nickel is accumulated by the plant.

1.8 *Mechanisms of Metal Tolerance and Storage in Berkheya coddii and Other Plants*

In the plant environment heavy metals tend to act as stress factors and can reduce or totally inhibit plant growth (Baker, 1987). Plants have, therefore, developed avoidance mechanisms, to protect them externally from the influences of the stress, and/or tolerance mechanisms, to protect them internally from the stress (Tomsett and Thurman, 1988). Every organism, regardless of whether it lives in a metal-enriched environment or not, is able to cope to a certain extent with non-essential metals as well as excessively available essential metals (Ernst *et al.*, 1992). Plants growing naturally on metalliferous soils, however, have better developed, largely internal, mechanisms allowing resistance to be achieved by true tolerance. Metal tolerance is a constitutive property present in every cell, tissue and organ of such plants.

Most metal tolerance appears to be genetically determined, although environmentally-induced cadmium tolerance has been seen in four grass species (Tomsett and Thurman, 1988). Initially it was thought that metal tolerances were independent of each other and that populations tended only to evolve tolerance mechanisms to specific metals present in their soils, although nickel and zinc tolerance mechanisms seemed to be linked (Macnair, 1993). This theory has, however, been challenged, as there are a number of cases where plant populations tolerant to one metal also show low level tolerance to another metal. This suggests that a number of tolerance mechanisms might be coded for by the same chromosome, although, as there is no unconditional co-tolerance, it is thought that different genes are required for a plant to be tolerant to more than one metal (Macnair, 1993).

Three types of plant-soil relationships (Figure 4) have been identified in areas of heavy metal contamination. These are:

1. *Accumulators* (Figure 4a): where metals are concentrated in the above-ground biomass (AGB) to levels greater than that present in the soil regardless of whether the soil metal levels are high or low e.g. the nickel hyperaccumulator *B. coddii*;

2. *'Indicators'* (Figure 4b): where the uptake and transport of metals to the AGB are regulated such that internal concentrations reflect external levels e.g. the copper indicator *Becium homblei* (Baker *et al.*, 1988);

3. *Excluders* (Figure 4c): where metal concentrations in the AGB are maintained constantly lower than that in the soil over a wide range of soil concentrations up to a critical soil value, above which the mechanism breaks down and unrestricted transport results e.g. many of the grasses found on serpentine soils (Baker, 1981).

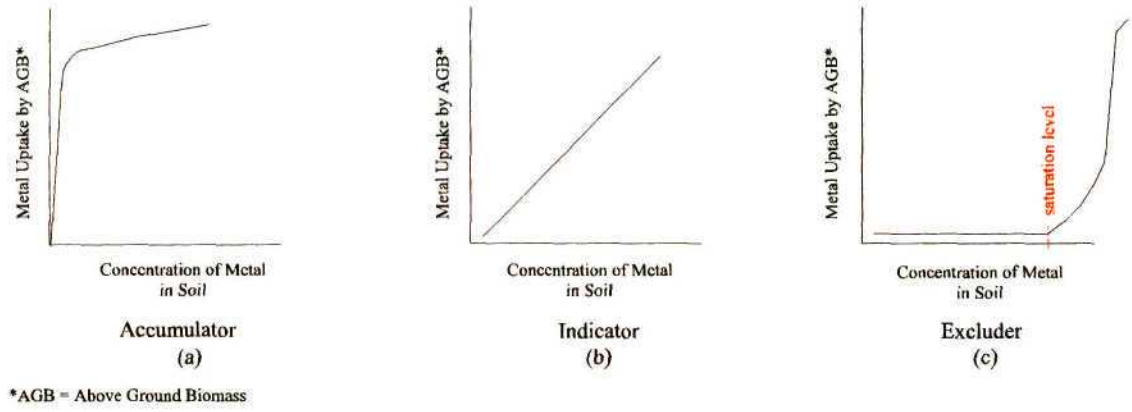


Figure 4: Typical Heavy Metal Uptake Profiles that Characterise Accumulators, Indicators and Excluders (modified from Baker, 1981)

Accumulators and excluders represent the extremes of the physiological response of plants to heavy metals. Plant species occurring on metalliferous soils tend to be accumulators while excluders are usually plant species, like grasses, that have developed races able to grow on both metalliferous and "normal" soils. Indicator behaviour is thought to be an intermediate type of response that may or may not reflect a direct link between metal uptake and metal tolerance (Baker, 1981). The mechanisms employed in the above responses differ amongst the different endemic plants. In the accumulators and, to a certain extent the indicators, the plants prevent the heavy metals from reaching their sites of toxic action within the plant through internal mechanisms, either by biochemical detoxification or by compartmentalisation of the metal within the cell or the plant. By contrast, the excluders rely on external tolerance mechanisms to prevent the metal from entering the plant (Antonovics *et al.*, 1971; Meharg,

1993). The external and internal tolerance mechanisms are discussed in sections 1.8.1 and 1.8.2 below.

The majority of serpentine plant species are neither accumulators nor hyperaccumulators as they are unable to tolerate high metal concentrations in their shoots and tend instead to accumulate toxic metals in the symplasm of their roots (Gabbrielli *et al.*, 1990). The low metal value in the xylem indicates that the endodermis functions as an extremely efficient physiological barrier (Ernst, 1972).

1.8.1 External Tolerance Mechanisms

Since very few plants exhibit external tolerance mechanisms such methods have not been widely explored. In external tolerance, heavy metals are confined to the apoplasm and are prevented from entering the plant and reaching sensitive metabolic sites. Most excluder mechanisms in plants have a saturation point (Figure 4c) beyond which they are unable to operate (Taylor, 1987). Furthermore, plants excluding heavy metals tend to have decreased root growth due to depressed mitotic activity in the root tips when exposed to certain levels of the heavy metal (Gabbrielli *et al.*, 1990). Some external tolerance mechanisms are not strictly "mechanisms" as they are not under the control of the plant. These "mechanisms" are as follows:

- The chemical form of the metal may not be readily soluble in water, or the surrounding water may rapidly dilute it. In both cases the effective concentration is too low to greatly affect the plant. In this regard, it has been noted that general plant growth increases on toxic soils where the soil is wettest (Antonovics *et al.*, 1971).
- The amounts of freely diffusable metal ions in the soil may be low causing the availability of the metal to the plants to be extremely low. For instance, if sulphate-reducing bacteria occur in the environment near the plants they may cause any free metal ions to be precipitated as the metal sulphide, rendering the metal unavailable to the plants (Antonovics *et al.*, 1971).
- Metal ion antagonisms may occur causing a reduction in the availability of a particular metal ion through, competitive effects in solution, the formation of metal complexes, or

through interference in metal uptake by competing for entry sites into the plants (Antonovics *et al.*, 1971; Brooks, 1987).

- Mycorrhizal fungi in, or on, the root system may cause metal exclusion or, at least restricted metal uptake (Baker, 1987). In some soils the metal acts as a fungicide and so no mycorrhizae are found.

Known excluder mechanisms where the plant exerts more direct control include:

- Immobilisation of the metal at the root cell wall (Taylor, 1987) causing a reduction in uptake of metals into the symplasm. This is not a general tolerance mechanism.

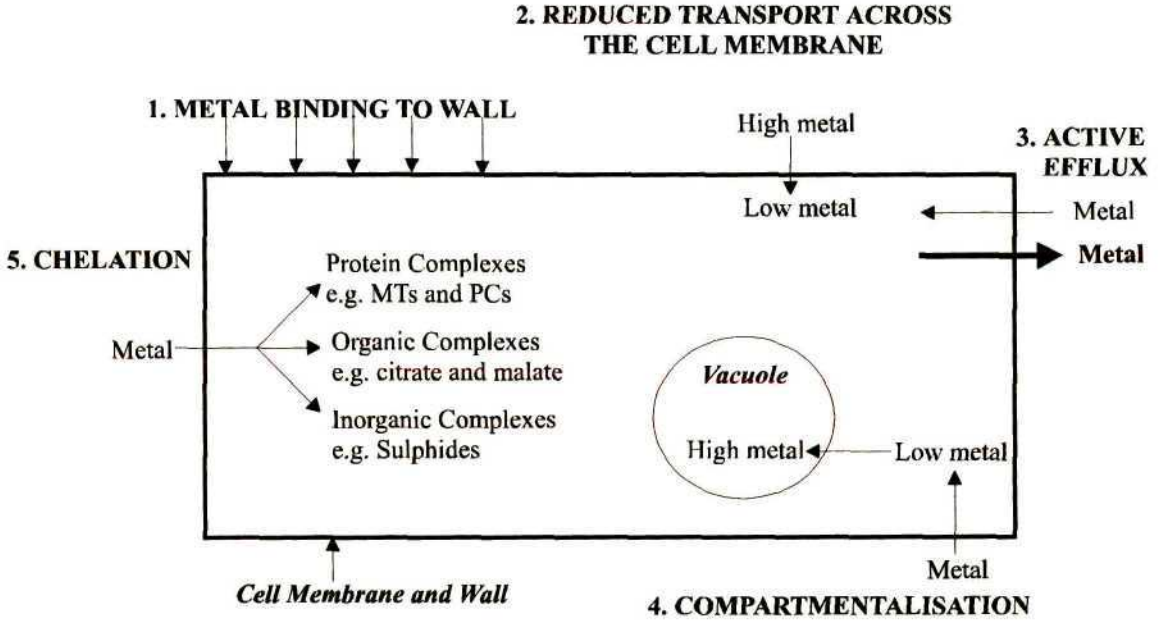
- Formation of a redox barrier at the plasma membrane (Taylor, 1987). Since the solubility of some metals depends on their redox state, plants growing on, for instance, reduced substrates might be more stressed by metal toxicity than those growing on more oxidised substrates. In this regard, some plants growing on reduced substrates have established a gradient of redox potential in the rhizosphere such that reduced ions in the soil must pass through an oxidised zone before entering the plant. This renders the metal less soluble and consequently less available and less toxic to the plant.

- The plant may create conditions that cause a lack of permeability to heavy metals (Antonovics *et al.*, 1971). For instance, if the cell lowers its pH to very acid levels it would, in theory, produce a net positive charge at the cell surface, which would reduce the uptake of cations through repulsion forces.

1.8.2 Internal Tolerance Mechanisms

Internal tolerance mechanisms can be defined as those where metals enter the plant and tolerance is achieved by cell-wall binding, active pumping of ions into vacuoles, complexing by organic acids, and possibly by specific metal-binding proteins, and the evolution of metal tolerant enzymes (Figure 5) (Taylor, 1987). Enzymatic adaptations and effects on membrane permeability can also be detected. Ernst and Weinert used an electron microscope to show that some metal accumulators deposit heavy metals in the cell wall (label **1** in Figure 5) thereby preventing the metals from entering the more sensitive sites in cell metabolism (Antonovics *et al.*, 1971; Ernst, 1972; Baker *et al.*, 1988). This was confirmed by other workers who found

that, in *Agrostis capillaris*, there was a correlation between zinc tolerance and the cell wall's zinc-binding capacity (Ernst *et al.*, 1992). This mechanism of detoxification by storage is one employed by many hyperaccumulators (Brooks, 1987). Most of the cell wall-associated heavy metals are bound to polygalacturonic acids, for which the affinity of metal ions decreases in the order, lead, chromium, copper, calcium and then zinc (Ernst *et al.*, 1992).



MTs = metallothionins; PCs = phytochelatins.

Figure 5: Possible Mechanisms of Metal Tolerance in Plants (Tomsett and Thurman, 1988)

Studies have been performed showing that, in all nickel hyperaccumulators, the nickel is not lost from the roots to the surrounding soil, but instead is moved to the shoots of the plant (Reeves and Baker, 1984). Nickel accumulation in *B. coddii* is similar to that of other nickel hyperaccumulators as studies have shown that the nickel concentration is low in the roots and stems of the plant but high in the leaves (Morrey *et al.*, 1989). This suggests that the tolerance mechanisms in *B. coddii*, and many other nickel hyperaccumulators, might involve the immobilisation of the nickel within the plant. This metal immobilisation is thought to involve the complexing of the metal with water-soluble compounds, which would also promote the transport of the metal-complex to other cellular compartments (Figure 5). Such complexes are

usually stored in vacuoles (Figure 5) or the epidermis and sclerenchymous areas between vascular bundles, where they are found as organic acids or sugar phosphates (Antonovics *et al.*, 1971; Ernst, 1972; Thurman and Hardwick, 1988). Two groups of compounds appear to act as complexing agents to promote transport to the vacuole for storage. These are:

- Phytochelatins, which are generally found in plants when exposed to increased concentrations of heavy metals e.g. *Silene cucubalus* has been found to activate phytochelatin synthase in the presence of cadmium, silver, bismuth, lead, zinc, copper, mercury and gold (Grill *et al.*, 1989); and

- Organic acids, which are generally found in zinc and nickel tolerant plants e.g. *Phyllanthus serpentinus* binds its nickel as 42% citrate and 40% malate (Vaquez *et al.*, 1992; Brooks, 1987).

"Phytochelatins" is a generic term used to cover substances that affect plant growth by acting as chelating agents (Kinnersley, 1993). Phytochelatins appear to be related to the metallothionins found in animals and fungi, and so are also classified as class III metallothionins. Like the metallothionins, phytochelatins are low-molecular weight peptides related to glutathione and rich in cysteine residues (Tomsett and Thurman 1988; Rauser, 1990; Thurman and Hardwick, 1988). Much work has been done on phytochelatins in many plants, including *Silene vulgaris* and *Rauvolfia serpentina*, and it appears that they are synthesised by plants in response to heavy metals, after which they bind to the metals. Their role in detoxification seems to be that of a carrier, rather than a buffer in the cytosol, as they transport the metal into the vacuole (Ernst *et al.*, 1992). As yet there is no concrete proof that their main role in the plant is metal detoxification since phytochelatins are also found in a wide range of non-tolerant plant species. However, tolerance to several metals in metal-tolerant plants has been shown to involve phytochelatins, but tolerance to all metals does not result from phytochelatins alone (Steffens, 1990). There is no available evidence for the involvement of phytochelatins in nickel tolerance in *B. coddii*.

In nickel and zinc hyperaccumulators the metal tends to be bound to simple organic acids such as malate, citrate and malonate or derivatives of these acids (Brooks, 1987). In this regard, Lee *et al.* (1978) have recorded higher concentrations of these organic acids in the nickel

hyperaccumulator *Serbertia accuminata* compared with non-accumulating plants. It is not yet known whether this increase is part of the primary tolerance mechanism or caused by secondary effects such as cation over anion uptake, a process known to stimulate organic acid synthesis in plants. In the present study the changes in malate concentration in *B. coddii* were compared with those of non-accumulating plants, the results of which are presented in 3.3.4. Nickel-citrate complexes have also been discovered in the mature leaves of fifteen New Caledonian nickel hyperaccumulators and two *Alyssum* species, although generally *Alyssum* hyperaccumulators bind the nickel as malate and malonate complexes (Baker and Brooks, 1989). In *Pearsonia metallifera* homocitric acid appears to be the major complexing agent (Homer *et al.*, 1991).

The complexing of nickel with ubiquitous metabolites such as malate and citrate cannot fully explain the hyperaccumulation of nickel within certain plant species as, if these were the only compounds required for tolerance, many more plant species would be able to at least tolerate, if not accumulate, nickel (Woolhouse, 1983). Perhaps coupling the mechanism of nickel uptake to the supply of organic acids, without draining the Tricarboxylic Acid Cycle, has limited the potential for tolerance in other plants; or perhaps non-tolerant plants are unable to store the organic nickel complexes. It has also been argued that if organic acids are responsible for metal tolerance then plants able to accumulate one metal should be able to accumulate another, because the organic ligands are not specific for one metal and metal-complexes formed with organic acids generally have similar stability constants. This, however, assumes that the metals have similar degrees of accessibility to the acids and, as this is not necessarily so, this might be why hyperaccumulators are unable to accumulate more than one metal (Thurman and Collins, 1983).

Another factor thought to contribute to metal tolerance is the synthesis of stress metabolites and/ or proteins (Neumann *et al.*, 1994). Neumann *et al.* (1994) showed that the most prominent metabolic difference between heavy-metal tolerant plants and those growing on "normal" soils is the induction of a certain two mRNAs for the synthesis of two small heat shock proteins. These proteins appear to play a role in lessening stress effects on membrane function caused by metal uptake. More work is required to determine the exact role of heat shock proteins in metal tolerance but it is thought that their role in protein folding is of

significance. Heat shock proteins have not, as yet, been reported to be synthesised by *B. coddii*, in response to metal treatment.

In bacteria, alterations to the influx and efflux systems in the plasmalemma play a role in metal tolerance by limiting the amount of metal that can be accumulated (Meharg, 1993). In higher plants little work has been performed on the effect of metal-uptake on the plasmalemma, although preliminary studies have shown that the plasmamembrane does play a role in metal tolerance. Kinetic studies, using short-term influx studies with appropriate desorption procedures, and appropriate biochemical and electrophysical studies on the role of maintenance of plasmalemma integrity would show whether the plasmamembrane plays a universal role in metal tolerance.

A full genetic study, to determine whether metal tolerance is polygenic or coded for by a single gene, would also shed more light on the tolerance mechanisms occurring in higher plants (Macnair, 1993). If a polygenic system is involved it would show tolerance as a complex mechanism involving changes to many different biochemical pathways and enzyme adaptations. If a single gene exists for tolerance it would imply that a primary physiological change allows plants to survive metal stress.

From the above it can be seen that hyperaccumulators, as well as non-tolerant plants, appear to have internal mechanisms in place allowing them to cope with increased metal concentrations. However, the full process of metal-detoxification has not been discussed, as it has not yet been elucidated in any accumulators, including *B. coddii*. Brooks (1987), however, has put forward the following unsubstantiated theory on the process of nickel accumulation. He proposes that initially the nickel is taken up by the root using a selective transport ligand, which occurs in the root membrane (Brooks, 1987). As the "selector" is thought to be linked to the membrane, other compounds, namely the organic acids, act as "transport" ligands. These transport ligands form mixed (ternary) ligand complexes with the selector-nickel complexes on the internal surface of the root membrane, which then break down, causing the transport-nickel complexes to be released into the xylem. The "selector" then remains in the membrane. The transport-nickel complexes then move through the xylem to the leaf cells, where they cross the plasmalemma, cytoplasm and tonoplast, and enter the vacuole where they act with terminal acceptor ligands to form, once again, ternary ligand complexes. These complexes then break

down, releasing the transport ligands, while the acceptor-nickel complexes accumulate in the vacuole, where they remain harmless to the cell. For these complexes to accumulate in the vacuole they must be impermeable to the tonoplast. The transport ligands, to which the tonoplast is permeable, then move out of the vacuole, through the cytoplasm and plasmalemma and into the phloem where they are transported to the roots and diffuse back into the xylem.

The above mechanism assumes that the transporter (e.g. malate) and acceptor (e.g. citrate) are different species, but it is conceivable that in some cases they are the same species. This combination of roles would mean that a non-cyclic system is in place and that the transport–nickel complex would accumulate in the vacuole. A fresh supply of ligand would then always be made available to the roots, and the vacuole tonoplast would always be permeable to the nickel complex, perhaps accounting for the fact that nickel is able to move freely through plants. The major advantage of the above proposal is that at no stage would there be free aquonickel[II] ions in the plant – the cause of nickel toxicity (Brooks, 1987). In hyperaccumulators, metal accumulation increases with age causing the demand for accumulation sites to increase to the point where cell metabolism is overcharged. At this stage, even a mechanism well adapted for heavy metal accumulation may not be able protect the plant from metal toxicity (Ernst, 1972) and so, in order to reduce the likelihood of metal toxicity the older leaves, having a higher metal content than the younger leaves, die off and are discarded. This enables new leaves to be almost continually produced.

From the above it can be seen that metal tolerance in angiosperms is complex and few generalisations can be made. However, generally the mechanisms are internal, as plants from metalliferous substrates rarely do not absorb metals. Resistance is thus achieved by true tolerance (Baker, 1987). The particular tolerance mechanism in *B. coddii* remains to be elucidated and is partially addressed in Chapter 3.

1.9 *Phytoremediation*

Most unnaturally, metal-contaminated sites occur due to: metal-rich mine tailings, metal smelting, electroplating, gas exhausts, energy and fuel production, down-wash from power lines, intensive agriculture (including spraying with insecticides) and sludge dumping (Raskin *et al.*, 1994). As mentioned in the introduction (section 1.1), the practical aspect, therefore, of

looking into hyperaccumulators is in their potential application for the remediation of these metal-contaminated sites, as most conventional remediation approaches provide unacceptable solutions for the removal of toxic metal pollution from soils and water (Salt *et al.*, 1995). The more conventional methods of treating soils contaminated with heavy metals are landfilling, fixation and leaching. All these processes are expensive and generally lead to soil sterility. Phytoremediation, on the other hand, tends to be cost effective and the soils, once clean, are again arable. Furthermore, as hyperaccumulators are non-invasive, due to the fact that they are uncompetitive as they have an intrinsically slow growth rate and they are easily destroyed by fungi, which are not normally present in mineralised soils, they can be used in phytoremediation without threat to the indigenous flora. Thus once a soil has been remediated indigenous plants will outgrow the hyperaccumulators and the fungi, returning to the remediated soil, will infect and kill them. This method of clean up could, however, take several years since it will depend on the level and depth of soil contamination.

Phytoremediation can be divided into (i) *phytoextraction*, where hyperaccumulators are used to concentrate the metals in the aerial parts of the plants for easy harvesting; (ii) *rhizofiltration*, in which plant roots absorb, precipitate and concentrate toxic metals from polluted effluents; and (iii) *phytostabilisation*, where heavy metal tolerant plants are used to reduce the mobility of heavy metals, thereby reducing the risk of further environmental degradation by leaching of the metals into the ground water or by airborne spread (Salt *et al.*, 1995). For the purposes of this project phytoremediation has been taken to mean phytoextraction as no work on phytostabilisation or rhizofiltration was performed with *B. coddii*.

The optimum plant for phytoextraction is one having a rapid growth rate and the ability to accumulate high levels of heavy metals in its harvestable parts and a high biomass in the field. Unfortunately most naturally occurring hyperaccumulators are relatively small in size and have a fairly slow growth rate. Thus, work is beginning on the engineering of plants to render them suitable for phytoextraction (McGrath *et al.*, 1993; Salt *et al.*, 1995). It will become apparent later that *B. coddii*, the subject of this thesis, is ideally suited for phytoextraction.

The ideal plant for rhizofiltration should have rapidly growing roots with the ability to remove toxic metals from solution over extended periods of time (Salt *et al.*, 1995). From tests, Salt *et al.* (1995) have shown that many "large-root" plant species e.g. sunflowers, are able to absorb

and precipitate heavy metals from solution, and thereby dramatically reduce heavy metal concentrations to acceptable discharge levels within 24 hours. The mechanisms for metal removal by plant roots involve, surface sorption (a mechanism not requiring biological activity), intracellular uptake, vacuolar deposition, and translocation to the shoots. Since metal transport to the shoots would render rhizofiltration less efficient, plants used in rhizofiltration would probably not be efficient translocators. Rhizofiltration is most effective and economically viable when low concentrations of metal contaminants and large volumes of water exist (Salt *et al.*, 1995).

The presence of accumulator plants has often been used to locate metal-polluted sites (Baker and Brooks, 1989). Such sites are otherwise generally fairly barren and prone to erosion and leaching. In order to reduce these effects they can be re-vegetated with other metal-tolerant plant species. Work has been performed on modelling the reduction of contaminant transport in surface flows when a site is re-vegetated (Green *et al.*, 1996). This work is useful for determining the type of cover that should be used on a particular site. Unfortunately, due to the barren nature of the land, heavy fertilisation is generally required when the phytostabilising plants are established, making the process a fairly expensive one.

As has been mentioned only those elements available to the plant can be taken up by the plant. Thus, in general, for phytoremediation to be effective, manipulation of the soil environment is required to enhance the availability of the metals. At present very little data is available on practices designed to enhance metal uptake. The work that has been done is outlined below.

1.9.1 Enhancing Metal Uptake from the Soil

Chelating agents are considered essential in plant and soil manipulations. They have been used as soil extractants, as a source for micronutrient fertilisers and to maintain the solubility of micronutrients in hydroponic solutions (Salt *et al.*, 1995). Generally, in forming metal-chelate complexes, precipitation and sorption of metals is prevented causing them to remain available for plant uptake. By adding chelates to the soil, metals may also be brought into solution by the desorption of sorbed species and the dissolution of iron and manganese oxides and other precipitated compounds. Metals can also become more plant-available if a moderately acid soil

pH can be maintained. Thus ammonium-containing fertilisers or soil acidifiers are generally used to render the metals more soluble and available for uptake (Salt *et al.*, 1995).

In soils heavy metals tend to be bound or sorbed on to oxides (Salt *et al.*, 1995). Thus, if these oxide materials could, in some way, be dissolved metal solubility would be enhanced. In this regard, many plants are known to release reductants from their roots to obtain insoluble metals, bound to oxides. Thus the artificial addition of reductants to the soil could be a feasible method of enhancing metal solubility and uptake. Unfortunately it is not always possible to alter the redox status of the soil (section 1.9.1) in a field, but the addition of reducing agents may confer a slightly increased metal solubility.

Finally, microorganisms appear to play a role in stimulating metal uptake by plants (Salt *et al.*, 1995). Thus, by populating the rhizosphere during phytoremediation with selected microorganisms (taken from heavy metal-contaminated sites), it should be possible to enhance metal uptake. The microorganisms could be applied via a seed treatment or added to the irrigation water.

In summary, various strategies might prove useful for enhancing metal solubility and uptake by *B. coddii* when it is employed for phytoremediation. These include the addition of chelators, acids, reductants, reducing agents, and microorganisms to the soil. As more is learnt as to the suitability of *B. coddii* for phytoremediation so some of these agents may be investigated.

1.9.2 Plants Suitable for Use in Phytoextraction

Plants able to accumulate high concentrations of metals are required for effective phytoextraction. A full list of the various metals accumulated by diverse plant species is given in Appendix 1. The following are examples of hyperaccumulators thought to be suitable for soil remediation. Probably the greatest hyperaccumulator of copper is *Aeollanthus biformifolius* (Table 30, Appendix 1), found on the Shaban Copper Arc in the Democratic Republic of the Congo (formerly Zaïre) (Baker and Brooks, 1989). It has been found to accumulate as much as 1.37 g copper per 100 g dry matter, and is also able to hyperaccumulate cobalt. It is interesting to note that the Becium genus, while containing copper indicator species, does not include any copper hyperaccumulators. Work is being done

on the suitability of *Haumaniastrum katangense* (Table 30, Appendix 1), found in Lubumbashi, as a tool for the remediation of soils containing copper (de Plaen *et al.*, 1982). The greatest hyperaccumulator of cobalt is *Haumaniastrum robertii* (Table 30, Appendix 1) which is able to accumulate up to 1.02 g cobalt per 100 g dry material, and up to 0.2 g copper per 100 g dry material (Baker and Brooks, 1989). It is also found in the Democratic Republic of the Congo, in the region between Lowezi and Likashi (de Plaen *et* al, 1982).

Chromium is not an essential element but there are reports that low concentrations of chromium in the surrounding medium (0.05 - 0.1 μg/ml) can stimulate plant growth (Baker and Brooks, 1989). The hyperaccumulation of chromium though, is a fairly controversial subject as many researchers feel that, due to its toxic nature, chromium cannot be accumulated in plants. Wild (1975), however, reported extraordinarily high levels of chromium in the leaves of *Dicoma niccolifera* Wild (0.15 g/ 100 g dry weight) and *Sutera fodina* Wild (≈ 0.24 g/ 100 g dry weight). Due to the proximity of both these plant species to the Noro chrome mine on the Great Dyke and, with other analyses showing chrome to be at least twenty times lower in these plants (Brooks, 1987), it is felt that the samples from the Noro mine may have simply been contaminated on the surface of the plant by wind blown ore. Many researchers feel so strongly that chromium cannot be accumulated by plants that any found in the metal analyses is said to be due to surface contamination of the leaves, and this can then be used to assess the degree to which a plant has been surface-contaminated by the surrounding soil (Brooks, 1987).

The accumulation and hyperaccumulation of lead, by plants, is extremely rare as lead can readily be precipitated as the insoluble sulphate in the rhizosphere, which minimises its uptake (Baker and Brooks, 1989). *Thlaspi rotundiflorum* subsp. *Cepaeifolium*, found in Central Europe (Table 31, Appendix 1), appears to be the most likely plant to be used in rehabilitation of soils containing lead. It is interesting to note that this species, found colonising a mine dump, is closely related to the non-tolerant *Thlaspi rotundiflorum* (L.) Gaud leading to the suggestion that the colonisation of mine wastes might be a neoendemic process.

Manganese is an essential micronutrient where, below concentrations of 1 - 2 mg/ 100 g dry matter, deficiency symptoms in plants can be detected (Baker and Brooks, 1989). At concentrations above about 0.15 g/ 100 g dry weight toxicity symptoms generally start to

appear. However, the manganese hyperaccumulators (hypermanganęsophores) have a manganese content of greater than 1 g/ 100 g dry weight (Table 32, Appendix 1).

Nickel hyperaccumulators (Table 34, Appendix 1) form by far the largest and most diverse group of hyperaccumulating plants. Thus, phytoremediation of soils containing nickel should be relatively simple as, from the large selection of plants, one should be able to find a plant suited to the given climatic conditions as well as the depth of contamination. For example, and depending on climatic conditions, if the nickel contamination is not very deep (approximately 10 cm) a fast-growing plant with a shallow root system such as *Streptanthus polygaloides*, could be used to clean up the site (Reeves *et al.*, 1995). If, on the other hand, the nickel contamination is very deep (over a metre), a slow growing plant with a deep root system, such as *Serbertia accuminata*, could be used. In the present project the use of *B. coddii* as a tool for phytoextraction was investigated since it appeared to have ideal characteristics for the task in hand. These included, *inter alia*, a shallow - medium root system (about 50 cm), an ability to accumulate relatively large nickel concentrations in its leaves (about 1 – 3 g/ 100 g dry matter), the fact that it is perennial, germinating vegetatively at the beginning of each season, and the fact that it grows relatively quickly producing a large biomass.

Zinc is an essential element in plant nutrition and is readily taken up from the soil. Plants growing in unmineralised soils typically contain only 0.001 - 0.002 g zinc / 100 g dry material (Baker *et al.*, 1988). In hyperaccumulators the zinc is translocated either in the free ionic form or as simple organic acid complexes to the shoots, where it may accumulate above 1 g/ 100 g dry material. Table 33 of Appendix 1 gives a list of the well-known zinc hyperaccumulators of which the most interesting is *Thlaspi calaminare* as it is able to accumulate both zinc and nickel.

Besides terrestrial plants, aquatic or semiaquatic vascular plants such as water hyacinth, duckweed and water velvet, are also able to accumulate heavy metals including lead, copper, cadmium, iron and mercury (Salt *et al.*, 1995). Generally these plants are used in constructed wetlands where, unfortunately, the heavy metals are moved to a different location, rather than being removed from the environment. Due to the fact that water hyacinth is almost a scourge in subtropical and tropical areas it is not recommended that the plant be used to remediate open waters. As a possible alternative, research is being performed to determine the possibility

of using dried parts of the plant as an inexpensive, non-threatening adsorbent for removing contaminating metals from polluted waters (Schneider *et al.*, 1995).

1.9.3 Phytoextraction Trials

As more hyperaccumulating plant species are identified so the interest in phytoremediation grows. Currently a number of workers are testing various plant species for their suitability for economically viable remediation projects. For example, Nicks and Chambers (1995) are testing the nickel hyperaccumulator *Strepthanthus polygaloides* in Nevada for its ability to concentrate nickel, whilst providing a high biomass. *S. polygaloides* is a fairly low biomass annual that occurs naturally on serpentine soils in California. While it has a low individual biomass, when planted out a dry mass of 1 kg/m^2 can be expected. As it is able to accumulate at least 1 g nickel per 100 g dry material it is a suitable crop for soil remediation. Baker *et al.* (1995) have also tested a number of hyperaccumulator plants from the Brassicaceae family for their suitability as tools in phytoremediation of soils contaminated with zinc and lead, in the United Kingdom. They found that the plants not only accumulated these metals from soils with high background heavy metal concentrations, but also from soils that were marginally polluted. Their results also showed that phytoremediation and rehabilitation of the polluted sites could be achieved after a number of croppings. Furthermore they found that the specificity of metal accumulation was not absolute and that the plants were able to accumulate high levels of metals not present in elevated concentrations in their native soils.

Some forage crops are fairly efficient at extracting heavy metals from acid mine spoils and, with successive cropping, the toxic concentrations of the metals can be removed from the area (Taylor *et al.*, 1993). However, unless the area in which the crop is planted can be completely sealed off, it is not recommended that these crops are planted as, any animals or humans eating the crop, may die from the toxic levels of heavy metals contained in the foliage. Furthermore, work done by Chaney *et al.* (1995) has shown that, so far, no wild or crop plants can be used cost-effectively to remediate soils contaminated with lead. However, remediation of wastewaters contaminated with lead can be achieved by rhizofiltration and this prevents the lead from leaching down into the soil profile. These workers have achieved similar results with chromium.

Non-living biomass such as that from the green algae, *Halimeda opuntia,* and the brown algae, *Sargassum natans,* are also being tested for their phytoextraction capabilities (Kuyucak and Volesky, 1988). The advantage of using a non-living biomass is that conditions allowing life to be sustained do not have to be provided – a huge plus in the industrial world (Modak and Natajaran, 1996). The disadvantage of the biosorption process is that it is only able to deal with low concentrations of metallic effluents whilst the hyperaccumulating plants can perform their function on both land and, perhaps, in water.

Thus, in conclusion, several trials have successfully been performed in the world, on various waste sites, employing a variety of hyperaccumulators. Ultimately it is hoped that the technology of phytoremediation will become a viable and economical method of decontaminating metal-polluted areas worldwide. Towards this end the present project focussed on testing the feasibility of employing *B. coddii* to remediate nickel waste at Amplats' Rustenburg Base Metal Refineries (RBMR).

1.10 *Mine Dump Stabilisation*

In order to complete the rehabilitation of a mining area, it is prudent to look into the stabilisation of mine dumps (phytostabilisation) through revegetation. In many cases it is difficult to revegetate a mine dump as the slope of the dump is too steep causing high water run-off and a large amount of wind damage to plants trying to grow. With these very steep slope angles, the roots don't anchor the plants very well, causing the vegetation to be lost. Thus, in order to overcome these problems either a plant with a very good rooting system should be used, or the slope-angle of the dumps should be changed.

In Australia, once an area has been mined, the mining company is required to restore the region to its natural state. In this case exotic plants cannot be used to stabilise a dump – only plants that were originally growing in the area may be planted. Thus, before the area is mined its topography is modelled and a seed bank taken so that all plants growing in the area can be identified. Any topsoil that would be lost in mining is collected and stored until required for the rehabilitation process. Once mining is complete, the tailings are distributed over the area in such a way as to resemble the original model of the area, and so mullock dumps (Figure 6a) are formed (Woolard, 1997). These mullock dumps have a natural slope angle allowing the

area to be easily revegetated without fear of plant loss. In order to reduce erosion, while the vegetation is growing, large logs and general plant litter is placed over the site. To protect the plants from wind damage small mounds or dumps are also formed on the mullock dump (Figure 6b).

(a) (b)

Figure 6: View of a Mullock Dump (a) at Kambalda Nickel Mine, Australia. The Small Mounds and General Plant Litter (b) Prevent Wind and Soil Erosion.

Unlike Australia, mining in South Africa tends to be more long term with much larger underground areas being covered. The mines are older and the tailings' dumps much greater than those in Australia. For these reasons the formation of mullock dumps in South Africa will take a long time and until then other solutions for stabilising the already formed high angle dumps are required. A grass that is currently being used to stabilise some of the tailings dumps produced by mines in South Africa is Vetiver (Knoll, 1997). It is tough and erect and has a stiff stem and a vast, finely structured root system that forms a solid mat. The roots generally reach a depth of 2-3 m making the plant drought resistant and difficult to dislodge. It appears that it is not a very invasive plant and will not destroy surrounding natural vegetation as it seldom seeds and, if it does, the seed is generally sterile. It is propagated via the replanting of broken clumps. In order to establish the grass, adequate nitrogen, phosphorus and soil moisture are required. It appears to tolerate adverse soil conditions, including a pH range of 3 to 9.5 and high levels of heavy metals. Vetiver is planted as a hedge to slow down run-off and to allow good soil penetration.

The Amplats dumps are generally not very toxic as there is little acid mine drainage and the ores mined do not contain arsenic. Thus, at present, it is only the stabilisation of the dumps that is really required. Some of these dumps have, in mining terms, fairly high levels of both base and precious metals and it is hoped that, if the dumps can be stabilised with *B. coddii*, some of these metals can be retrieved by harvesting and processing the above-ground biomass from the plant, each season (section 5.3.6). Eventually it is hoped that these dumps will be rehabilitated and that some of the natural fauna and flora within the areas will begin, once again, to find their niche.

With the formation of mullock dumps and an attitude towards restoration rather than just rehabilitation, Australia is setting out to retain their ecosystems, and thereby maintain biodiversity. World-wide, biodiversity is decreasing and, with this in mind, it is hoped that eventually South African industries will turn to a process of restoration, keeping our wonderfully diverse fauna and flora intact (Bradshaw, 1997).

From the above discussion it can be seen that hyperaccumulator plants constitute a viable proposition for the decontamination and rehabilitation of areas polluted with heavy metals. With this in mind the suitability of *Berkheya coddii* was assessed in terms of removing nickel from a contaminated soil at an Amplats mine site in Rustenburg (see Chapter 5).

1.11 *Objectives of this Thesis*

The objectives of this thesis are as follows:

1. To assess the ability of *B. coddii* to accumulate nickel and other heavy metals from specific Amplats materials and to evaluate their effect on nickel uptake (Chapter 3);
2. To trace the path of nickel within *B. coddii* using radioactive nickel (Chapter 3);
3. To determine the role of malate in nickel storage and tolerance in *B. coddii* and whether malate concentrations in *B. coddii* are greater than in non-tolerant plants (Chapter 3);
4. To assess the climatic and soil suitability of the Rustenburg sites for *B. coddii* growth, (Chapter 4);

5. To determine the natural growth-cycle of *B. coddii* at Barberton so that the seedlings may be planted at Rustenburg at the correct time of year for maximum growth and nickel uptake (Chapter 4);

6. To establish the germination rate of the *B. coddii* seeds and to develop a suitable method for propagating large numbers of plants for phytoremediation (Chapter 4);

7. To determine the nutrient requirements of *B. coddii* in order that growth and nickel uptake may be maximised (Chapter 4);

8. To develop a method for removing the nickel accumulated within the above ground biomass, once the plants have been harvested from the Rustenburg sites (Chapter 5); and

9. To produce metallic nickel from the base metal precipitate, formed from the extraction process, thereby proving that *B. coddii* can remove significant amounts of nickel from the soil and that phytoremediation is a viable process (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2.1 *Introduction*

This chapter describes the various experimental sites and facilities and details the materials used in the heavy metal uptake experiments presented in this thesis. The more generally applicable procedures (e.g. nickel analysis) are also included in this chapter, while procedures pertaining to specific experiments (e.g. radioactive nickel uptake) are presented in the relevant results chapters.

2.2 *Experimental Sites and Facilities*

The experiments described in this thesis were performed at various facilities and locations. These are described in this section.

2.2.1 Natal University

2.2.1.1 Phytotron

The phytotron used at the University of Natal, Pietermaritzburg (UNP) was a temperature and humidity controlled growth room in which the night-time temperature was set at 16°C and the daytime temperature at 28°C. Since *B. coddii* is sensitive to the fungal growth that occurs in areas of high humidity, the humidity controller was set to zero, enabling the growth room to be maintained at the humidity levels occurring naturally in the air. The radiant flux density was set at 200 $\mu E.m^{-2}s^{-1}$, with a light cycle of 16 hours. This facility was used for the nutrient trials performed on *B. coddii*, described in section 4.2.5.5.

2.2.1.2 Greenhouse

The greenhouse used at UNP was not temperature controlled. The temperatures in Barberton are fairly high and it was found that, by not heating or cooling the greenhouse, temperatures similar to those found in Barberton could be maintained. The daytime temperatures generally

varied between 30°C and 38°C, although temperatures of 45°C were recorded on some days. The night-time temperatures were usually in the region of 20°C to 25°C. This facility was used for the experiments involving the uptake of radioactive nickel by *B. coddii* (section 3.2.2.2) and for the experiments to determine the germination rate of *B. coddii* seeds (section 4.2.4.2)

2.2.1.3 Light Incubator

A Conviron EF7H light incubator was used to cultivate the explants grown in tissue culture (section 4.2.4.1). The temperature was set at a constant 25°C and the plants were incubated with a radiant flux density of 250 $\mu E.m^{-2}s^{-1}$. The light cycle was set at 16 hours.

2.2.2 Barberton Site

The site is described in detail in sections 1.6 and 4.2.1. In order to perform the heavy metal uptake experiments (section 3.2.1) and their effect on malate concentration (section 3.2.4) in *B. coddii*, *in situ* experiments were performed on the plants. An area of about 0.5 hectares was fenced off at the Queens River sawmill site (Figure 3) and selected plants within this area were used for experimentation. Other than adding the materials under investigation, the plants were not interfered with in any other way. The reason for fencing off the area was that cows tended to wander through the field destroying plants thereby rendering the experiments useless. All seeds and serpentine soil required for the experiments described in sections 4.2.4 and 5.2.3 were obtained from this site.

2.2.3 Rustenburg Base Metal Refineries Contaminated Sites

Three sites A, B and C (described in section 5.2.4) were chosen at Rustenburg Base Metal Refineries (RBMR) for the phytoremediation experiments. These areas were fenced off and access was prohibited.

2.2.4 Amplats Research Laboratories

All the metal analyses performed on plant material were done at Amplats Research Laboratories (ARC). The author prepared the samples but, due to time constraints and a lack

of training, people within the Analytical Chemistry Department (see Acknowledgements) performed the actual instrument analyses.

2.3 *Plant Materials*

2.3.1 *Berkheya coddii* Seeds

All seeds used to germinate *B. coddii* seedlings (section 2.3.2) were taken from mature plants growing on the Barberton site at the Queens River sawmill. The seeds were germinated in the greenhouse (section 2.2.1.2) on damp cotton wool (section 4.2.4.2) and then grown up for three weeks before being transferred to the required media.

2.3.2 *Berkheya coddii* Seedlings

For the nickel uptake experiments (section 3.2.1) the three-week-old seedlings were transferred into 10-cm plastic pots containing serpentine soil. They were grown up for a further 5 weeks before they were used in the nickel uptake experiments (section 3.2.1.1).

The seedlings germinated for the nutrient trials on acid-washed sand and serpentine soil (section 4.2.5.5) were directly transferred, after three weeks, from the cotton wool to containers filled with acid-washed sand and serpentine soil.

2.4 *Metal Uptake in Berkheya coddii*

The materials used to determine the effect of various heavy metals on *B. coddii* (section 3.2.1.1), as well as the radioactive nickel that was used to try and elucidate nickel transport (section 3.2.2) within the plant, are described below.

2.4.1 Final Insoluble Concentrate (FIC)

FIC is a very high value fine black powder-concentrate containing the insoluble salts of the precious platinum group metals (PGM) namely, platinum (Pt), palladium (Pd), rhodium (Rh), ruthenium (Ru), gold (Au), osmium (Os) and iridium (Ir). It also contains, in lower concentrations, the base metals copper (Cu), nickel (Ni), cobalt (Co) and iron (Fe). Sulphur

(S) and silicates (SiO_2) are also present. It is from this concentrate that the precious metals are separated and pure Pt, Pd and Rh produced at the refinery. It is a more reactive substance than the metallics fraction (section 2.4.2) as the metals are present as salts rather than as stable metals. Unfortunately, due to the value and economic importance of this material, Amplats requires the specific composition of FIC to remain confidential. However, the composition of FIC is discussed in more detail in section 3.3.1, where a comparison of the PGM and nickel concentrations is given.

2.4.2 Metallics (Met)

The metallics fraction is fairly coarse and comprises the magnetic fraction of the precious metals that are mined. It is produced by passing an ore concentrate over shaking tables known as Jameson tables. The metallics fraction contains copper (Cu), nickel (Ni), iron (Fe), platinum (Pt), palladium (Pd), rhodium (Rh), gold (Au) and ruthenium (Ru) and these stable metals are fairly unreactive, compared with FIC. A number of high-pressure leaches under various conditions are performed on the material to separate out the base metals and to produce 'pure' Pt, Pd, Rh and Ru. Once again, due to the economic value of this material, details of the analyses cannot be given but the material is discussed and compared in more detail in section 3.3.1.

2.4.3 Radioactive ^{63}Ni Solutions and Standards

2.4.3.1 Materials

Radioactive Nickel Solution

A solution of ^{63}nickel chloride, in 0.5 M HCl, prepared by Du Pont, France, was obtained from Scientific Separations. The solution contained 74 x 10^6 Bequerel (Bq) (2.0 mCi) ^{63}Ni. It was diluted to 74 ml with dist. H_2O to give a final concentration of 10^6 Bq/ml (6 x 10^8 disintegrations per minute (dpm)/ ml). A working solution was then made from this stock solution by removing 1 ml and making it up to 100 ml with dist. H_2O. The concentration of the working solution was 10^4 Bq/ml (600 000 dpm/ml). This solution was used to prepare the ^{63}nickel standards (section 2.4.3.2).

Digestion Mixture

In order to analyse for any radioactive nickel (^{63}Ni) taken up by the plant, it was necessary to digest the plant material to release the ^{63}Ni. Thus a digestion mixture containing 1 : 1 (v : v), 30% hydrogen peroxide (H_2O_2) : glacial acetic acid was prepared and stored at 4°C until required.

Scintillant

Beckman Ready Solv™ MP was used in the preparation of all cocktails analysed on the liquid scintillation counter.

2.4.3.2 Procedure

Standards

A standard curve was constructed by making up a series of standard solutions (Table 1) of ^{63}Ni to be read on the Packard Tri-Carb™ 1 500 Liquid Scintillation Analyser. So as to minimise differences in quenching and, therefore, counting efficiencies, the standards were treated in a similar fashion to the samples. A leaf of *B. coddii*, grown in serpentine soil that was free of radioactive nickel, was placed in each of 6 polythene scintillation vials. To each tube 1 ml of digestion solution was added and the samples were left to digest for 48 hours. The lids were removed and the samples were boiled at 100°C for 1 hour, to complete the digestion process, and then left to cool to room temperature. The required amounts (Table 1) of the 10^4 Bq working solution (section 2.4.3.1), followed by 4 ml of scintillant, were then added to each vial and mixed thoroughly. The vials were left at room temperature for 5 min, 25 min, 35 min, 45 min, 60 min, 75 min and 90 min respectively, and then counted for 1 minute (or to a sigma error of 2%) on the scintillation analyser. The standards were read against a reagent blank containing water instead of ^{63}Ni. The readings were recorded as disintegrations per minute. The reason for reading the vials at the various times was to investigate the stability of the scintillant, thereby determining at which counting time the fluorescence was most stable.

Table 1: Composition of ^{63}Nickel Chloride Standards

N° Disintegrations per Minute Required	Volume of 10^4 Bq Solution Required
50 000	83.0 µl
40 000	66.6 µl
30 000	50.0 µl
20 000	33.3 µl
10 000	16.6 µl
5 000	8.3 µl

Once the standard curves had been constructed it was seen that they all gave very similar scintillant stabilities. However, some previous work (Howes, 1990) had shown that the scintillant was most stable after 90 minutes and therefore the standard curve at 90 min is shown (Figure 7). All subsequent samples were incubated with scintillant for 90 minutes, before being read on the liquid scintillation counter.

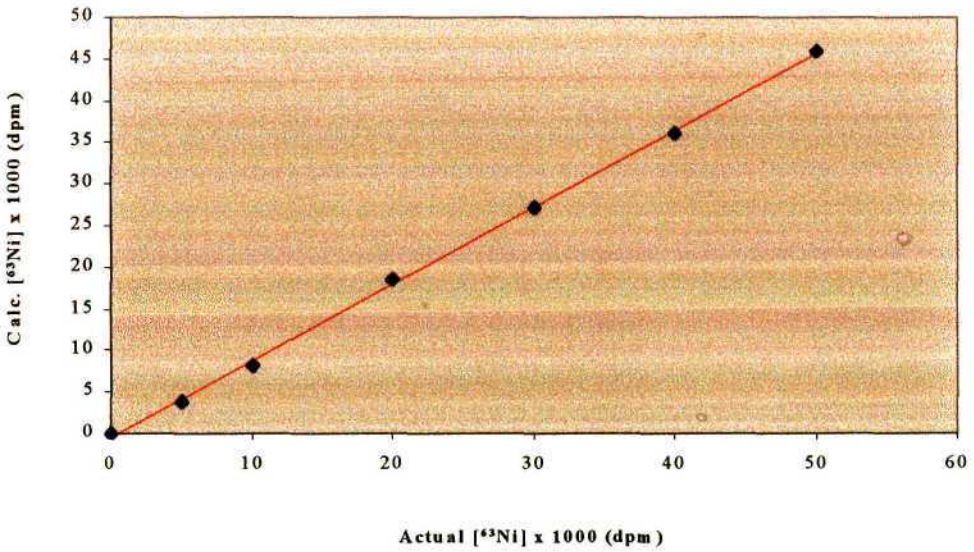


Figure 7: Standard Curve for ^{63}Nickel Chloride

(formed by plotting the calculated ^{63}Ni against the actual ^{63}Ni concentration, which was read off the liquid scintillation counter after a reaction time of 90 min.)

Determination of Quenching Capacity

Due to the quenching effect of certain materials within the sample not all of the radioactivity could be read and so a quench correction curve was set up using a standard known quenching agent, chloroform.

To 1 ml of the digestion mixture 0 µl, 20 µl, 50 µl and 100 µl of chloroform were added to separate polythene scintillation vials. ^{63}Ni, at an activity of 20 000 dpm (33.3 µl of the 10^4 Bq working solution), was then added to each vial along with 4 ml of scintillant. The solutions were left to stand for 1½ hours after which they were counted on the scintillation analyser. The quenching calibration curve was then calculated using the fact that 20 000 dpm existed in each sample. The calibration curve was stored in the memory of the scintillation analyser, which automatically corrected for quenching in all the experimental samples studied.

2.5 *The Extraction and Analysis of Malic Acid in Berkheya coddii* Organs

2.5.1 Materials

50% Methanol

500 ml analytical methanol, supplied by NT Laboratories, RSA, was combined with 500 ml dist. H_2O and mixed in a 1-litre volumetric flask.

Kit Reagents (Supplied in the Boehringer-Mannheim Kit for L-Malic Acid Analysis)

Solution 1: glycylglycine buffer at pH 10.00 containing 440 mg L-glutamic acid and stabilisers.

Solution 2: dist. H_2O containing 210 mg NAD lyophilisate.

Solution 3: glutamate-oxaloacetatetransaminase suspension, 160 units.

Solution 4: L-malate dehydrogenase solution, 2 400units.

A C_2 Bond Elut column, by Varian, Cat. No. 1210-2027, with a packing of 500 mg/ 2.8 ml was employed.

The sodium azide used was of general-purpose reagent grade and supplied by BDH, RSA.

2.5.2 Calibration Curve for L-Malic Acid

To determine the range in which the spectrophotometer read most accurately a calibration curve of the absorbances obtained at concentrations of 0.2 g/l, 0.5 g/l and 1.0 g/l L-malic acid was plotted. The calibration curve (Figure 8) shows that the spectrophotometer is accurate across a wide range of absorbances. This gave confidence in the readings – even those where the absorbance was high, which meant that dilutions of samples were generally not required.

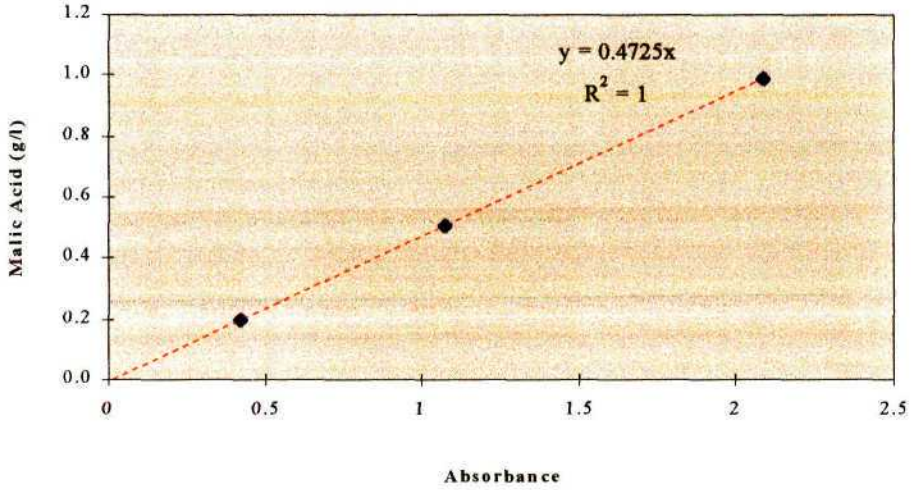


Figure 8: Calibration Curve for Various Concentrations of L-Malic Acid

2.5.3 Procedure

The plant material was oven dried overnight at 50°C to remove all the water. The material was weighed and then blended with an Ultra-Turrax® in a suitable volume of 50% methanol (usually about 100x the mass of the plant material). The homogenate was left to stand for 1 hour at 60°C after which it was re-blended. The methanol was then completely evaporated off in the draught oven at 60°C. The remaining water-soluble extract was centrifuged at 15 000 rpm and 4°C for 20 min in a Sigma 3K20 centrifuge with a 12156 rotor. The pellet was discarded and the solution volume reduced to approximately 10 ml, at 60°C. The solution was made up to 25 ml with dist. H_2O containing 0.2% sodium azide as a preservative.

A Varian Bond Elut® C_2 column was primed with one volume of 50% methanol (a polar solvent) and rinsed with two volumes of dist. H_2O, containing 0.2% sodium azide. A known sample volume (usually 5 ml) was passed through the column. The column was rinsed with dist. H_2O containing 0.2% sodium azide. The rinse solution was collected with the sample until a volume of 10 ml was reached. This solution was then assayed for L-malate using the L-malic acid analysis kit.

The sample solution for the L-malate determination was made up by placing 1.00 ml Solution 1 (section 2.5.1) in a test tube. Into this tube was added 0.20 ml Solution 2, 0.9 ml dist. H_2O, 0.01 ml Solution 3 and 0.10 ml sample solution. A blank solution containing no sample and 1.00 ml dist. H_2O was also made up. The solutions were mixed thoroughly on a vortex and left to stand for 5 min. A calibrated LKB Biochrom Ultrospec IIE spectrophotometer, from Cambridge in the UK, was set at 340 nm and zeroed with the blank. The sample was then read and the reading recorded as Reading A. To the sample and blank solutions 0.01 ml Solution 4 was added. The contents of the tubes were again mixed, left to stand for 10 min and the spectrophotometer zeroed with the blank. The sample was read and the reading recorded as Reading B. The concentration of L-malic acid in the sample was then calculated as described below.

Calculation:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\,[g/l] \quad \text{where,}$$

V	=	final volume [ml]
v	=	sample volume [ml]
MW	=	molecular weight of substance to be assayed [g/mol]
d	=	light path [cm]
ε	=	absorption coefficient of NADH at 340 nm
	=	6.3 [1 x $mmol^{-1}$ x cm^{-1}]
ΔA	=	Reading A – Reading B

For L-malic acid:

$$c = \frac{2.997}{\varepsilon} \times \Delta A\,[g\ L\text{-malic acid}/l\ \text{sample solution}]$$

2.6 *Metal Analysis of Plant Material*

2.6.1 Materials

Washing Solution

10 ml of Contrad, supplied by Merck, RSA, was added to 1 000 ml dist. H_2O, giving a 1% (v/v) concentration.

Digestion Mix

A digestion mixture comprising 5:2 (v/v) nitric acid (HNO_3) : perchloric acid ($HClO_4$) was carefully made up in the fume cupboard. The digestion mixture was always made up fresh and usually 500 ml of the mixture was required to fully digest 25 g of dry plant material.

10 % (v/v) Nitric Acid

One litre of 10% nitric acid (HNO_3) was made up by adding 100 ml concentrated (conc.) HNO_3 to 900 ml dist. H_2O and mixing thoroughly.

Analytical Instruments

1. Atomic Absorption Spectroscope: Varian 1275, using an air/ acetylene mix as the carrier gas.
2. Inductively Coupled Plasma Spectroscope: a hybrid instrument comprising a Hilger E1000 ICPS (made in England) and a RFPP radio frequency generator (made in the USA).
3. Inductively Coupled Plasma Mass Spectroscope: VG PlasmaQuad PQ2, using PQ Vision software.

2.6.2 Procedure

Before the plant material could be digested it needed to be decontaminated of any experimental metals. Thus immediately after collection, the plant material was thoroughly washed in the 1% Contrad™ solution for 30 min with agitation on a GFL 3015 shaker (made in West Germany). The material was then rinsed twice in distilled water for 15 min, with agitation on the shaker, after which it was placed in beakers and dried overnight at 60°C. The dry material was weighed and wet ashed with 20 ml digestion mix per 1 g material, on an electric hot-plate at

about 300°C (Gabbrielli *et al.*, 1991). The acid mixture was evaporated until almost dry and left to cool. The salts were dissolved in 10% nitric acid and made up to a known volume with 10% HNO_3. Quantitative determination of metals was then performed by atomic absorption spectroscopy (AAS) (section 2.6.2.1), Inductively Coupled Plasma Spectroscopy (ICPS) (section 2.6.2.2) or Inductively Coupled Plasma Mass Spectroscopy (ICPMS) (section 2.6.2.2).

AAS was generally used to determine a single metal (usually nickel), in low concentrations (approx. 500 mg/l) in a sample. ICPS was used for samples containing several metals, occurring in high concentrations (> 500 mg/l). The ICPMS was used where only trace amounts (parts per billion) of various metals were found in the plant tissue, such as in the case of precious metals.

2.6.2.1 Atomic Absorption Spectroscopy (AAS)

AAS determines the concentration of atoms in a sample, regardless of how they are combined (Skoog *et al.*, 1992). An aqueous solution of the sample is nebulised as a fine spray and then mixed with gaseous fuel and oxidant that carries it to the burner. The solvent evaporates in the base region of the flame (located just above the burner) and the finely divided solid particles that result are carried to the centre of the flame. In this, the hottest part of the flame, gaseous atoms and elementary ions are formed from the solid particles. Atomic absorption occurs when a gaseous atom or ion absorbs a photon of radiation from a hollow-cathode lamp and becomes excited. As the excited atom or ion returns to the ground state, characteristic wavelengths are emitted. This radiation is then absorbed by the carrier gas. Any unabsorbed radiation passes through a monochromator to the surface of a radiation detector. The monochromator isolates the excited spectral line of the light source. The absorption of radiation is proportional to the concentration of the metal in the sample solution.

Since nickel has a number of emission peaks the monochromator was set at the wavelength that corresponded to the estimated nickel concentration, reflected in Table 2 (Varian, 1979). To prevent overlap of interfering metals, a narrow slit width of 0.2 nm was used (the normal slit width is 0.5 nm). At wavelength 232.0 nm non-atomic species in the flame may absorb strongly, otherwise few interferences have been documented (Howes, 1990).

Table 2: Optimum AAS Wavelength and Slit Width Settings for Various Nickel Concentrations (Varian, 1979)

WAVELENGTH (nm)	SPECTRAL BAND PASS (nm)	OPTIMUM WORKING RANGE (µg/ml)
232.0	0.2	3-12
341.5	0.2	15-60
352.4	0.5	15-60
351.5	0.5	35-140

In the determination of nickel at ARC, a nickel lamp was used as the light source, with the wavelength set at 232.0 nm, as most of the samples contained nickel at a concentration of between 5 and 11 µg/ml (Table 2). The accuracy of the instrument was determined by plotting a calibration curve (Figure 9) for standard nickel solutions read at 232.0 nm. Figure 9 shows that nickel could be read accurately by AAS up to a concentration of at least 30 mg/l. This technique was used to analyse the nickel content in the seeds, leaves and stems of *B. coddii* after treatment with FIC and metallics (section 3.2.1); to determine the nickel concentration in the leaves of *B. coddii* plants growing on serpentine soils treated with various nutrients (section 4.2.5.5); and to determine nickel in the leaves of *B. coddii* plants growing on the RBMR sites (sections 5.2.4 and 5.2.7). AAS was also used to determine the concentration of residual base metals in the leached ash (section 5.2.10).

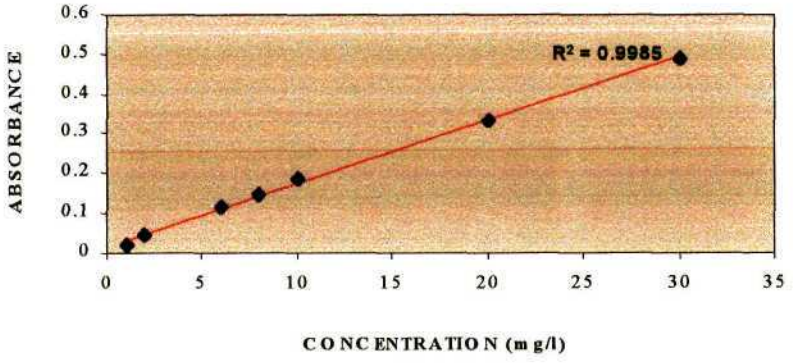


Figure 9: Calibration Curve of Standard Nickel Solutions Read at 232.0 nm on the AAS

2.6.2.2 Inductively Coupled Plasma Spectroscopy (ICPS) and Inductively Coupled Plasma Mass Spectrometry (ICPMS)

In conventional inductively coupled plasma spectroscopy (ICPS), inductively coupled argon plasma is used as an effective source of atomic emission in the quantitative determination of all elements, other than argon (Thompson and Walsh, 1983). The argon plasma gas is inductively heated to about 10 000K and then an aerosol of the sample material is injected at a flow rate of about 1 litre.min^{-1}. The atoms within the sample then become highly excited and partially ionised. Light, produced from the excited material spontaneously reverting to a lower energy state, is separated into its component radiation in the spectrophotometer, and recorded by a photodetector (Skoog *et al.*, 1992; Thompson and Walsh, 1983). For quantitative analysis it is assumed that the emitted energy is proportional to the concentration of atoms or ions in the sample. This technique was used to determine the nickel content in the leaves of harvested plants (section 5.2.7), to assess some of the base metals contained in the ashed plant material (section 5.2.8), and to determine the base metal content in the leachate of the leached ash solution (sections 5.2.9 and 5.2.10)

In inductively coupled plasma mass spectroscopy (ICPMS), the sample is again excited by inductively coupled plasma but, instead of quantifying the emitted energy, ion mass is measured. After excitation the plasma core, containing the sample ions, is extracted into a reduced pressure region (VG PlasmaQuad, 1989). A portion of this extracted plasma is then passed through an orifice, accompanied by a further drop in pressure. The positively charged ions are then extracted and transported to a quadropole mass filter, which only transmits ions of a selected mass to charge ratio. Since each naturally occurring element has a unique pattern of nearly integer mass to charge ratios corresponding to its stable isotopes, an ion detector is able to register the transmitted ions and identify them. The number of registered ions depends directly on the concentration of the relevant element in the sample, allowing easy quantification using specific computer software. ICPMS was used to analyse the low concentrations of platinum, palladium, rhodium and gold (PGM) in the seeds, leaves and stems of *B. coddii* plants that had been treated with FIC and metallics (section 3.2.1.2), to determine the PGM in the leaves of the *B. coddii* plants growing at RBMR (section 5.2.7), and to analyse the base-metal-containing hydroxide precipitate for PGM (section 5.2.10).

CHAPTER 3

UPTAKE, DISTRIBUTION AND STORAGE OF NICKEL AND SOME PLATINUM GROUP METALS IN *BERKHEYA CODDII*

3.1 *Introduction*

Limited data are available on the typical amount of nickel accumulated by *B. coddii*. Morrey *et al.* (1989) published the first data for nickel accumulation by *B. coddii* plants growing at the Agnes Mine site near Barberton. These results, along with those subsequently reported by Howes (1990), for plants at the Queens River site near Barberton, are given in Table 3. From these results it can be seen that the plants at the Queens River site contain much more nickel than those at the Agnes Mine site, probably because the nickel in the Queens River soil is more available to *B. coddii*.

Table 3: Nickel Content in *B. coddii* Plants Growing at the Agnes Mine and Queens River Valley Sites, near Barberton (Anderson *et al.*, 1997)

***Berkheya coddii* Site**	**Organ**	**Ni Content***	**Ni Content****
		(mg/kg dry weight)	**(mg/kg dry weight)**
Agnes Mine[1]	Leaf	13 130	11 637
	Stem	2 302	4 344
	Root	3 000	nd
Queens River Valley[2]	Leaf	27 811	nd
	Stem	7 606	nd
	Root	3 000	nd

* Howes, 1990.

** Morrey *et al.*, 1989.

[1] 491 mg of EDTA-available nickel per kg soil.

[2] 385 mg of EDTA-available nickel per kg soil.

Table 3 also shows that *B. coddii*, like most nickel hyperaccumulators, does not retain the nickel in its roots (Morrey *et al.*, 1989). Instead, the nickel is transported from the roots to the leaves where the greatest accumulation of nickel occurs. In support of this, Baker and Brooks (1989) found that in some of the *Alyssum* nickel hyperaccumulating species the lower and

middle stem zones, which are brown and woody, contain lower nickel concentrations than the upper and lower lateral stems, which are green and soft. This suggests that nickel is preferentially accumulated in photosynthetic tissues. The location of nickel in the tissue and cells of hyperaccumulating plants is thought to be related to the detoxification mechanism of nickel hyperaccumulation (Mesjasz-Przybylowicz *et al.*, 1997). Research has shown that nickel hyperaccumulators tend to utilise organic acids such as citric, malic and malonic acids in their internal tolerance mechanisms (Antonovics *et al.*, 1971; Ernst, 1972; Vaquez *et al.*, 1992; Brooks, 1987). These water-soluble organic acids, that exist as anions at physiological pH, bind to the toxic nickel ions, after which the nickel-organic acid complexes are transported across the tonoplast (Brooks, 1987). The metal is then rendered harmless to the plant by being stored, in many cases, in the vacuole. Plants tolerant to metals other than nickel and zinc tend to use phytochelatins as their complexing agents (Grill *et al.*, 1985; Tomsett and Thurman, 1988). Since preliminary studies by Howes (pers. comm.) have suggested that malate might be implicated in the nickel tolerance mechanism in *B. coddii*, the present project involved searching for any correlation between malate and nickel levels in the plant (Table 8 and Table 9 section 3.3.3). This aspect of the project also involved determining whether the malate levels in *B. coddii* were higher than the malate levels associated with non-accumulating plants (Table 8 section 3.3.3).

It has been found (Baker and Brooks, 1989) that the nickel concentration in leaves of nickel hyperaccumulators tends to correlate positively with that of cobalt, chromium, manganese, sodium and zinc, but not with any of the other plant nutrient elements in the leaves. Thus in the case of *Berkheya coddii* it was important to fully substantiate exactly where in the plant the nickel (and other metals) is optimally accumulated. In this way the plant parts containing the highest levels of nickel could be harvested and maximum use made of the growing season in a phytoremediation process (Chapter 5). Although Morrey *et al.* (1989) showed that *B. coddii* is unable to accumulate the chrome associated with serpentine soils, it was considered possible that *B. coddii* might also be able to accumulate other heavy metals. Since the Amplats mines have tailings dams and dumps containing very low, but valuable, levels of platinum, palladium, rhodium and gold, it was important to test whether *B. coddii* was able to accumulate these platinum group metals (PGM) and, therefore, could be used for the biomining of such precious metals.

The experiments reported in this chapter were designed to establish:

a. Whether *B. coddii* can hyperaccumulate and tolerate sufficient nickel, and possibly some of the PGM, to make phytoremediation a viable proposition (see results, section 3.3.1);

b. The distribution of the heavy metals within the plant and, therefore, which plant parts should be harvested for optimal metal recovery (section 3.3.1);

c. The path taken by absorbed nickel within the plant, employing radio-tracer studies (section 3.3.2); and

d. Whether malate is involved in the mechanism of storage and tolerance of nickel in *B. coddii* (sections 3.3.3 and 3.3.4).

3.2 *Materials and Methods*

3.2.1 Nickel and PGM Content of Seeds, Leaves and Stems of *Berkheya coddii* Treated with Final Insoluble Concentrate and Metallics

3.2.1.1 Materials

Twenty six, three-month-old *B. coddii* plants growing at the Barberton site (section 2.2.2).

One hundred and eleven grams of FIC, supplied by Amplats (section 2.4.1).

One hundred and eleven grams of metallics, supplied by Amplats (section 2.4.2).

5:2 (v/v) nitric (HNO_3) : perchloric acid ($HClO_4$) mixture (section 2.6.1).

10% HNO_3 made up by placing 100 ml conc. HCl in a 1-litre volumetric flask and making up to volume with dist. H_2O (section 2.6.1).

3.2.1.2 Procedure

Twenty-six, three-month old *B. coddii* plants of similar size were selected at the Barberton site (section 2.2.2). The earth around the base of the stems was gently turned over. A certain mass of metallics or FIC, Table 4, was placed in the loose soil and each plant was watered with approximately 1 litre tap water. The plants were left to grow for 1 month after which the same mass of metallics or FIC (Table 4) was again added. This process was repeated once more

giving a total of three inoculations over a three-month growing period. At the end of the third month the above-ground biomass (AGB) of the plants was harvested and rinsed in water. The leaves were stripped from the stems and dried overnight in a draught oven at 60°C. The leaves from duplicate experimental plants (a and b) were combined, as were the stems. The stems and leaves were then divided into quarters and half were used for duplicate malate assays (section 3.2.4.2). The remaining halves were used to assay for nickel (Ni), platinum (Pt), palladium (Pd), rhodium (Rh) and gold (Au) by AAS and ICPMS as described in sections 2.6.2.1 and 2.6.2.2.

Table 4: Mass of FIC and Metallics Placed in the Soil around the Experimental Plants

Plant Number	Material Added per Inoculation		Material Added to Plants after 3 Months	
	Mass FIC (g)	Mass Metallics (g)	Mass FIC (g)	Mass Metallics (g)
Control	-	-	-	-
1a and b	2.0	-	6.0	-
2a and b	5.0	-	15.0	-
3a and b	10.0	-	30.0	
4a and b	20.0	-	60.0	
5a and b	-	2.0	-	6.0
6a and b	-	5.0	-	15.0
7a and b	-	10.0	-	30.0
8a and b	-	20.0	-	60.0

At the Barberton site, one of the plants on which the experiments with precious metals had been performed flowered before the end of the experimentation period and seeds were produced. The seeds were collected, dried overnight in a draught oven at 60°C and weighed. They were wet ashed in a 5:2 (v/v) HNO_3 : $HClO_4$ mixture on an electric hotplate, at about 300°C, and made up in 10% nitric acid (section 2.6). The solutions were then analysed for nickel by AAS (section 2.6.2.1) and for PGM by ICPMS (section 2.6.2.2). All AAS and ICPMS results were expressed in units of either mg/ kg "dry tissue" or mg of actual metal accumulated.

3.2.2 Path of Absorbed Nickel in *Berkheya coddii*

3.2.2.1 Materials

Twenty, 10-cm diameter plastic pots containing serpentine soil obtained from Barberton.

Twenty, 10-cm diameter plastic pots containing topsoil obtained from a local Pietermaritzburg supplier.

Forty, two-month-old *B. coddii* seedlings, germinated on cotton wool (sections 2.3.2 and 4.3.4.2) and then transplanted into 5 cm diameter plastic pots containing serpentine soil.

2% (v/v) Contrad™ (obtained from Merck) solution.

Digestion mixture and scintillant was prepared as described in section 2.4.3.1.

Radioactive Nickel Tracer

200µl of dist. H_2O were placed in 40 eppendorf tubes. To each of these tubes, 41 µl of the 10^6 Bq ^{63}Ni (in the form of $NiCl_2$) stock solution (section 2.4.3) was added and mixed thoroughly.

3.2.2.2 Procedure

Twenty of the two-month-old *B. coddii* seedlings were transplanted into 10-cm diameter plastic pots containing serpentine soil while the other 20 plants were transplanted into 10-cm diameter plastic pots containing topsoil. All the plants were then grown in these pots for 1 week, to allow them to heal fully.

The plants were then treated with ^{63}Ni (in the form of $NiCl_2$) as described in section 2.4.3. Since optimal counting occurred at 50 000 dpm and the plants would, on average, have about 5 leaves to harvest, it was estimated that plant levels of 250 000 dpm of ^{63}Ni would be suitable to achieve optimal counting. Since the roots were expected to take up only about a tenth of the ^{63}Ni added, it was estimated that the soil should be inoculated with 2 500 000 dpm ^{63}Ni, which is equivalent to 4.1×10^4 Bq. Thus 41 µl of the stock solution (10^6 Bq) was added to 200 µl dist. H_2O for each plant. This solution was then poured around the base of the plant, 1 cm from the stem. To ensure consistency in results between the plants harvested early in the experiment and those harvested later, only one inoculation was performed.

The experiments were performed in duplicate for each soil type (topsoil and serpentine soil). The duplicate plants for each soil type were harvested at intervals of 36 hours, 3 days, 5 days, 7 days, 10 days, 13 days, 17 days, 21 days and 28 days, respectively.

After harvesting, the plants were washed for 15 min, with shaking on the GFL Shaker 3015, in a 2% (v/v) solution of Contrad™. The plants were then rinsed three times in dist. H_2O, for 15 min, with shaking. The roots, leaves and stems were separated, dried with tissue paper and weighed. The plant material was placed in separate vials and the digestion mixture (section 2.4.3.1) added. Where the plant material weighed less than 0.05 g, 1 ml digestion mix was added; between 0.06 g and 0.14 g, 2 ml digestion mixture was used; and between 0.15 g and 0.25 g, 3 ml digestion mixture was added. All material was digested for at least 48 hours although, as all the samples were read together, the plant material harvested first digested for nearly four weeks. Once all the material had been digested, the samples were boiled at 100°C for 1 hour, cooled and then 4 ml scintillant added. Each sample was shaken, left to stand for 1½ hours and read in the liquid scintillation analyser for 1 min, as described in section 2.4.3.2.

The data for the graphs given in Figure 11 and Figure 12 (section 3.3.2) was expressed as the average of the ^{63}Ni uptake into the stems, leaves and roots for each set of replicates. By doing this slight errors in the results were corrected. Radioactive nickel uptake was calculated as the absolute ^{63}Ni (in dpm) in each plant part and this was then calculated as a percentage of the total ^{63}Ni taken up by the plant (dpm).

3.2.3 Development of a Procedure for L-Malic Acid Determination in *Berkheya coddii* Tissue

Difficulties were encountered with the extraction and analysis of malic acid in plant tissue. An HPLC method was developed by the author and extensively tested but, due to resolution problems, it was decided that an enzyme assay should be used instead. A Boehringer Mannheim L-Malic acid test kit[1] was selected for this purpose, the principle of which follows.

[1] Boehringer Mannheim Biochemical and Food Analysis kit; Cat. No. 139 068.

Nicotinamide-adenine dinucleotide (NAD) oxidises L-malate to oxaloacetate in the presence of L-malate dehydrogenase (L-MDH) (Boehringer Mannheim, 1992):

$$\text{L-Malate} + \text{NAD}^+ \xrightleftharpoons{\text{L-MDH}} \text{oxaloacetate} + \text{NADH} + \text{H}^+$$

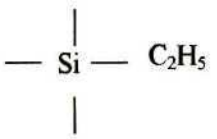
The equilibrium of this reaction lies on the side of L-malate. By removing the oxaloacetate from the reaction system, the equilibrium is displaced towards the oxaloacetate. In the reaction catalysed by the glutamate-oxaloacetate transaminase enzyme (GOT), oxaloacetate is converted to L-aspartate in the presence of L-glutamate:

$$\text{Oxaloacetate} + \text{L-glutamate} \xrightleftharpoons{\text{GOT}} \text{L-aspartate} + \text{2-oxoglutarate}$$

The amount of NADH formed is stoichiometric with the amount of L-malate. By measuring NADH, therefore, the amount of L-malate present can be determined. NADH is measured by means of its absorbance at 340 nm.

After extensive experimentation by the present author, it was found that the most efficient way of extracting the L-malic acid into solution was to homogenise the plant material with methanol at 60°C. Unfortunately, as both chlorophyll a and chlorophyll b are soluble in methanol (CRC Handbook, 1975) they were both extracted into the methanol, along with the L-malate. It was found that the absorbances of chlorophylls a and b interfered with the absorbance of NADH at 340 nm, and so these substances were removed from the extract using a C2 ethyl column[2].

The C2 ethyl column has a C_2 group bound to a chain of silicates as follows:



[2] Ethyl column made by Bond Elut®

This packing is only slightly non-polar due to the fact that the C_2 group is in close proximity to the polar silicates. Malic acid is a small molecule and so is able to pass through the column packing. Chlorophyll, on the other hand, has large non-polar groups and these tend to interact with the C_2 group of the packing material. Chlorophyll is also trapped on the column by the interaction of its polar groups with the more polar silicates. Thus, by passing the plant extract through a C_2 column, the chlorophyll can be removed without the L-malate being affected. The method given in section 2.5.3 was used in all L-malic acid determinations.

3.2.4 Comparison of L-Malic Acid Levels in *Berkheya coddii* with those in some Non-Accumulating Plants

3.2.4.1 Materials

Berkheya coddii seeds obtained from plants growing on the Barberton site (section 2.3.1).

Mature five-month-old *B. coddii* leaves taken from plants growing in the Barberton area.

Three-month-old *Gazania* hybrids, grown on potting soil, obtained from Keith Kirsten's Bush Hill nursery.

Three-month-old lettuce seedlings, grown on potting soil, obtained from Keith Kirsten's Bush Hill nursery.

Mature five-month *Gerbera jamesonii* leaves – taken from plants grown in KwaZulu-Natal.

Mature five-month *Senecio oxyrifolius* leaves – taken from plants growing in the Pietermaritzburg Botanical Gardens[3].

Mature five-month *Berkheya* species growing in the wild outside Pietermaritzburg.

10 ml Contrad (supplied by Merck Laboratories, RSA) per litre tap water solution for washing the plant material.

Boehringer Mannheim L-Malic acid test kit (section 2.5.1).

A standard malic acid solution was supplied with the L-malic acid test kit at a concentration of 0.203 g/l.

[3] Kind permission of Mr B Tarr.

3.2.4.2 Procedure

All the *B. coddii* seeds were germinated in a greenhouse at the Tobacco and Cotton Research Institute (TCRI) in the germination mix described in section 5.2.3. After 1 month, ten seedlings were transferred to serpentine soil, ten to RBMR soil and ten to topsoil. These plants were then grown up for a further two months.

All the plant material was thoroughly washed in Contrad solution by gently agitating on the GFL 3015 shaker for 30 min. The material was then rinsed twice in tap water and soaked in tap water for 15 min with agitation. All the plant material was dried overnight at 60°C and weighed. The plant material was halved to allow nickel (section 3.2.1) and L-malic acid (section 3.2.4) assays to be performed. All assays on the dried material were performed in duplicate.

Plant preparation for L-malic acid assays were carried out as described in section 2.5.3. To determine the range in which the spectrophotometer read most accurately a calibration curve of 0.2 g/l, 0.5 g/l and 1.0 g/l L-malic acid solutions was plotted, section 2.5.2. Plant material was prepared for nickel determination as described in section 2.6.

3.3 *Results and Discussion*

3.3.1 Nickel and PGM Content of Seeds, Leaves and Stems of *Berkheya coddii* Treated with Final Insoluble Concentrate (FIC) and Metallics (Met)

The areas incorporating the Amplats mines are generally mined for platinum, palladium and rhodium (collectively called platinum group metals or PGM) with the base metals, nickel and copper, forming by-products. Due to past spillages, the areas are mainly contaminated with nickel but PGM contamination also occurs. To test whether *B. coddii* could be used in the phytoremediation of all, or some, of these metals, experiments were designed to address the following questions:

a. Which, and how much, of the PGM and base metals is *B. coddii* able to accumulate?

b. In which parts of the plant are the metals accumulated and what is the relative distribution of metal in each part?

c. Is the ability of *B. coddii* to take up the metals affected by the particular chemical form of the metals on the soil? and

d. What levels of metal treatment can the plant tolerate and at what stage does the tolerance mechanism break down?

To obtain this information *B. coddii* plants growing at the Queens River site were treated (section 3.2.1.2) with increasing amounts of two different Amplats preparations of nickel and PGM (FIC and Met; sections 2.4.1 and 2.4.2) and then analysed for the extent of metal uptake into various parts of the plant.

The results in Table 5 show that *B. coddii* can accumulate and concentrate detectable amounts of various PGM from both FIC and Met. It can be seen that significant levels of platinum are accumulated in *B. coddii*, although notably more platinum is accumulated from the FIC fraction (146 – 3 116 mg/kg) than from the Met fraction (13.21 – 71.78 mg/kg). Palladium also appears to be accumulated in the above ground biomass (AGB) of *B. coddii* and again more palladium is taken up from FIC (152 – 3 021 mg/kg) than from metallics (3 – 11 mg/kg). It is interesting to note that much lower levels of palladium, compared with platinum, are accumulated from the metallics fraction but this is not mirrored in the FIC fraction, where platinum and palladium are accumulated to similar levels. Table 5 shows that no rhodium is taken up by the plant from metallics but that it is taken up from FIC (36 – 966 mg/kg). Only 10 mg/kg of gold is concentrated in the AGB of *B. coddii* from the FIC fraction, but 6 - 11 mg/kg gold is accumulated from the metallics fraction. These results suggest that Pt, Pd and Rh are more plant-available in the FIC compared with the metallics fraction, but that more plant-available gold is found in the metallics than in FIC. The base metal, nickel, from both FIC and Met, is accumulated to much higher levels compared with PGM. Approximately 22 500 mg/kg of nickel was found in the AGB of the plants growing on both FIC and Met.

From Table 5 it can be seen that an order of magnitude more PGM is accumulated in the AGB of the plants treated with 60 g FIC than with 5 g, 15 g or 30 g FIC. It was also noted that the plants treated with 60 g FIC were very unhealthy by the time they were harvested and that they

were going to die. This suggests that this concentration of FIC is toxic to *B. coddii* and that more metals could therefore be accumulated, compared with the lower treatments, due to membrane breakdown and indiscriminate movement into the plant tissue. As the plant dies with these concentrations of PGM it is no use in the phytoremediation of a site contaminated with these levels of PGM. However, at these PGM levels biomining would be a viable proposition and, as the plant would only be used as a tool to recover the metals, it would not matter that it would need to be planted every three months. However, biomining soils containing these levels of PGM is not a feasible proposition as, firstly, no such soils contain this concentration of PGM and, secondly, if they did, the mine would process the soil as fast as possible before it was stolen for illegal PGM recovery.

Table 5: Concentration of Platinum Group- and Base Metals Accumulated in the Above Ground Biomass (AGB) of *B. coddii* Treated with FIC and Met

	CONCENTRATION OF METAL [mg per kg AGB (n = 2)]				
TREATMENT	**Ni**	**Pt**	**Pd**	**Rh**	**Au**
MET - Control	25 012	4.74	3.41	1.14	0.00
6 g	25 304	13.21	3.16	0.00	0.66
15 g	24 291	41.60	10.07	0.79	6.22
30 g	22 251	71.78	11.40	0.00	11.64
60 g	24 078	56.25	11.13	0.00	6.95
FIC - Control	25 012	4.74	3.41	1.14	0.00
6 g	22 346	287	263	56.30	2.22
15 g	18 981	146	152	36.04	0.00
30 g	19 292	347	315	81.77	0.85
60 g	19 312	3116.14	3021	966	10.07

Table 6 gives the average harvestable mass of metals accumulated in the AGB of each *B. coddii* plant exposed to the various levels of FIC and Met. In terms of PGM contaminated soils, it was found that the soils around RBMR do not contain more than 6 g PGM per kg soil (Howes, *pers. comm.*) and therefore, in terms of PGM phytoremediation, *B. coddii* should only be assessed on the results achieved for the 6 g inoculations (Table 6). Therefore, from Table 6, in terms of harvestable metal per hectare, approximately 1 453 g of nickel, 9.2 g of platinum, 8.1 g palladium, 1.7 g rhodium and 0.1 g of gold could be obtained from 15 000 plants, with a dry mass of 61 kg, growing on a hectare of soil contaminated equally with metallics and FIC.

Thus these results suggest that *B. coddii* would be a viable tool for the remediation of soil contaminated with nickel and, possibly platinum and palladium, but not rhodium and gold.

Table 6: Absolute Mass of Metals Accumulated in the Above Ground Biomass of *B. coddii* Treated with FIC and Met

	ABSOLUTE METAL UPTAKE BY *B. coddii* [n = 2 (mg)]				
TREATMENT	**Ni**	**Pt**	**Pd**	**Rh**	**Au**
FIC- Control	162.45	0.03	0.02	0.007	0.004
6 g	56.36	0.42	0.37	0.092	0.007
15 g	136.03	0.26	0.25	0.063	0.006
30 g	79.34	0.97	0.86	0.233	0.010
60 g	140.88	9.49	8.85	2.784	0.050
Met - Control	162.45	0.03	0.02	0.007	0.004
6 g	137.95	0.07	0.01	0.004	0.006
15 g	67.18	0.10	0.02	0.005	0.009
30 g	64.21	0.11	0.02	0.007	0.013
60 g	108.30	0.17	0.02	0.009	0.015

Figure 10 shows that the majority of all the metal accumulated in the AGB is located in the leaves of *B. coddii*. The percentage distribution between the leaf and stem is, however, different for the different metals. About 80% of the nickel is found in the leaf, while only 20% occurs in the stem. In the accumulation of platinum, however, about 60% of the metal occurs in the leaf, with 40% being found in the stem. For palladium the relative percentages are 80% and 20% respectively; for rhodium, 75% and 25% respectively and for gold, 65% and 35% respectively. The fact that the nickel content in the stems is much lower than it is in the leaves of plants treated with FIC and metallics, agrees with the current knowledge (Brooks, 1987) that the nickel is stored in the leaves while the stems are used mainly as a transport vehicle. In terms of harvesting this indicates that if only the leaves, and not the stems, were harvested then 1 165 g of nickel, 5.52 g of platinum, 6.48 g of palladium, 1.28 g of rhodium and 0.07 g of gold would be removed by phytoremediation. These constitute important considerations (see Chapter 5) since the additional ashing of stems significantly affects the efficiency of the ashing process (section 5.3.7).

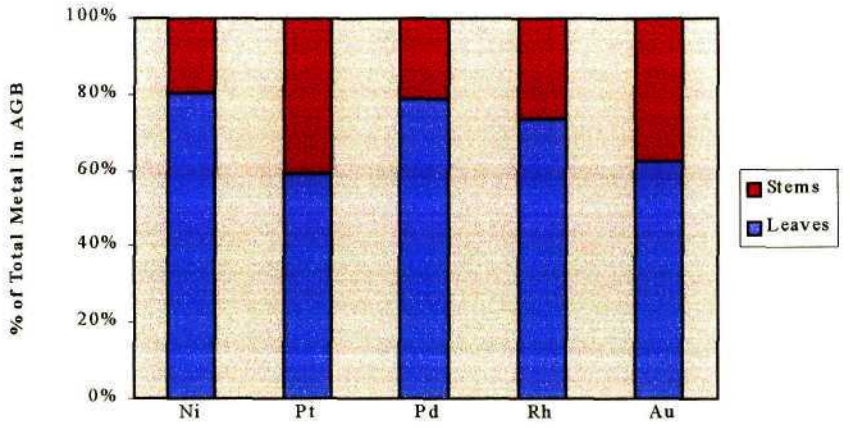


Figure 10: The Relative Distribution of Nickel and the Platinum Group Metals in the Leaf and Stem Tissue of *Berkheya coddii*

Table 7 gives the actual concentration of metals found in the seeds, stems and leaves of *B. coddii* plants treated with 15 g of FIC. From these results it can be seen that a significant amount of nickel, platinum and palladium, but no rhodium or gold, passes from the main plant to the seeds of *B. coddii*. Furthermore, *B. coddii* seeds tend to contain a greater concentration (not absolute amount) of nickel than do their stems. This result is in contrast with the findings of Ernst *et al.* (1992) who found that seeds from the metal tolerant plants *Thlaspi coerulescens* and *Silene vulgaris,* had a lower metal concentration than any other plant part. However, the present results are in agreement with those of Gabbrielli *et al.* (1997) who found fairly high concentrations of nickel in the seeds and flowers of *Alyssum bertolonii*. The present findings also show that nickel accumulation apparently does not affect reproduction in *B. coddii* and that inoculation with FIC does not increase nickel uptake above the control levels, probably because the seeds were already saturated with nickel before FIC treatment. Table 7 also shows that both the control seeds and the experimental seeds appeared to take up platinum and palladium. Although the control plants were not treated with either FIC or metallics, the soil surrounding them probably became contaminated with these substances as a result of run off.

Table 7 shows that small amounts of rhodium were taken up by the leaves and stems of the experimental plants treated with 15g FIC. In all these plants no rhodium is found in the seeds indicating that rhodium is mobile within the stems of *B. coddii* but that it is immobilised within the leaves of the plant. No gold is present within the seeds of *B. coddii* probably because very

little gold is available to the plant; no gold was accumulated in the stems or leaves of the control plants or the experimental plants treated with FIC.

Table 7: Concentrations of Nickel, Platinum, Palladium, Rhodium and Gold Found in the Seeds and Tissues of *B. coddii*

SAMPLE	TOTAL Ni (mg metal/ kg plant material)	TOTAL Pt (mg metal/ kg plant material)	TOTAL Pd (mg metal/ kg plant material)	TOTAL Rh (mg metal/ kg plant material)	TOTAL Au (mg metal/ kg plant material)
Control Seeds	7.01	0.43	0.15	0.00	0.00
15 g FIC Seeds	6.69	0.12	0.10	0.00	0.00
Control Leaves	20.33	0.00	0.00	0.00	0.00
Control Stems	4.68	0.00	0.00	0.00	0.00
15 g FIC Leaves	15.83	0.09	0.09	0.03	0.00
15 g FIC stems	3.15	0.06	0.06	0.01	0.00

From the results given in Table 5, Table 6 and Table 7 it appears that more platinum, palladium and rhodium is taken up by *B. coddii* plants growing on FIC, compared with those growing on metallics, with a general increase in uptake as the level of FIC or metallics increased. Not withstanding the fact that FIC contains 30.6 % more platinum, 85.8 % more palladium and 99.6 % more rhodium than metallics (with all metals except the rhodium in metallics found at a concentration of greater than 1%), the results in Table 5 clearly show that the metals in the FIC fraction were significantly more available to the plant than the same metals in the metallics preparation. Conversely, there appears to be slightly more plant-available gold in metallics than in FIC, despite the fact that FIC contains 39.4% more gold than metallics. Unfortunately no comparison of plant-available nickel could be made between the two substances as the plants were already saturated with nickel from the surrounding serpentine soil, as demonstrated by no increase in nickel concentration in the AGB with increasing levels of FIC or metallics. By contrast, platinum, palladium and, to a certain extent, rhodium in the FIC treatments, all showed significant increases in concentration in the AGB, with an increase in the levels of FIC and metallics. These increases can be attributed to low levels of these metals occurring in serpentine and, therefore, in the plant tissues at the start of the experiment. The above results are in agreement with the intrinsic properties of the two preparations. The chemical forms in which the PGM and base metals are found in FIC tend to be more available/ soluble, in

aqueous solution, than the metallic forms of the metals found in the metallics preparation, which tend to be more insoluble in aqueous media (Reinecke, 1998). Thus treatment of *B. coddii* with the two different preparations yielded information about the plant's preference for different chemical forms of the metal. Such information could prove useful when growing *B. coddii* on the RBMR waste sites (Chapter 5).

Inoculation of the plants with different levels of FIC and metallics also served to test the tolerance limit of *B. coddii* to the various metals tested. From the observed growth of *B. coddii* it was noted that exposure to FIC and metallics, at the levels given in Table 4, did not appear to affect the plants in any way, until 60 g of FIC had been added. At this point the plant growth was observed to be fairly stunted, resulting in them having a lower biomass than the control plants. This indicates that FIC becomes toxic to plants if a mass of greater than 30 g is added. The plants inoculated with metallics and FIC up to a level of 30 g, were all healthy and their observed growth and biomass was similar to that seen in the control plants. This indicates that *B. coddii* is able to tolerate the PGM, probably using the same mechanism as that for nickel tolerance since it is unlikely that the plant had developed a separate tolerance mechanism for PGM having not been previously exposed to these metals.

From the above results it can be concluded that *B. coddii* shows great potential as a tool for the phytoremediation of nickel-contaminated soils and that, in order to maximise the efficiency of the entire process, only the leaves should be harvested as they contain 80% of the nickel while the stems contain only 20% of the nickel. Due to the fact that the roots of *B. coddii* contain very little nickel, Table 3, and that they are used for the vegetative growth of the plant, it was felt that harvesting the roots would not be a viable proposition and thus no in depth studies were performed on them, either for nickel or PGM accumulation. The above results also show that *B. coddii* is able to accumulate, but not hyperaccumulate, platinum, palladium and rhodium and that the accumulation of these metals appears to have very little effect on the growth of the plant. It should be noted that, with an increase in PGM concentration in the AGB of *B. coddii*, there tends to be a decrease in the nickel concentration. In the case of the plants inoculated with 60 g FIC this effect could be attributed to metal toxicity. However, in the case of plants treated with 6 g, 15 g and 30 g of FIC, it is thought that this effect may be due to competition in uptake between nickel and the PGM. Competition for metal uptake

would occur both at the root level, where the metal is taken up, and at the leaf level, where the metal is stored. Before any conclusions as to the reason for the apparent decrease in nickel accumulation can be made, more studies on the effect and uptake of PGM in solution need to be made. The nickel concentrations in *B. coddii* appear not to be affected by the addition of metallics. It is thought that this is because little or no uptake competition occurs between nickel and the PGM since very little PGM in metallics are plant available.

3.3.2 Path of Absorbed Nickel in *Berkheya coddii*

The question of which path the absorbed nickel takes through the plant was more of academic interest than of practical application for phytoremediation. To determine the path of a compound or element within a plant, the compound needs to be administered in an available form and in a form that can easily be traced. Tracer elements that are commonly used are radioisotopes of the element being determined (Dighton *et al.*, 1990). To investigate the distribution of nickel in *B. coddii* the ^{63}Ni radioisotope was used. This isotope is a β^- emitter and is relatively environmentally safe, although it is strong enough to be counted, using liquid scintillation, as it has an energy of 0.067 MeV (Dyer, 1974). The half-life of ^{63}Ni is 92 years (Howes, 1990).

It should be noted that both serpentine soil and topsoil were used in the radio-tracer experiments as it was felt that the quantity of stored nickel in *B. coddii* plants resulting from growth on the two different soils could influence the path of ^{63}Ni through the plant. Plants growing on serpentine would contain significant quantities of nickel, whilst those growing on topsoil would have insignificant amounts of Ni ions stored in their tissues.

The relative uptake of ^{63}Ni into the leaves, stems and roots of *Berkheya coddii*, growing on topsoil and serpentine soil over a period of 29 days, is shown in Figure 11 and Figure 12, respectively. Both histograms show that after 1.5 days most (40 – 60 %) of the ^{63}Ni is in the middle leaves of the plant with significantly less (about 30%) in the stems. More ^{63}Ni occurs in the roots of the plants growing in topsoil (about 17%) compared with those growing in serpentine soil (about 10%). Little or no ^{63}Ni is found in the apical and bottom leaves of the plants after 1.5 days. On day 3 it can be seen that the bottom leaves of the plants growing on topsoil contain more ^{63}Ni (about 30%) than those growing in serpentine soil (about 1.5%).

Similar amounts of ^{63}Ni (about 15%) occur in the stems of the plants growing on both serpentine and topsoil on day 3. Other than that the roots of the plants growing in topsoil contain double the ^{63}Ni (about 17%) compared with the roots of the serpentine soil plants, while the serpentine soil plants contain more ^{63}Ni in the middle (about 53%) and apical (about 22%) compared with the topsoil plants (about 37% and 3 % respectively).

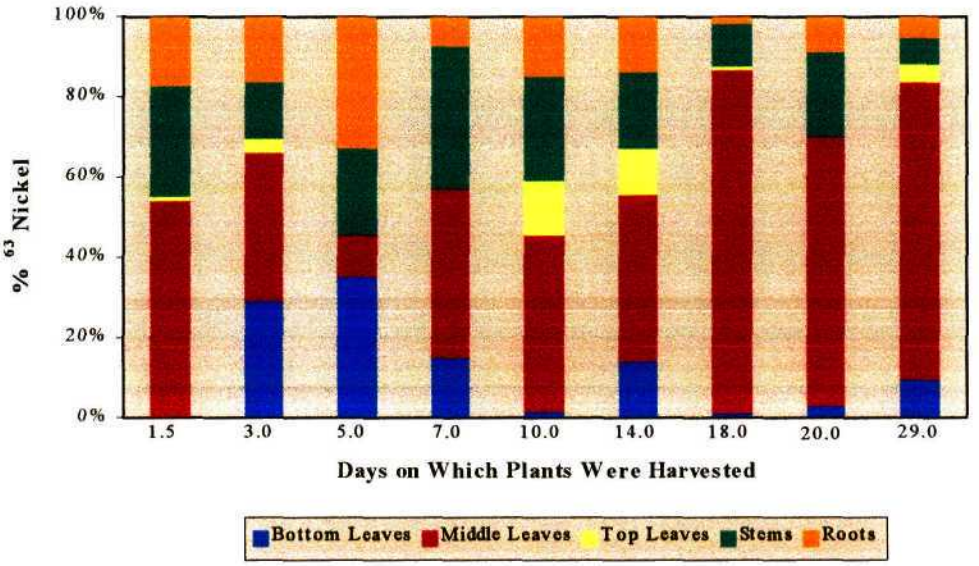


Figure 11: Relative ^{63}Ni Uptake in the Leaves, Stems and Roots of *Berkheya coddii* Plants Growing in Topsoil

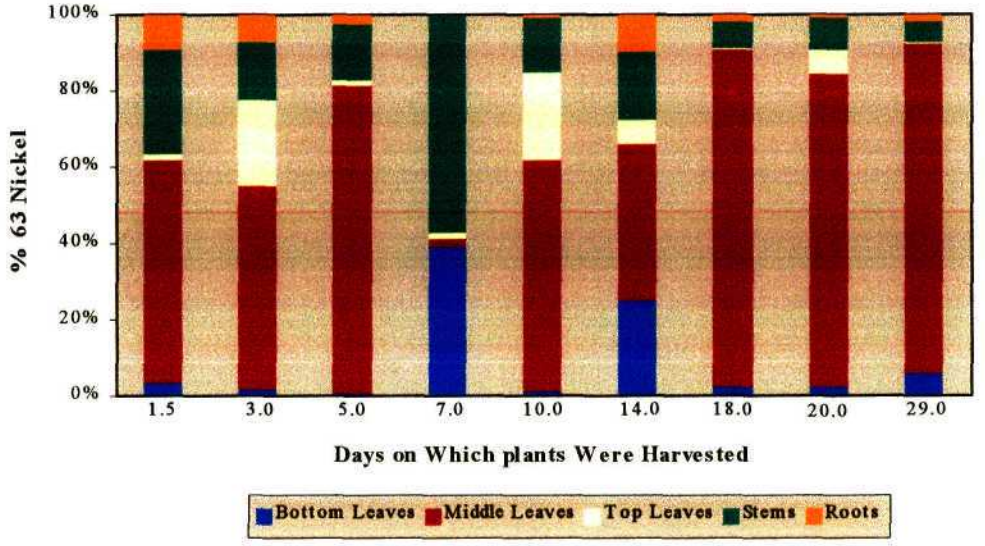


Figure 12: Relative ^{63}Ni Uptake in the Leaves, Stems and Roots of *Berkheya coddii* Plants Growing in Serpentine Soil

On day 5 more ^{63}Ni is again contained in the bottom leaves, the stems and the roots of the plants growing on topsoil (about 35%, 21% and 33% respectively) compared with those growing on the serpentine soil (about 1%, 15% and 2% respectively). The plants growing on serpentine soil show greater levels of ^{63}Ni in their middle and top leaves (about 80% and 1%) compared with the topsoil plants (10% and 0% respectively). On day 7, while the ^{63}Ni increases to about 39% in the bottom leaves of the serpentine plants, it decreases to about 15% in the topsoil plants. No ^{63}Ni is seen in the roots of the serpentine plants as, it appears, most of it has been transported to the stems (about 57%). About 8% ^{63}Ni is still seen in the roots of the topsoil plants with 36% ^{63}Ni occurring in the stems. Very little ^{63}Ni is observed in the apical and middle leaves of the serpentine plants (about 2 % in each) and this trend is followed in the topsoil plants for the apical leaves where no ^{63}Ni is observed. However, 15% ^{63}Ni still occurs in the bottom leaves of the topsoil plants. From day 7 onwards, the trends of ^{63}Ni uptake observed in the plants growing on serpentine and soil are very similar although more ^{63}Ni is generally found in the roots of the plants growing on topsoil.

Figure 11 and Figure 12 are very similar and from both of them a very general path for ^{63}Ni can be mapped. As expected the nickel is initially taken into the roots of the plant and immediately transported to the stems. From the stems most of the nickel moves into the middle leaves but whether this is via the apical leaves downwards through the plant still needs to be ascertained. It is thought that there is a flow of nickel through the plant as some ^{63}Ni was found in the apical leaves whilst none, or very little, was seen in the bottom leaves. Whether this flow is downwards or upwards still needs to be ascertained. The data also suggest that the middle leaves are used as the storage areas for nickel as this is where the most ^{63}Ni occurs. The stems and roots appear to be transport media as, with time, there appears to be a general decrease in the levels of ^{63}Ni found in the roots and stems, as the finite amount of ^{63}Ni is distributed throughout the plant. More work is clearly required to fully establish the path of nickel through the plant and one way in which this could done is to perform a "pulse-chase" experiment in which the plant is initially pulsed with ^{63}Ni and immediately "chased" with non-radioactive nickel. It is interesting to note that, even though the plants growing in topsoil did not have any nickel in their tissues at the start of the experiment, the general trends of nickel uptake in plants growing in the different media was fairly similar.

3.3.3 Possible Involvement of Malate in Nickel and PGM Storage and Tolerance in *Berkheya coddii*

Since *Berkheya coddii* is a true nickel hyperaccumulator, capable of accumulating nickel up to 3% dry weight in its leaves (Table 3; Howes, 1990), it was assumed to have a tolerance mechanism similar to those seen in other nickel hyperaccumulators. Work was thus done by Howes (*pers. comm.*) to isolate one or more of the organic acids normally associated with nickel hyperaccumulators. Initially no such organic acid could be isolated and it was assumed that *B. coddii* used a different mechanism to detoxify nickel (Howes, 1990). However, more work by Howes, using gel chromatography and molecular exclusion chromatography, finally led to the isolation of a nickel-malate complex from *B. coddii* (Anderson *et al.*, 1997; Howes *et al.*, in preparation). This strongly suggested that malate may be utilised by *B. coddii* for nickel tolerance, although further work is required to fully substantiate this possibility. Towards this end, attempts were made in the present study to check for a correlation between malate levels and nickel, as well as between malate and the platinum, palladium, rhodium and gold (section 3.3.3) accumulated by *B. coddii*. When checking for possible correlations it was important to bear in mind that the majority of the malate would be engaged in the Tricarboxylic Acid Cycle and that such analyses and correlations would need to be sensitive enough to detect small changes in the additional amount of malate that might be involved in metal tolerance and storage. It should also be noted that only naturally occurring free L-malic acid was assayed.

Table 8 and Table 9 give the free L-malic acid levels, the corresponding metal concentrations and the correlation coefficients between the free L-malic acid and metal data for the leaf and stem tissue, respectively. It should be noted that the PGM values obtained from analysis of the stem and leaf tissue inoculated with 60 g FIC have not been included in the calculation for the correlation coefficients as these plants were dying due to the toxic levels of PGM found in the soil. Two separate tables (Table 8 and Table 9) were drawn up for the comparison between L-malic acid and metals, in the leaves and stems respectively, in order to be in a position to detect any differences in the roles of the two tissues. From Table 8 it can be seen that there is a better correlation between the L-malic acid and metal concentration in the leaves than in the stems (Table 9). This suggests that L-malic acid might play a role in metal storage in the leaves but not in the stems. It appears that, in the leaves (Table 8), the L-malic acid concentration is

proportional to the concentration of all the metals except gold, where very little of the metal is accumulated.

Table 8: Comparison of L-Malic Acid and Metal Concentrations on *B. coddii* Leaves treated with FIC or Metallics

TREATMENT	L-MALIC ACID	METALS [mg/kg "dry tissue"(n=2)]					
	mg/kg "wet tissue"	Ni	Pt	Pd	Rh	Au	Total Metals
FIC - Control	85.78	20 329	3.73	2.34	0.78	1.25	20 337
6 g	22.63	18 326	99.57	102.1	29.09	1.42	18 558
15 g	32.75	15 835	87.52	94.88	26.73	1.30	16 045
30 g	12.81	15 223	211.2	209.2	58.98	1.27	15 703
60 g	22.36	15 482	1 842	2 387	711.5	2.91	20 426
Met – Control	85.78	20 329	3.73	2.34	0.78	1.25	20 337
6 g	36.58	20 714	8.60	1.60	0.58	0.81	20 726
15 g	47.26	19 144	29.14	5.01	0.88	4.66	19 184
30 g	26.96	17 550	11.70	3.17	1.68	1.73	17 568
60 g	29.60	19 514	15.92	1.59	0.67	1.12	19 533
Correlation (n = 20)	-	0.3697	0.3461	0.3182	0.3238	0.0225	0.3596

Table 9: Comparison of L-Malic Acid and Metal Concentrations in *B. coddii* Stems Treated with FIC or Metallics

TREATMENT	L-MALIC ACID	METAL [mg/kg "dry tissue" (n = 2)]					
	mg/kg "wet tissue"	Ni	Pt	Pd	Rh	Au	Total Metals
FIC - Control	71.30	4 683	1.01	1.07	0.36	0.50	4 686
6 g	69.64	4 020	187.0	160.4	27.21	8.38	4 403
15 g	64.21	3 147	58.84	57.08	9.31	4.71	3 277
30 g	105.10	4 069	136.2	105.7	22.79	2.67	4 336
60 g	79.22	3 830	1 274	634.3	254.2	3.78	5 996
Met - Control	71.30	4 683	1.01	1.07	0.36	0.50	4 686
6 g	104.64	4 590	4.61	1.56	0.65	0.65	4 597
15 g	69.22	5 147	12.46	5.06	2.70	2.79	5 170
30 g	98.88	4 701	60.08	8.23	4.05	5.85	4 779
60 g	71.49	4 564	40.33	9.54	2.35	5.83	4 622
Correlation (n = 20)	-	0.0362	0.0024	0.0044	0.002	0.1012	0.0438

The above results, therefore, constitute further evidence for the role of L-malic acid in the storage of metals in the leaves of *B. coddii*. As some free L-malic acid was also found in the

stems it is thought that malate may have a role to play in the transport of nickel in *B. coddii*. More work, however needs to be performed to ascertain the presence of D-malic acid and complexed malate within the plant and then to develop methods for the determination of free D-malic acid and complexed malic acid in order to fully substantiate the storage and tolerance mechanism for nickel in *B. coddii*.

3.3.4 Comparison of L-Malic Acid Levels in *Berkheya coddii* with those in some Non-Accumulating Plants

Malate is an essential component of the Tricarboxylic Acid Cycle and it is thus found in all cells. It was therefore suggested that if malate was in fact used in nickel tolerance mechanisms in *B. coddii*, then higher levels of malate might be found in *B. coddii* than in plants having little or no tolerance to nickel. Thus experiments were performed to determine the differences in malate concentration between nickel tolerant *B. coddii* and various non-tolerant plants.

Lettuce (*Lactuca sativa* L.) was chosen as one of the plants for comparison as it was thought to have little tolerance to heavy metals. It has been found, however, that lettuce appears to contain an abundance of low-molecular weight trace metal complexes (Walker and Welch, 1987). Unlike *B. coddii*, the trace metals in lettuce appear not to be associated with organic acids but rather with phytochelatins. It was, therefore, felt that the free L-malic acid concentrations in lettuce would be similar to those of most other non-tolerant plants. The free L-malic acid levels in *Gazania* hybrids were also compared with *B. coddii* as *Gazania* is extremely closely related to *Berkheya* and appears not to take up heavy metals. *Arctotis*, *Berkheya*, *Gazania* and the cultivated ornamentals of *Gazania* and *Arctotis* make up the four sub-tribes of Arctotideae, belonging to the Compositae family (Mabberley, 1987). Another species of *Berkheya*, indigenous to Natal, was also used for free L-malic acid comparison. Unfortunately this plant could not be further identified. *Senecio oxyrifolius* was also used in the free L-malic acid comparison as it belongs to the Compositae family but is part of the Asteroideae group, as opposed to the Arctotideae group (Mabberley, 1987). Again *Senecio oxyrifolius* has not been reported to take up, or show tolerance to, heavy metals. Finally *Gerbera jamesonii* was used in the free L-malic acid comparison. This plant (commonly known as the Barberton daisy) is indigenous to the Barberton area (as is *B. coddii*) and it was felt that a comparison of its free L-malic acid levels would be interesting as this plant not only

belongs to the Compositae family but is a member of the subfamily, Arctotideae, to which *B. coddii* also belongs (Mabberley, 1987). Unlike *B. coddii*, *G. jamesonii* is not known to accumulate any heavy metals.

Table 10 reflects the data for the free L-malic acid concentration found in the dry tissues of *B. coddii*, growing on various soils, as well as the free L-malic acid concentration found in the dry tissues of other, non-tolerant plants. These results indicate that the less related a plant is to *B. coddii* the greater are its concentrations of free L-malic acid in the AGB. This is illustrated by the fact that *Lactuca*, which, in these studies, is the plant least related to *B. coddii*, contains the greatest concentrations of free L-malic acid in its AGB. Conversely, the unknown *Berkheya* species contains the least amount of free L-malic acid in its AGB. Thus this result is not consistent with work done on nickel hyperaccumulators by Lee *et al.* (1978) where it has been found that nickel and zinc accumulators show higher levels of the simple organic acids when compared with non-accumulating plants. Also, in work done by Pelosi *et al.* (1976), to confirm that malic and malonic acids were the nickel binders in *Alyssum bertolonii*, it was shown that the plants grown on ordinary soil contained malic and malonic acid concentrations an order of magnitude lower than those grown on serpentine soils. The results obtained for this project, however, were for free L-malic acid only, whilst those of the above workers were for total malate. It was thus felt that the malate assays in *B. coddii* may have been low due to the assay the kit being unable to assay D-malic acid and complexed malate, although it was felt that the harshness of the extraction method would have caused the complexed malate to dissociate, probably to the natural L-malic acid form. Another factor that may have contributed to the low free L-malic acid levels in *B. coddii* may have been that the extraction method did not allow all the free L-malic acid to be released from the plant cells. As *Berkheya* tissue is tougher than that of *Lactuca, Senecio, Gerbera* or *Gazania* less free L-malic acid may have been released from this tissue compared with the others.

It should also be remembered that *Lactuca* grows much more actively than *B. coddii* and therefore it probably has a more active Tricarboxylic Acid Cycle than *B. coddii*, as it requires more energy, causing more free L-malic acid to be found in *Lactuca* than in *B. coddii*. It is thought that, of all the plants analysed the *Berkheya* plants grew the slowest and so they would naturally have less free L-malic acid. Thus, the only true comparison of free L-malic acid levels

between an accumulator and a non-accumulator species is between *B. coddii* and the unknown *Berkheya* species. From these results it can be seen that the leaves of the non-accumulating *Berkheya* species contains about 55% less free L-malic acid than do the leaves of *B. coddii*, occurring naturally on serpentine soil. This result is, therefore, in accordance with those of Lee *et al.* (1978) and Pelosi *et al.* (1976) and constitutes further evidence for the involvement of malate in the mechanism of storage of nickel within *B. coddii*.

Table 10: Comparison of the L-Malic Acid Levels in the Different Tissues of Various Plants

PLANT SPECIES	L-MALIC ACID CONC. (mg/ kg "dry tissue")			
	Leaf	**Stem**	**Root**	**AGB**
Lactuca sativa L.	36.84	13.43	5.93	50.27
Gazania hybrid	16.47	13.25	16.51	29.72
Senecio oxyrifolius	23.97	Nd	Nd	Nd
Gerbera jamesonii	9.28	Nd	Nd	Nd
Berkheya (sp. unknown)	1.95	Nd	Nd	Nd
Average L-Malic Acid	*17.70*	*13.34*	*11.22*	*40.00*
***B. coddii* on Various Soils**				
Serpentine	4.30	5.55	6.52	9.85
RBMR	7.58	4.25	3.57	21.08
Topsoil	1.50	13.50	9.46	5.75
Average L-Malic Acid	*4.46*	*7.77*	*6.52*	*12.23*

To try to determine whether malic acid was produced only in response to growth on soils containing heavy metals, *B. coddii* was grown up on metal-free topsoil and the free L-malic acid levels were measured. The free L-malic acid levels of *B. coddii* grown on RBMR soil were also compared with those of *B. coddii* grown on serpentine soil. The composition of these soils is given in section 4.3.2. The free L-malic acid level in the leaves of *B. coddii* grown on topsoil is much lower than the levels found in the plants grown on either serpentine or RBMR soil. This constitutes a significant result in that it suggests that larger amounts of L-malic acid are only produced in *B. coddii* in response to heavy metal contamination. Thus it is possible that some sort of activation mechanism is involved in the storage process with malate. It is interesting to note that the levels in the leaves of *B. coddii* grown in topsoil are similar to

the levels seen in the unknown *Berkheya* species. This *Berkheya* species was growing on uncontaminated soils and so no tolerance mechanism would be required. Thus it would be interesting to see the response of this plant to contaminated soils and to determine if it produced greater amounts of malic acid when under stress.

Less free L-malic acid appears to be found in *B. coddii* growing on serpentine soil than in *B. coddii* growing on RBMR soil. This is to be expected as both sets of plants produce large amounts of L-malic acid in response to metal contamination but there is less plant-available metal in the RBMR soil compared with the serpentine soil (section 4.3.2).

Although the above differences in free L-malic acid levels between accumulators and non-accumulators give no conclusive evidence that malate is the binding ligand for nickel in *B. coddii*, the influence of metals in soil on the free L-malic acid levels in *B. coddii* alone, constitute strong evidence for the activation of malic acid synthesis in the presence of increased metals taken up from the soil. Thus this in turn strengthens the argument that malate is involved in nickel storage and tolerance in *B. coddii*. More work to elucidate a method for measuring the total bound malate might also show that malate in *B. coddii* would be higher than in the non-accumulating plants.

3.4 *Conclusions*

All the above results show that *B. coddii* is able to efficiently accumulate nickel, both from serpentine soil and RBMR soil, which makes *B. coddii* an extremely useful tool in the phytoremediation of nickel contaminated soils. From these results it was proposed that the plant be used in a field trial of phytoremediation at RBMR (Chapter 5). It was also seen that, in the process of accumulating nickel, *B. coddii* could accumulate platinum, palladium and, to a certain extent, rhodium. It was thus felt that if the soils also showed PGM contamination the plant would pick up these metals and that they could then be recovered. This would make the process more economically viable but the success with nickel was the major reason for the commencement of the field trials.

The above results also confirmed that nickel is accumulated in the greatest levels in the leaves of the plants and not in the stems. This means that, in order to obtain about 80% of the nickel

accumulated by the plant, only the leaves need to be harvested. The nickel distribution experiments were not conclusive as they were not run for long enough and so, although it appears that most of the nickel is concentrated in the middle leaves of the plant, it was felt that leaves should not be selectively harvested, until the nickel distribution for an entire growing season had been mapped. In terms of ashing (section 5.3.7), it is more efficient to ash leaves than stems, however, in terms of harvesting it is less work to cut off the base of the stems and to shred the leaf and stem material. As labour is more expensive than ashing it was decided that the entire plant (excluding the roots) would be harvested.

The levels of free L-malic acid appear to differ amongst the various plant species but it is thought that the growth rates of the different plants and, therefore the activity of the respective Tricarboxylic Acid Cycles, is responsible for this anomaly. It is thought, however, that greater metal binding, resulting in lower free L-malic acid levels, occurs in *Berkheya coddii* compared with the other plant species examined. This observation provides more evidence that malate is involved in the metal tolerance properties seen in *B. coddii*.

The above results show, fairly conclusively, that L-malic acid does indeed appear to play a role in metal tolerance in *B. coddii* as differences in free L-malic acid levels are seen in *B. coddii* plants grown on different soils, containing distinct levels of plant-available heavy metals. However, these results need to be confirmed by analysing the malate concentrations *in vivo* as, what may be happening in the *in vitro* extraction procedure, is that the malate may be artificially binding to the released nickel, thereby falsely indicating that malate binds to nickel.

From the above results however, it could not be fully substantiated whether malate is the only organic acid involved with nickel tolerance or whether another acid is used as a transport ligand for nickel. It is thought that malate acts as the storage ligand in the leaves as the decrease in free L-malic acid levels between *B. coddii* and other plant species is very marked, perhaps suggesting that more of the malic acid is bound in *B. coddii* than in the other plants. Nickel uptake, at the root level, needs to be examined in order to determine the compound used by *B. coddii* to take up nickel from the soil. Work also needs to be done to determine the compound that transports nickel to the leaves via the stem. The results given for the concentration of L-malic acid contained within the stems of *B. coddii* are inconclusive and

more accurate work on extracting L-malic acid from the stems would need to be performed before conclusive results could be obtained.

CHAPTER 4

FEASIBILITY OF USING *BERKHEYA CODDII* FOR PHYTOREMEDIATION

4.1 *Introduction*

Although the results presented in Chapter 3 confirmed that *B. coddii* is able to hyperaccumulate significant quantities of nickel from various metal preparations, test soils and sites, it was considered vitally important to first investigate the feasibility of growing the plant in the highly toxic waste sites of Rustenburg before developing any phytoremediation process. In this regard, of particular importance were considerations of the plant's nutrient requirements and nutritional tolerance, and its ability to tolerate the Rustenburg climate and accumulate nickel from the toxic soil conditions. Furthermore, for the purpose of harvesting the accumulated nickel, it was important to study the morphology and growth-cycle of the plant, and to perform field trials in the Rustenburg site to optimise *B. coddii*'s ability to grow on the site. Since the field trials, as well as any future population of waste areas, would require large numbers of plants, various approaches for plant and seed propagation and production were investigated.

In this chapter, the results obtained from the above investigations are presented and discussed in the light of the possibility of using *B. coddii* in a phytoremediation process for cleaning up the Rustenburg site.

4.2 *Materials and Methods*

4.2.1 Comparison of Climatic Factors in the Barberton and Rustenburg Sites

The six serpentine sites populated by *B. coddii* near Barberton, were compared with the Rustenburg sites, with respect to various climatic factors, including rainfall, temperature and incidence of hailstorms. Data resources investigated included information supplied by the Institute for Tropical and Sub-tropical Crops in Nelspruit, as well as from Rustenburg Base Metal Refineries (RBMR).

4.2.2 Comparison of Soil Types on which *Berkheya coddii* was Grown

Since it is the physical and chemical compositions of soils that ensure plant growth, it was imperative that soil from the Rustenburg site and soil from the serpentine site were fully analysed and compared. Due to time constraints, and a lack of equipment, the soil samples were sent to the Agricultural Research Council[4] for analysis (Steyn *et al.*, 1995). In order to establish the differences in these soils from a "normal" soil, a topsoil sample taken from the Rustenburg area was also analysed.

In order that the elements under analysis can be properly extracted it is important to understand the forms in which available nutrient and metal elements are held in soil, for uptake by plants. Although there is still much debate over the extraction of nutrients to determine those that are available to plants, generally elements are extracted as follows:

1. By water extraction to remove elements that are in solution in ionic or combined form;
2. By neutral salts such as ammonium acetate to remove the readily exchangeable ions in inorganic or organic exchange-active complexes;
3. By dilute acetic acid to remove the more firmly bound ions in the exchange complexes;
4. By EDTA to remove the insoluble organic or organo-mineral complexes; and
5. By acid ammonium oxalate to remove precipitated oxides or other insoluble salts (Levison, 1974).

All three soil samples were air-dried away from sunlight, to prevent the fixation and release of compounds. They were then sieved to < 2 mm, whilst ensuring that they were not contaminated with metals from the sieves (Barnard *et al.*, 1990; Baker *et al.*, 1994). The analyses that were then performed on the prepared serpentine soil, RBMR soil and topsoil are listed below:

[4] Agricultural Research Council: Institute for Soil, Climate and Water, Arcadia, Pretoria, RSA

4.2.2.1 pH

Two pH readings were taken from the soils. In the first, pH (KCl), the soil samples were suspended in a 1 mol dm^{-3} potassium chloride (KCl). In the second, pH (H_2O), the samples were suspended in a 1:2.5, soil : water ratio, on a mass basis. Carbon dioxide (CO_2) was excluded from the suspensions as it lowers the pH in calcareous soils (Barnard *et al.*, 1990).

4.2.2.2 Extractable Cations

Steyn *et al.* (1995) used ammonium acetate to remove the extractable cations Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} from the soils, allowing the nutrient status of each soil to be determined. As the rate of extraction is a function of temperature all extractions were performed at 20 ± 2°C (Baker *et al.*, 1994; Barnard *et al.*, 1990). The cations in solution were analysed using AAS (section 2.6.2.1).

4.2.2.3 Soil Leachability

Steyn *et al.* (1995) made up a water-saturated soil paste of each sample, using the method of Barnard *et al.* (1990), in order to determine the leachability of each soil. The extracted elements were then analysed.

4.2.2.4 Plant-Available Micro-Nutrients

The micronutrients were determined by Steyn *et al.* (1995) using the di-ammonium EDTA method of Barnard *et al.* (1990), although there is still a large amount of debate over ways of determining the availability of the micro-elements to plants (Howes, AW *in press*).

4.2.2.5 Extractable Phosphorus

The book by Barnard *et al.* (1990) describes the Bray-1 method that Steyn *et al.* (1995) used to determine the extractable phosphorus. This method extracts more soluble phosphorus, thought to be the phosphorus easily available to plants, than does the Bray-2 method (Barnard *et al.*, 1990).

4.2.2.6 Total Nitrogen

Steyn *et al.* (1995) used a digestion method to determine the plant available nitrogen.

4.2.2.7 Particle Size Distribution

From the method of Barnard *et al.* (1990), which uses chemical and mechanical means, Steyn *et al.* (1995) analysed the particle size distribution of RBMR soil, serpentine soil and topsoil. It is an expensive analysis as it is labour intensive, requiring skilled labour. The particle size distribution of a soil allows a soil to be classified according to the soil classes found, Table 11, (Barnard *et al.*, 1990).

Table 11: Soil Particle Size Classes and the Methods of Separation Used

(Barnard *et al.*, 1990)

CLASS	DIAMETER	SEPARATION METHOD
Gravel	> 2	Sieve
Coarse sand	2.0 – 0.5	Sieve
Medium sand	0.5 - 0.25	Sieve
Fine sand	0.25 - 0.106	Sieve
Very fine sand	0.106 - 0.05	Sieve
Coarse silt	0.05 - 0.02	Sedimentation
Fine silt	0.02 – 0.002	Sedimentation
Clay	< 0.002	Sedimentation

4.2.3 Observed Growth-Cycle of *Berkheya coddii*

In order that *B. coddii* could be planted, its seeds collected, and its leaves harvested at the correct intervals, its growth-cycle was observed at the Queens River site in Barberton over a period of twelve months. The growth-cycle was also documented photographically using a Minotola Automatic camera with a zoom lens.

4.2.4 Cultivation of *Berkheya coddii* Plants for the Nutrient Experiments and Phytoremediation

Two methods of cultivating *B. coddii* plants for the phytoremediation of contaminated sites were investigated and are detailed below.

4.2.4.1 Culturing of *Berkheya coddii* by Direct Organogenesis

In propagating *B. coddii* plants in tissue culture by direct organogenesis three separate areas were required (Dixon, 1985):

1. An area where the medium was prepared containing chemicals, glassware, a fridge, a balance, a pH meter, a stirrer and an autoclave;
2. A laminar-flow bench where work requiring sterile technique could be performed; and
3. A light incubator (Conviron EF7H) with a temperature control, where the explants could be grown.

All experimentation was performed at room temperature and the cultured plants were incubated at 25°C with a light cycle of 16 hours and a radiant flux density of 250 $\mu E.m^{-2}s^{-1}$. All the solutions given in Table 12 were made up in high quality water containing low salts (Hartmann *et al.*, 1990). The inorganic stock solutions were stored at 4°C for 4 weeks while the vitamins were dispensed as 10 ml aliquots and frozen at 20°C (Dixon, 1985). All the reagents used were analytical grade. Common solutions (numbered 1 to 6 under the "Solution" column in Table 12) were made up where no precipitation or other interactions between the reagents occurred (Hartmann *et al.*, 1990).

In order to germinate *B. coddii* seeds in tissue culture a solid Murashige and Skoog (MS) medium was prepared, using the components given in Table 12. Once the medium had been made up and the Gelrite® added, 10 ml of the culture medium was dispensed into glass tubes (100 mm length and 24.5 mm diameter) using a cattle doser. The tubes were then capped with well fitting clear perspex lids and autoclaved for 20 min at a pressure of 1.5 $kgf.cm^{-2}$ and a temperature of 121°C (the conditions used to autoclave all material in these experiments). The

tubes were removed while the media was still liquid and swirled gently to allow even solidification of the media. These tubes were stored at 4°C until required for seed germination.

Two empty 500 ml glass beakers, covered with aluminium foil, 1 x 500 ml glass beaker containing 200 ml dist. water and covered with aluminium foil, 15 x 500 ml glass beakers containing 400 ml dist. water and covered with aluminium foil, 5 tea strainers, 2 pairs long nosed forceps and a scalpel, all wrapped in aluminium foil, were autoclaved as above and then taken to the laminar-flow bench. Pipette tips for automatic pipettes were autoclaved along with a 100-ml bottle containing dist. water. The tubes containing the seed germination medium were also taken to the laminar-flow bench, which had been turned on 10 min before use and swabbed down with 65% (v/v) ethanol to reduce contamination.

Table 12: Murashige and Skoog Nutrient Medium (Hartmann *et al.*, 1990)

SOLUTION	SALT	STOCK SOLN. (g/l)	VOL. (ml) STOCK/ litre CULTURE MEDIUM
1	KH_4NO_3 KNO_3	16.5 19.0	100
2	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$	37.0 1.56 0.86 0.0025	10
3	$CaCl_2.2H_2O$ KI $CoCl_2.6H_2O$ $Na_2MoO_4.2H_2O$	44.0 0.083 0.0025 0.0250	10
4	KH_2PO_4 H_3BO_3	17.00 0.62	10
5*	$FeSO_4.7H_2O$ Na_2EDTA	3.73 2.78	10
6	Myo-inositol Glycine Nicotinic acid Pyridoxine-HCl Thiamine-HCl	10.00 0.20 0.005 0.050 0.005	10
7	Sucrose Agar	10 g/l for a 1 % concentration. 10 mg/l agar or 6 mg/l Gelrite ® for a solid medium. No agar or Gelrite® is used for a liquid medium.	
8	pH	Adjust to 5.8 using 1M NaOH or 1M HCl prior to autoclaving.	

*The iron solution should be stored in a dark glass bottle

Before the *B. coddii* seeds were taken to the laminar-flow bench, excess plant material was removed and the seeds were washed in tap water. They were then placed in a 500-ml glass beaker, containing 65% (v/v) ethanol, and soaked for 1 min. The beaker containing the seeds and ethanol was then taken to the laminar-flow bench. At the laminar-flow bench the ethanol was poured into an empty sterile beaker, whilst the seeds were captured in a sterile tea strainer. The seeds were then surface sterilised in a beaker containing 200 ml sterile dist. water, 300 ml commercial JIK™ (3.5% sodium hypochlorite) and 5 drops Tween™-20. The seeds were removed from the sterilising solution in batches. The first batch was removed after 1 min, the next after 5 min, the following after 10 min, another after 15 min and the final batch after 20 min. This ensured that optimum sterilisation of the seeds occurred with least seed damage.

Each set of seeds was then placed in a separate glass beaker containing sterile dist. water for 15 min. This process was repeated three times in order to ensure that all the sodium hypochlorite was removed from the seeds.

Three seeds from each sterilisation batch were placed into separate tubes containing the MS media, using sterile forceps flamed in 65% (v/v) ethanol. A drop of sterile dist. water was then placed onto each set of seeds using an automatic pipette and sterile tips. Enough water was added to enhance seed germination by imbibition of the water, whilst ensuring that oxygen starvation did not occur causing a lack of germination. The open tubes were then flamed using a Bunsen burner and the perspex lids replaced. One tube was left with its lid open, on the laminar-flow bench for the entire working period. Once all the seeds had been sterilised and placed in their respective tubes, the lid was replaced on the seed-free tube and this tube was then incubated, in the light incubator, with the others in order to determine the sterility on the laminar-flow bench. During growth in the light incubator, any tubes that became contaminated were immediately disposed of. Once the seeds had germinated the plants were left to grow to a stage at which they could be subcultured.

A liquid medium was used for direct organogenesis as *B. coddii* is extremely sensitive to water stress. The liquid medium was made up from the components given in Table 12, after which 10 ml of medium was dispensed into 100-ml glass jars (obtained from Consol Glass, RSA) with perspex screw caps, using the cattle doser. Perspex screw caps (obtained from Kirstenbosch Botanical Gardens, Cape Town) were loosely placed on the jars before

autoclaving. After the jars containing the media had been autoclaved, the lids were tightened and the jars stored at 4°C until required for direct organogenesis.

In direct organogenesis the sterile explants, contained in tissue culture medium, are cut into sections (Figure 13) and replaced in fresh culture medium. In this way explant growth becomes exponential. In order to produce explants by direct organogenesis, the laminar-flow bench was again sterilised with 65% (v/v) ethanol. Forceps, scalpel and glass petri dishes were all wrapped in aluminium foil and autoclaved as above, while sterile blades were used with the scalpel. The glass jars containing the MS media were sprayed with 65% ethanol before being placed on the laminar-flow bench. The tubes (jars) containing the sterile plants were also sterilised with ethanol before being opened.

After flaming the mouth of the tube (jar) containing the sterile plant, the plant was removed from the tube, with flamed, sterile forceps, and placed on a sterile petri dish. The stem was then cut into nodal sections (sections with axillary buds) with the flamed scalpel, Figure 13. These nodal sections were transferred into the liquid culture medium after which the mouth of the jar was flamed and then sealed. All the jars were then incubated, in the light incubator, on an orbital shaker that was agitated at a speed of 40 – 100 rpm, to allow aeration of the plant material. The explants were grown up for a period of two to three weeks after which they were again subcultured. Subculturing continued until 400 plants had been obtained.

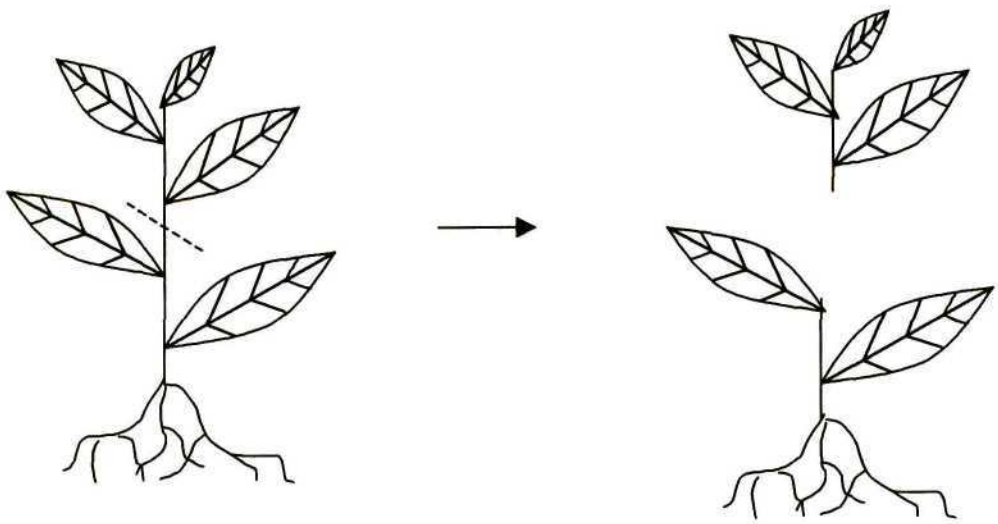


Figure 13: Diagrammatic Representation of Explant Production for Direct Organogenesis

As some of the *B. coddii* explants appeared to have a systemic infection, which caused media contamination, it was necessary to identify the contaminant. In this regard a sample of the contaminated media was plated onto 2 nutrient agar (Table 13) plates. One plate was incubated overnight at 37°C, the other was incubated overnight at room temperature.

Table 13: Composition of Nutrient Agar

COMPOUND	MASS (g per litre)
Sodium chloride (NaCl)	5
Tryptone	10
Yeast Extract	5
Agar	15

A Gram stain (Schlegal, 1988) was performed on a colony picked off the plate incubated at room temperature. For the Gram stain the colony was fixed to a slide by passing the slide over the flame of a Bunsen burner. A solution of crystal violet was added to the fixed bacteria and left for 1 min. The solution was washed off with 65% (v/v) ethanol. Iodine solution was then added and left for 30 sec. Again it was washed off with 65% (v/v) ethanol. The bacteria were then counter-stained with fuchsin, for 1 min. This solution was again washed off. The slide was left to dry and then viewed under a light microscope at a 1 000x magnification.

Once the contaminant had been identified as being Gram-negative, antibiotics affecting these bacteria were tested. An antibiogram test was performed (Lancini and Parenti, 1982), in which antibiogram test discs, inoculated with known concentrations of the antibiotics nalidixic acid, kanamycin, streptomycin, polymyxin B, tetracycline, rifampicin, gentomycin and chloramphenicol, were placed on nutrient agar plates, inoculated with the contaminating bacteria. These plates were incubated overnight, at room temperature, and then checked for halo formation. Halos form when bacteria, sensitive to a specific antibiotic, are unable to grow within a certain radius of the disc; the larger the halo the more sensitive the bacteria to the particular antibiotic. The diameters of the halos were measured and the antibiotic conferring greatest sensitivity identified.

As the plated bacteria appeared to be most sensitive to kanamycin it was important to determine the concentration of kanamycin at which the bacteria first became sensitive. Thus a

gradient plate was poured. To make up the gradient plate a solution of 10 mg/ml kanamycin was prepared in dist. H_2O. As all antibiotics are thermolabile the kanamycin stock was sterilised by filtering it at 0.22 μm, into a sterile glass bottle. The 10 ml of kanamycin stock was added to 90 ml of liquid (± 45°C) sterile nutrient agar, thus giving a concentration of 1 000 μg/ml. The plate was then placed at a 25° angle and the nutrient agar containing kanamycin poured into it (about 30 ml). Once the agar had set, a layer of nutrient agar, containing no antibiotic, was poured over it in such a way as to leave a small section of agar containing kanamycin exposed. Thus, at one end of the plate the highest concentration of kanamycin of 1 000 μg/ml was found, while at the other end the kanamycin concentration was 0 μg/ml. The concentration of kanamycin decreased linearly between 1 000 μg/ml and 0 μg/ml on the plate. The contaminating bacteria were plated onto the gradient plate and incubated at room temperature for 36 hr. The plate was then examined for bacterial growth and the point at which growth stopped was said to be the kanamycin concentration at which the bacteria were sensitive. The corresponding concentration of sterile kanamycin could then be added to the nutrient media of the contaminated plants.

4.2.4.2 Germination of *Berkheya coddii* Seeds on Cotton Wool

In order to determine the germination rate of *B. coddii*, three hundred 8 month-old seeds (2.3.1) were planted in rows on wet cotton wool in Tupperware™ containers (Anderson *et al.*, 1997). The containers were then covered with Clingwrap™, which allowed the entry of light but prevented moisture loss. The containers were placed in the greenhouse at UNP (section 2.2.1.2) and the seeds left to germinate.

The germination trials showed that the seeds of *B. coddii* had a very low germination rate and that the rate decreased rapidly within a year (Anderson *et al.*, 1997). Seeds that were seven months old were dissected, under a dissection microscope, to determine the presence or absence of an embryo within the seed casings.

4.2.5 Optimisation of the Nutrient Requirements of *Berkheya coddii*

4.2.5.1 Introduction

Ideally nutrient trials should be performed on plants growing in tissue culture as, due to the fact that they are all clones, any variation in response can be attributed solely to nutrient affects. Unfortunately, problems were experienced with performing the nutrient trials on the plants in tissue culture, and so the final nutrient trials were performed on whole *B. coddii* plants growing in pots in the greenhouse at UNP. In order to minimise differences in nutritional requirements due to genetic effects, a number of plants were used for each experiment (replicates) and the experiments were performed in duplicate.

The pot trials were performed on seedlings that had been germinated on cotton wool (section 4.2.4.2). In each experiment three replicates were used, and each experiment was performed in duplicate. The effects on the replicates were then averaged whilst all the material from the replicates was combined for analysis of metal uptake.

4.2.5.2 Nutrient Trials Performed on Fully-Formed *Berkheya coddii* Explants Growing in Tissue Culture

Before making up the various culture solutions to test the nutrient requirements of *B. coddii* it was decided that, as the explants had not been exposed to any nickel, growth of the cultured plants on MS media containing nickel should be tested. It was felt that a soluble nickel compound should be used in the trials rather than serpentine nickel, as the effects of autoclaving on serpentine are not known. As approximately 4 000 mg/kg nickel is found in serpentine soil it was decided that the media should contain 2 000 mg/l nickel in the form of nickel sulphate ($NiSO_4.6H_2O$) (Joughin, 1992). Thus 150 ml of liquid MS media (Table 12), containing 9.5684 g of $NiSO_4.6H_2O$, was added to each of ten 100-ml glass jars. The jars were autoclaved and sterile explants placed in the media. The plants were grown up for one week in the Conviron (section 2.2.1.3).

4.2.5.3 Disease Control

Disease in plants used in pot trials can be a problem and so, as plant diseases are usually carried and transmitted by vectors, the vector population is generally removed by sprays toxic to the vector but not to the plant. *B. coddii* plants growing in the greenhouse (section 2.2.1.2) appeared to be susceptible to whitefly and so two insecticides were used for their control. Whiteflies have a five-day life-cycle with six life stages: egg, crawler, second instar, third instar, pupa and adult (Gill, 1990). The insecticide acts on all the stages thereby interrupting the life-cycle. To ensure complete removal of whitefly the plants need to be sprayed every four days and the insecticides need to be alternated thereby ensuring that the whiteflies do not become resistant to either product (Lifestyle[5]). Spraying should be continued until no more whiteflies are seen, after which the plants are sprayed once a week. An alternative to removing whiteflies from greenhouses is to use a household vacuum cleaner.

The *B. coddii* plants growing in the greenhouse were sprayed alternately with 5 ml of Dursban per 10 l tap water and 80 ml of Vydate per 10 l tap water. Both the insecticides were purchased from Macdonalds Nursery, Pietermaritzburg. As *B. coddii* is a fairly hardy plant and the whitefly infestation in the greenhouse was particularly bad, the plants were sprayed every two days for 10 days. This effectively removed the whitefly without harming the plants.

4.2.5.4 Nutrient Solutions

Various nutrient solutions were prepared for the nutrient trials on *B. coddii*. In these nutrient trials the effects of various concentrations of the macronutrients nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) were examined. Three different concentrations of each nutrient were tested, in duplicate, on three replicate plants; a control experiment was also performed in duplicate on three replicate plants.

Table 14 shows the concentration of each nutrient tested. It should be noted that only the concentration of the nutrient being tested was changed in each experiment and that the control values of the remaining nutrients were used to make up the remainder of the solution. This

---

[5] Information on spraying to eliminate whitefly infestations was obtained from the information centre at Lifestyle Nursery, Randburg, RSA.

meant that in each experiment the plants were treated with composite nutrient solutions and not just the nutrient under consideration e.g. a solution to test the effect of 250 mg/l nitrogen on the plants also comprised 150 mg/l P, 300 mg/l K, 500 mg/ l Ca and 250 mg/l Mg. Table 15 gives the concentrations of the compounds used to make up each separate stock solution. Table 16 gives the volumes of each stock solution (given in Table 15) required to make up the experimental solutions. From Table 15 and Table 16 it can be seen that the experimental solutions also contained micro-nutrients as no nutrients are contained in acid-washed sand and, without the micronutrients, the plants would not be able to grow at all.

Table 14: Concentrations of Macronutrients Tested in the Nutrient Trials

TREATMENT	MACRONUTRIENT CONCENTRATIONS USED IN NUTRIENT EXPERIMENTS* (mg/l)				
	1	2	3	4	5
	N	P	K	Ca	Mg
	NH_4NO_3	$Ca(H_2PO_4)_2$	KCl	$CaCl_2.2H_2O$	$MgSO_4.7H_2O$
Control	1000	150	300	500	250
1	250	37.5	75	125	62.5
2	500	75	150	250	125
3	2000	300	600	1000	500
4	4000	600	1200	2000	1000

*For each experiment only the concentration of the specified macronutrient was adjusted. The remaining nutrient concentrations remained the same as for the control.

Please Turn to Page 89 for Table 15.

Table 15: Basic Stock Solutions Required in making up the Experimental Solutions for the Nutrient Trials

SOLUTION	SALT	STOCK SOLUTION CONC. (g/l)	STANDARD CULTURE MEDIUM	
			Concentration (mg/l)	Stock Soln in Medium (ml)
1	NH_4NO_3	10	1000	100
2	$Ca(H_2PO_4)_2$	15	150	10
3	KCl	30	300	10
4	$CaCl_2.2H_2O$	50	500	10
5	$MgSO_4.7H_2O$	25	250	10
6	$FeSO_4.7H_2O$	4	40	10
	Na_2EDTA	3	30	
7 (Micronutrients)	$MnSO_4.4H_2O$	10.0	10	100
	H_3BO_3	10.0	10	
	$ZnSO_4.7H_2O$	1.0	1.0	
	KI	1.0	1.0	
	$CuSO_4.5H_2O$	0.1	0.1	
	$NaMoO_4.2H_2O$	0.1	0.1	
	$CoSO_4.7H_2O$	0.01	0.01	
8 (Vitamins)	Inositol	100	100	10
	Glycine	2.0	2.0	
	Nicotinic acid	0.05	0.05	
	Pyridoxine HCl	0.5	0.5	
	Thiamine HCl	0.05	0.05	
9	Sucrose	10 g/l for a 1 % concentration.		
	Agar	No agar or Gelrite® is used for a liquid medium.		
10	pH	Adjust to 5.8 using 1M NaOH or 1M HCl prior to autoclaving.		

Please Turn to Page 90 for Table 16.

Table 16: Volumes of each Stock Solution Required to Provide the Correct Macronutrient Concentrations as given in Table 14

	VOLUME STOCK SOLUTION (ml) TO ADD PER 200 ml CULTURE MEDIA																			
		Nitrogen				Phosphate				Potassium				Calcium				Magnesium			
Expt. N°	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Soln. N°																					
1	10.0	**5**	**20**	**40**	**80**	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
2	1.0	2	2	2	2	**0.5**	**2**	**4**	**8**	2	2	2	2	2	2	2	2	2	2	2	2
3	1.0	2	2	2	2	2	2	2	2	**0.5**	**2**	**4**	**8**	2	2	2	2	2	2	2	2
4	1.0	2	2	2	2	2	2	2	2	2	2	2	2	**0.5**	**2**	**4**	**8**	2	2	2	2
5	1.0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	**0.5**	**2**	**4**	**8**
6	1.0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
7	10.0	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
8	1.0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
9	10.0	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Sucrose (g)	0.5	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

*Experiment 1 is the control.

4.2.5.5 Nutrient Trials on Acid-Washed Sand and Serpentine Soil

The nutrient trials were initially performed on *B. coddii* plants growing in acid-washed sand. A 40-kg packet of river-sand, purchased from a local pool shop, was thoroughly washed with tap water in a large plastic bin, the base of which was lined with muslin cloth to prevent loss of sand through the three holes, which had been punched in it. A volume of 6 M HCl, equalling the volume of sand in the bin, was then run over the sand. The sand was washed with tap water until the effluent water ran clean, after which it was rinsed with 3 volumes of distilled water. The sand was distributed into 500 ml polystyrene containers, purchased from Game, Pietermaritzburg, which had holes punched in the base for drainage. The seedlings germinated on cotton wool (section 2.3.2) were then planted into the containers of acid-washed sand and watered daily with their respective MS-based nutrient solutions, Table 16. All the plants were left to grow in the greenhouse at UNP for the duration of the experiment.

As *B. coddii* seedlings could not be maintained in the acid-washed sand the nutrient experiments were repeated using serpentine soil as the base material. For each of the 21 experiments (Table 16), 3 replicates were performed and the experiments were performed in duplicate. Serpentine soil, obtained from Barberton, was placed in 126 x 12 cm diameter plastic plant pots, with drainage holes and individual plastic trays for solution capture. A three-week-old seedling was then placed in each pot, after it had been weighed. The pots were set up in the temperature-controlled phytotron at UNP (section 2.2.1.1). Each plant was then watered, once every three days, with 100 ml of its respective MS-based nutrient solution, Table 16. After two months the plants were carefully harvested, ensuring that no root material was lost. Each plant was then thoroughly washed in tap water, blotted with tissue paper to remove any excess water and weighed. The roots were removed, weighed and, for convenience, termed below-ground biomass (BGB). The stem and leaf material (AGB) was also weighed. The material was then prepared for nickel analysis by AAS (section 2.6).

4.3 *Results and Discussion*

4.3.1 Comparison of the Climatic Factors in the Barberton and Rustenburg Sites

In considering the feasibility of using *B. coddii* at RBMR, one of the first questions that was asked was whether the Barberton and Rustenburg climates are similar enough to make growing *B. coddii* at RBMR feasible. Of the six serpentine sites near Barberton on which *B. coddii* was found to grow, the area near the sawmill on the Queens River (16) (Figure 3, section 1.1), was chosen as the site most similar to that of Rustenburg. The geographical location of the Queens River, 30°55' (longitude) and 25°46' (latitude), and Rustenburg, 27°09' (longitude) and 24°56' (latitude) (Philips Atlas, 1986) sites are sufficiently similar to not warrant any concern about significant variations in day/ night length. Furthermore, since the altitude of the Barberton site is 920-1320 m, which is similar to that of Rustenburg (1150 m) (Balkwill *et al.*, 1997), no adverse effects of altitude on *B. coddii* were anticipated if grown in Rustenburg.

A comparison of average yearly rainfall and temperature between the two areas is given in Table 17. Although it can be seen that Rustenburg is significantly hotter than Barberton, this temperature difference was not expected to affect *B. coddii*, as the plant is fairly sturdy and has been grown successfully up to temperatures of 45°C in greenhouses in Pietermaritzburg. It was thought, however, that the drier, lower rainfall conditions of Rustenburg might be a problem, particularly when establishing the plants on the waste site. Thus irrigation would be probably be required for the period taken to establish the plants. However, it was hoped that the plants would adapt to the drier climate and that they would not require continuous irrigation.

The only other area of concern with regards climate was hail. Barberton is in a relatively hail-free band while Rustenburg, being on the edge of the highveld, tends to have two or three bad hailstorms annually. The effect of hail on the plants was unknown but would be determined once the plants had been established in Rustenburg.

Table 17: Average Rainfall, Minimum Daily Temperature and Maximum Daily Temperature of Barberton and Rustenburg

MONTH	BARBERTON*			RUSTENBURG**		
	Rainfall (mm)	Min. Temp. (°C)	Max. Temp. (°C)	Rainfall (mm)	Min. Temp. (°C)	Max. Temp. (°C)
January	188.2	15.7	25.2	124	18.5	31.4
February	167.4	15.7	24.9	88	18.2	30.6
March	137.3	14.7	24.1	80	16.1	29.4
April	54.0	12.4	22.9	52	12.8	26.4
May	15.8	9.7	21.6	15	9.4	23.8
June	12.3	7.0	19.3	6	5.1	20.8
July	10.1	6.9	19.6	2	5.5	21.1
August	22.0	8.1	20.9	4	7.8	23.7
September	48.3	10.6	22.8	13	12.6	29.0
October	114.3	12.1	22.6	49	15.4	29.8
November	154.8	13.7	23.5	81	16.9	30.6
December	181.1	14.9	24.6	108	17.1	30.3

* Information supplied by the "Institute for Tropical and Subtropical Crops", Nelspruit.

** Information supplied by RBMR from data collected over the past 40 years.

Thus, at this stage of the investigations, the climatic conditions of the Rustenburg waste site were considered sufficiently similar to that of the Barberton area to make growing *B. coddii* in Rustenburg a viable proposition, although the effect of drought and hail would have to be monitored. Further studies, however, on the soil types (4.3.2), growth-cycle (4.3.3) and the nutrient requirements of *B. coddii* (4.3.5), needed to be performed to fully establish the usefulness of the plant for phytoremediation.

4.3.2 Comparison of Soil Types on which *Berkheya coddii* was Grown

Another question that required answering, before *B. coddii* could be deemed suitable as a tool for the phytoremediation of the contaminated RBMR sites, was whether the soil at RBMR was physically and chemically similar enough to serpentine soil to allow the plant to adapt to it. Thus samples of the contaminated RBMR soil and serpentine soil were taken and fully analysed. A sample of topsoil, taken from the Rustenburg area, was also analysed in order that the level of contamination at the RBMR site could be determined.

The analyses of serpentine soil (Barberton), RBMR soil and topsoil are given in Tables 18, 19 and 20 and they were all performed by Steyn *et al.*, 1995, at the Institute of Soil, Climate and Water, Pretoria. From Table 18 it can be seen that serpentine and RBMR soils have a similar particle size distribution, although serpentine soil has more coarse- and medium-grained sand and less silt and fine sand than the RBMR soil. This indicates that the serpentine soil is better drained than RBMR soil, and this is confirmed by the fact that the RBMR soil has a greater clay content than serpentine soil. It was thought that both these factors would benefit *B. coddii* growth at RBMR as they should ensure that more water is retained in the soil, allowing for slight compensation of the drier climate (section 4.3.1). *B. coddii* was therefore not expected to have any difficulty in adapting to the physical properties of the RBMR soil. The particle size distribution was not determined for topsoil due to the expense of the analysis, which is fairly labour intensive (Table 18, section 4.2.2.7).

The pH (Table 18) of the RBMR soil and the serpentine soil was fairly similar with both occurring within the expected pH range for serpentine soils. Interestingly, the topsoil was more acidic in the water determination, where its pH was about 6.00, than were serpentine (at a pH of 6.35) and RBMR (at a pH of 6.21) soils. In the KCl determination the pH was similar in all three (5.22; 5.32 and 4.99 respectively). According to Steyn *et al.* (1995), the similarity of the pH (KCl) readings indicates that there is a fair amount of organic material in all three soils.

Table 18 shows that the phosphate levels in the three soils were identical and so it was felt that *B. coddii,* grown in Rustenburg, would not be affected by phosphates. Figure 23, (section 4.3.5.1) shows that *B. coddii* growth improved with the addition of phosphates and so it was felt that, in order to enhance *B. coddii* growth on the RBMR sites, phosphates should be added as a fertiliser.

The nutrient trials (section 4.3.5.1) show that nitrogen appears to have little effect on the growth of *B. coddii* plants growing in serpentine soils, which may be due to the fact that the nitrogen levels are high in serpentine soil (0.13%), as normally nitrogen is present in soils at a concentration of about 0.025%. The RBMR soil has a much lower nitrogen content (0.032%) than serpentine soil and it is not known what this effect will be on the growth of *B. coddii*. It is suspected, however, that these low levels may decrease the growth rate of the plant and, if

Brooks' opinion (Howes, *pers. comm.*) that nitrogen enhances nickel uptake is correct, the nickel uptake into *B. coddii* plants growing on RBMR soils may also decrease.

Table 18: Physical and Chemical Properties of Serpentine Soil, RBMR Soil and Topsoil (Steyn *et al.*, 1995)

TEST	SERPENTINE SOIL	RBMR SOIL	TOPSOIL
PARTICLE SIZE DBN. (%)			
Coarse grain sand (2 - 0.5 mm)	8.9	3.2	-
Medium sand (0.5 - 2.5 mm)	9.1	4.9	-
Fine sand (0.25 - 0.106)	6.5	6.1	-
Very fine sand (0.106 - 0.05 mm)	3.4	4.6	-
Coarse grain silt (0.05 - 0.02 mm)	4.9	5.6	-
Fine silt (0.02 - 0.002 mm)	16.9	17.9	-
Clay (<0.002 mm)	47.3	54.9	-
Texture	Clay	Clay	-
CHEMICAL ANALYSIS			
pH(H_2O)	6.35	6.21	6.00
pH(KCl)	5.32	4.99	5.22
PLANT AVAILABLE NUTRIENTS			
Phosphorus (ppm)	1.0	1.0	1.0
Nitrogen (%)	0.128	0.032	0.018

The data in Table 19 gives an indication of the leachability of the soils, as it shows the concentrations of elements that can be removed from the soils by water alone. Obviously, all these elements are plant available as they are water-soluble. Serpentine soil appears to be more leachable than RBMR soil since less magnesium (Mg), copper (Cu) and zinc (Zn) were removed from it. Topsoil appears to be less leachable than either RBMR or serpentine soil and this is to be expected as, overall, it contains fewer mineral elements than RBMR and serpentine soils.

From Table 19 it can be seen that the RBMR soil shows a low Ca/ Mg ratio of about 0.25, which is typical of most serpentine soils (Brooks, 1987). The Ca/ Mg ratio of the serpentine soil from Barberton is fairly high for serpentine soils at about 0.47. This high ratio in the Barberton soil may explain why *B. coddii* is a fairly lush plant. The Ca/ Mg ratio for the topsoil from Rustenburg is lower than normal at 0.8; most topsoils have a Ca/ Mg ratio of 1.0 or more and this may explain why the general vegetation in the Rustenburg area is fairly sparse. It was

felt that the slightly lower Ca/ Mg ratio of the Rustenburg soil would not greatly affect *B. coddii* growth on the RBMR site since the nutrient trials (section 4.3.5.1) showed that calcium and magnesium do not appear to individually affect plant growth.

Since the nutrient trials (section 4.3.5.1) showed that increasing the potassium levels up to a certain concentration, increased plant growth it was felt that the low potassium content seen in the RBMR soil might have the greatest effect on the growth of *B. coddii*. Thus, with the lower levels of potassium in RBMR soil compared with serpentine soil, it was felt that the plants at RBMR might not grow as fast or as large as the plants on the serpentine site. However, it was felt that the plants would still grow and that, that growth could be improved if the soil was fertilised with potassium.

Table 19: Water Soluble Cations in the Three Soils

	Ca	Mg	Na	K	Fe	Ni	Co	Cu	Mn	Zn	Cr
	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
Serpentine Soil	16.17	34.22	8.48	4.05	n/d	0.419	0.011	0.002	0.869	0.073	0.003
RBMR Soil	12.85	48.45	5.04	1.73	n/d	0.08	0.022	0.033	0.511	0.116	0.01
Top Soil	1.47	1.86	2.91	2.03	n/d	0.017	0.003	0.001	0.062	0.018	0.007

*nd = not detected

Table 20 gives the concentrations of the plant-available micronutrients. These analyses were performed in order to determine the contamination levels of RBMR soil and to see if *B. coddii* would be able to cope with any elevated levels of micronutrients not found in serpentine soil. From Table 20 it can be seen that serpentine soil contains more plant-available metals than do either RBMR soil or topsoil. It was hoped that the slightly elevated levels of copper, iron and chrome in RBMR soil would not adversely affect *B. coddii* growth. Table 20 also shows that there is approximately four times less plant available nickel in the RBMR soil than in the serpentine soil. This suggests that *B. coddii* growing on the RBMR soil will probably accumulate less nickel each season compared with plants growing on the serpentine soil. Indeed, the Agnes Mine site at Barberton has less plant available nickel than does the site at Queens River and it has been shown that the plants growing on the former site accumulate significantly less nickel per season than those growing on the latter site (Morrey *at al.*, 1989).

Table 20: Extractable Micro-Elements Available to the Plant

	Zn	Mn	Cu	Fe	Co	Cr	Ni
	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
Serpentine Soil	1.47	636	12.52	239.4	33.39	0.057	225
RBMR Soil	0.39	532.5	4.33	246.3	51.39	0.132	57.3
Top Soil	0.22	39.6	1.33	12.09	4.91	0.061	2.4

The above comparisons between serpentine soil and RBMR soil indicate that *B. coddii* should be able to grow on the RBMR soil although, with the decreased nitrogen and potassium levels, the RBMR site may need to be fertilised with these nutrients. Phosphate might also need to be added to the RBMR soil as *B. coddii* responded positively to phosphate, as seen in the experiments described in section 4.3.5.1. Thus, the fertiliser of choice would be 2:3:2 (N:P:K) as it is a general fertiliser that contains the elements found to enhance *B. coddii* growth.

4.3.3 The Observed Growth-Cycle of *Berkheya coddii*

Having established (section 3.3.1) that the AGB accumulates the greatest amount of nickel and, therefore, should be harvested, another question that required answering was when the plants should be planted and when the biomass should be harvested in order that the accumulated nickel could be retrieved. Thus, the growth-cycle of *B. coddii* was observed and documented in plants growing naturally in Barberton for a period of almost twelve months. From this growth-cycle, essential information for both the establishment of the plants at Rustenburg and the timing for optimal harvesting of the biomass, for nickel recovery, was gathered. It was also important to assess the natural germination of the plants in order to establish the most effective method of ensuring their continued growth on the sites at Rustenburg (section 5.3.2).

B. coddii, like many plants found in warm subtropical regions such as Barberton, was observed to not respond to changes in temperature but rather to changes in moisture. In spring, at the onset of the rainy season (generally towards the end of August), the plants start to germinate. Most germination occurs by vegetative budding from the rootstock where a small eye initially develops (Howes, 1990). It then reveals small leaves that grow into the mature plant by the

end of December (after 4 months growth), Figure 15. At this stage the yellow flowers are produced and seed production occurs. The seed heads then begin to dry out and ripening of the seeds is usually complete towards the end of February (after 6 months growth). The seeds are then dispersed and the plants, with no further growth, begin to dry out. It is at this stage of the growth-cycle that the leaves and stems should be harvested (section 5.3.6) in the phytoremediation process, otherwise the leaves drop off and return the nickel to the soil. The mature seeds should also be collected (section 5.3.5) at this stage for germination and further population of other waste sites. Should no seed harvesting occur, they dry out and are wind dispersed. The seeds of *B. coddii* are parachute-like to aid in wind dispersion, Figure 14. Vast numbers of these simple seeds are produced (about 5 000 per plant), the viability of which is discussed in section 4.3.4.2.

During this "drying out" period, March to end-April, the plant accumulates carbohydrates in its large tuber, found just beneath the soil. This tuber is the only living part of the plant during the dry winter months (May – end August) and it is from this that the plant obtains the energy required for vegetative germination. Seed germination also occurs but this appears to be a back-up mechanism and, if vegetative growth is successful, most of the germinating plants tend to die off due to competition for nutrients.

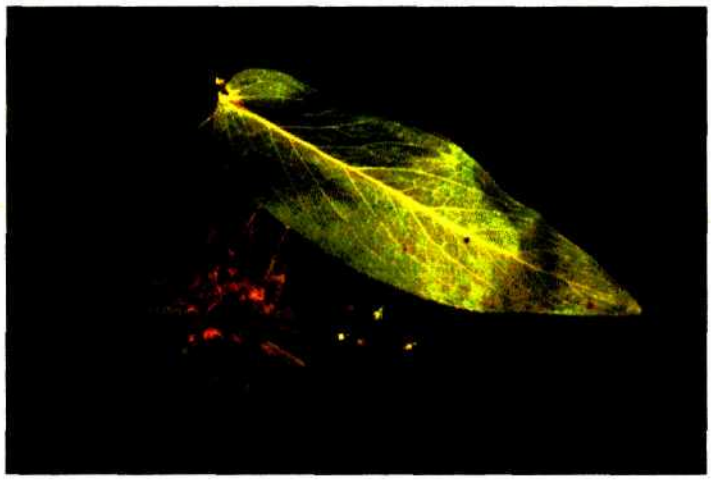


Figure 14: Structure of the Seeds and Seed-Head of *Berkheya coddii* (Howes, 1990)

1) *B. coddii* Plants at 2 Months

2) *B. coddii* Plant at 3 Months

3) *B. coddii* Plant at 4 Months

4) A Field of Flowering *B. coddii* Plants at 5 Months

5) *B. coddii* Plants at 7 Months Drying Out

Figure 15: The Growth-Cycle of *Berkheya coddii* Observed at the Sawmill Site in Barberton

Seeds of *B. coddii* are produced when cross-pollination between flowers occurs. Originally it was thought that pollination was effected by butterflies as the flowers have no smell, have a sturdy structure and are yellow in colour – the preferred conditions for butterfly pollination (Northington and Goodin, 1984). It was noted, however, that very few butterflies were in existence in the area and that more prevalent were a black beetle with iridescent blue and red colouring, and a black beetle with yellow colouring. As very few butterflies were observed in the Barberton area it is thought that the beetles are responsible for pollination. Preliminary studies on the beetles have identified them as belonging to the Melyridae family of the Coleoptera order. These beetles are better known as "soft-winged flower beetles" (Booth *et al.*, 1990). The beetles are usually 15 mm in length and fairly narrow in width. They tend to be predators but may also feed on pollen. The beetles have been observed in the Rustenburg area suggesting that pollination of *B. coddii* in Rustenburg would not be a problem.

From investigation of the growth-cycle it was found that *B. coddii* growth responds to moisture content, rather than to temperature changes. As the rainy seasons of Rustenburg and Barberton are similar (Table 17) it is expected that the growth-cycle of the plants growing in Rustenburg will follow the same monthly pattern as was observed in the Barberton plants. Observations of the growth-cycle suggested that, at least for the first season, 5-month-old *B. coddii* plants should be planted in the RBMR sites (sections 5.2.4 and 5.3.3) at the beginning of the Rustenburg rainy season, which is generally at the end of September. The plants in their first season are not expected to reach maturity and will probably grow until the end of the rainy season, usually at the end of March, after which they will begin to dry out. If this happens, the above-ground plant material will be harvested at the beginning of May (section 5.2.7 and 5.3.6). Once the plants reach their second growing season at RBMR it is expected that they will vegetatively germinate at the onset of the rainy season (the end of September), that they will flower towards the end of December and that seeds will be produced from mid to end of January. The seeds will be collected once they are dry, which is expected to be mid-February. Again the dry AGB will be harvested about one month after the rains have ended, which is about the end of April or the beginning of May.

4.3.4 Cultivation of *Berkheya coddii* Plants for the Nutrient Experiments and Phytoremediation

Another important question to be answered before phytoremediation of the RBMR sites could commence was whether or not we would be able to produce sufficient plants to populate the RBMR sites. The production of plants was also important for investigating the nutrient requirements of *B. coddii*. Thus a protocol was developed for the mass production of plants under controlled conditions. To achieve this, plant production was studied both in tissue culture, by direct organogenesis (section 4.2.4.1), and with seeds germinating on cotton wool in Tupperware® containers (section 4.2.4.2).

4.3.4.1 Culturing of *Berkheya coddii* by Direct Organogenesis

In tissue culture numerous plants with the identical genetic make-up can be produced and the conditions in which the plants are grown can be monitored (Allen, 1991). It was these factors that made the production of *B. coddii* in tissue culture attractive. In putting *B. coddii* into culture the effects of nutrients could be monitored as, any change in phenotypic appearance could be attributed only to nutrients. In the present study, *B. coddii* plants were produced by direct organogenesis from a single explant grown up from seed in culture. In this process growth of the apical meristem is inhibited whilst the lateral growing points on the explant are stimulated at the nodes below the apical meristem. Growth from these axillary shoots is rapid and the number of potential plants can be exponentially increased by repeated subculturing.

Germinating *B. coddii* seeds in tissue culture was extremely difficult due to low seed fertility as well as high levels of bacterial and fungal contamination. Of the 1 200 seeds placed in tissue culture only 20% germinated. This germination rate is in line with the seed analysis results, presented in 4.3.4.2, in which only 22.5% of the seeds were found to contain embryos. Of the seeds that germinated in culture, only two seedlings survived; the remainder were destroyed by fungal and bacterial contamination. The high contamination rate was thought to have been caused by difficulty in sterilising the seeds, due to their small size and the parachute-like structure used for wind dispersal, as no contamination was observed in the control, seed-free tubes. It was felt that in future tissue culture work the parachute-like structure should be removed before sterilisation, to reduce the levels of contamination. The two surviving

seedlings were those that had been sterilised in sodium hypochlorite for 20 min suggesting that the sterilisation procedures were not too harsh.

Of the two surviving seedlings, one plant was healthy and showed no sign of fungal or bacterial infections while the other plant was weak and appeared to have a systemic bacterial infection. The healthy plant grew at a fairly rapid rate and after 22 weeks 400 fully formed explants (such as the one in Figure 16) had been produced. No auxin or cytokinin was added to the media as root and shoot initiation occurred in *B. coddii* without the addition of these hormones respectively. In some plants the formation of roots was fairly slow. It has since been suggested that to speed up root formation the plants should be grown on a nutrient deficient medium (Meney, *pers. comm.*). This causes the plants to "seek" nutrients, which, in turn, means that roots are produced more quickly.

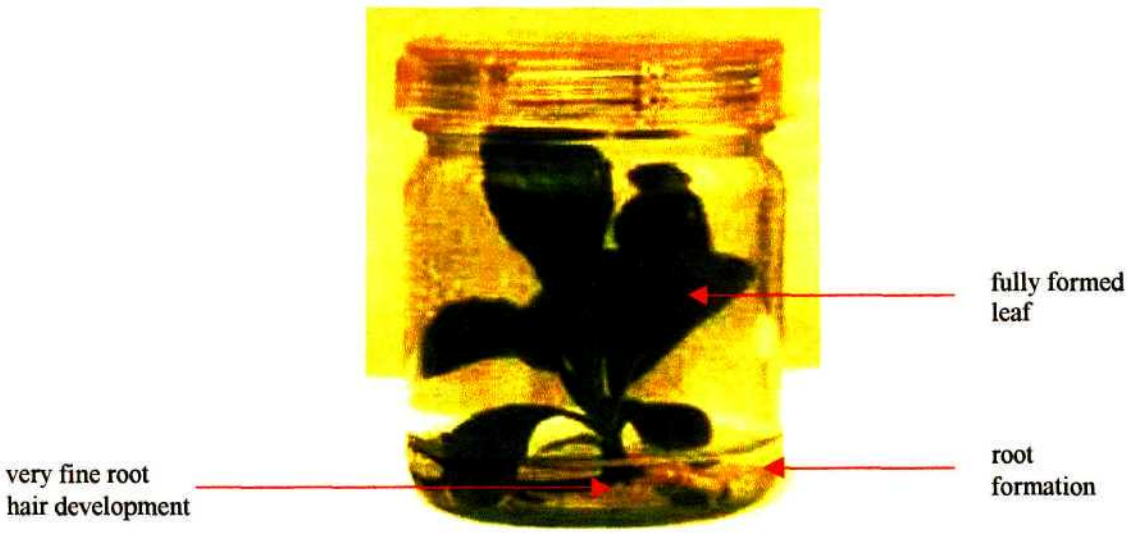
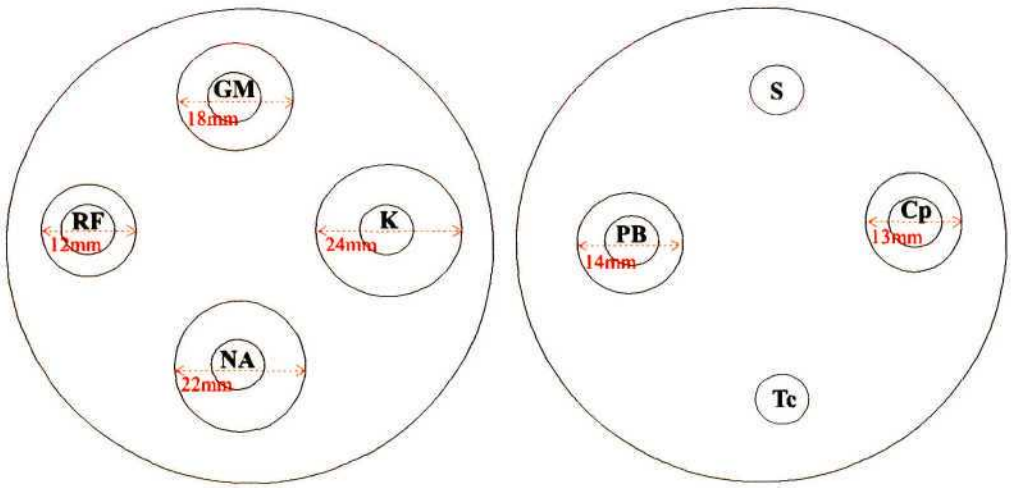


Figure 16: A Fully Formed *Berkheya coddii* Explant in Tissue Culture

The weak plant was suspected to have a bacterial infection as there was no typical fungal growth and the MS media had a very sweet honey-like smell. After plating out a sample of the tissue culture media on nutrient agar plates it was found that no bacterial growth occurred on the nutrient agar plate incubated overnight at 37°C. The plate incubated overnight at room temperature, however, was covered with a bacterial monolayer. This indicated that the contaminant was plant or soil based, rather than of human origin. The colonies were smooth and circular in shape, and had a creamy colour. The Gram stain showed the bacterium as being a Gram-negative rod. As the bacteria grew in oxygenated media it was assumed that they were

aerobic. All these observations fit the rather general description for the Agrobacterium genus, of the Rhizobiaceae family (Krieg and Holt, 1984). As Agrobacterium is a common plant "pathogen" it was realistic to assume that this contaminant was an Agrobacterium. No further studies were performed to fully identify the bacterium.

Having concluded that the contaminating bacterium was Gram negative, discs of antibiotics, affecting mainly Gram negative bacteria, were chosen for the antibiogram test. The sensitivity of the bacterium to the various antibiotics was determined by measuring the halo formation around the antibiotic discs. From these measurements, Figure 17, it can be seen that the bacterium was most sensitive to kanamycin at a concentration of 1 000 µg. Kanamycin was readily available and so was chosen for plant decontamination. Figure 17 also shows that the bacterium was resistant to streptomycin, which has the same mode of action as kanamycin, gentamicin and tetracycline.



K = 1000 µg kanamycin
NA = 30 µg nalidixic acid
RF = 15 µg rifampicin
GM = 10 µg gentamicin

Cp = 30 µg chloramphenicol
Tc = 30 µg tetracycline
PB = 300 µg polymyxin B
S = 25 µg streptomycin

Figure 17: Antibiogram Tests Showing Halos of Antibiotic Resistance

The concentration of kanamycin to which the bacterium was sensitive was seen to be 500 µg/ml. Large cream colonies were observed on the gradient plate up to a concentration of about 350 µg/ml kanamycin. Thereafter the colonies became small and translucent in appearance. No colonies grew above 450 µg/ml kanamycin.

When the contaminated plant was subcultured and treated with 500 µg/ml kanamycin, it was found to be more sensitive to the antibiotic than the bacterium and so all the subcultured plants died. This suggests that kanamycin should not be used as a decontamination agent; instead an antibiotic such as nalidixic acid, to which the bacterium is sensitive at lower concentrations should be used with the hope that explants will be able to survive the lower doses. Explants also need to be tested for antibiotic toxicity before a particular antibiotic is used on all the subcultured plants.

From the above results, it can be seen that the germination rate of *B. coddii* seeds in tissue culture matched the typical germination rate achieved for *B. coddii* plants (section 4.3.4.2). This shows that once a sterilisation procedure for the seeds has been worked out, or a suitable antibiotic found that destroys the bacteria without harming the plants, *B. coddii* can be propagated in tissue culture. Once a single sterile explant had been produced it appeared that *B. coddii* was extremely easy to propagate further in tissue culture as no hormone addition was required. Thus, although fairly expensive in terms of labour and equipment, it would be possible to propagate *B. coddii* on a large scale using direct organogenesis.

4.3.4.2 Germination of *Berkheya coddii* Seeds on Cotton Wool

In view of the difficulties and general expense encountered in employing tissue culture (section 4.3.4.1) for *B. coddii* production, plants for the nutrient trials (section 4.3.5) were grown from seeds germinated on cotton wool. This germination procedure proved highly effective in that the 25% germination rate of 6 - 8 month-old seeds (Anderson *et al.*, 1997) compared well with the 22.5% fertility rate obtained from the dissection of 200 seeds. Of the 200 seeds dissected under the dissection microscope, only 45 were found to contain an embryo. Of those 45 seeds, 40 were the larger seeds and 5 were the smaller seeds generally found in *B. coddii* seed cases. The loss of embryo was thought to be a result of the seeds becoming dehydrated and it therefore appears that the larger seeds do not dehydrate as easily as the smaller seeds. It was thus felt that the storage method for *B. coddii* seeds needs examining.

These results suggest that in germinating plants for the phytoremediation project only large seeds, collected from the previous season, should be used in order to establish a good germination rate, thus ensuring that the germination process is efficient and relatively inexpensive.

The above germination process successfully supplied sufficient plants for the nutrient requirement trials, the results of which are presented in section 4.3.5 below. However, for phytoremediation more bulk germination was necessary and this was done in zeolite, as reported in section 5.3.2.

4.3.5 Optimisation of the Nutrient Requirements of *Berkheya coddii*

Having established that the AGB of *B. coddii* could take up significant amounts of nickel (section 3.3.1), it was important to perform nutrient requirement trials on the plant to ascertain whether or not the addition of various nutrients could improve growth and nickel uptake within the plant. Such information would be useful when growing *B. coddii* on the RBMR sites. Unfortunately the nutrient trials were performed on plants growing in serpentine soil, when actually they should have been performed on plants growing in RBMR soil, so as to maximise their potential for phytoremediation. Thus the results obtained need to be extrapolated for growth on RBMR soil and this extrapolation requires that the soil analyses of serpentine and RBMR soils be taken into account. The relative compositions of these two soil types are given in section 4.3.2. It should also be noted that serpentine soil already contained calcium, phosphorus, nitrogen, magnesium and potassium and thus these concentrations need to be taken into account when extrapolating the data obtained from the nutrient additions for the enhancement of *B. coddii* growth in phytoremediation.

Not only were the nutritional requirements of *B. coddii* considered to be essential for the optimisation of growth and nickel uptake on the Rustenburg waste sites (section 5.2.4), they were also required for seedling cultivation. The nutrient requirements were designed and performed on seedlings, germinated on cotton wool (section 4.3.4.2), and grown up on acid-washed sand and serpentine soil.

4.3.5.1 Nutrient Trials on Acid-Washed Sand and Serpentine Soil

Usually nutrient trials are performed on seedlings growing in acid-washed sand as no nutrients are provided to the plants by acid-washed sand, thus allowing the effects of the nutrients to be easily seen. Unfortunately, *B. coddii* seedlings could not be maintained in acid-washed sand and so nutrient trials were performed on seedlings growing in serpentine soil instead. This was not thought to be ideal but it was felt that the effects of the nutrients would be seen if there

were enough replicate plants, per duplicate set of trials. An advantage of growing the plants on serpentine soil was that the effects of the various minerals on nickel uptake could be evaluated.

No results were obtained from the nutrient trials performed on *B. coddii* grown in acid-washed sand. All the plants died after 4 weeks of being fed with the nutrient solutions. The reason for their death is unknown although the roots might have rotted due to moisture being retained at the base of the container, even though drainage holes were drilled in the pots. Most of the plants turned a yellow colour, which suggests that a lack of a particular nutrient might have killed the plants. As the explants in tissue culture were grown on MS media, and showed no sign of a deficiency, this was thought unlikely.

The nutrient trials performed on seedlings grown in serpentine soil were highly successful as all the plants survived and all were healthy when harvested. The nutrients tested in the trials were all the plant macronutrients i.e. nitrogen, phosphorus, potassium, calcium and magnesium, as it is these nutrients which affect plant growth (Table 36 in Appendix 2 gives all the documented effects of each of the macronutrients on plant growth). It was felt that, by determining the optimal macronutrient requirements of *B. coddii*, optimal growth could be established. It was also important for phytoremediation purposes (Chapter 5) to determine the effect the various macronutrient concentrations had on nickel uptake. It should be noted that as the nutrient trials were performed on plants germinated on cotton wool, all the nickel taken up from the serpentine soil by the plants, was during the course of the experiment. The graphs in Figure 18 to Figure 28 show the effects of the various macronutrients on both the growth and the nickel uptake in *B. coddii*. Unfortunately no control was set up for the experiments showing the effect of nutrients on the growth of *B. coddii*, which means that no comparisons with the natural growth of the plant could be made. These experiments need to be repeated with a control in order that the effect of nutrients on plant growth can be fully assessed.

Figure 18 and Figure 19 show the effect of various concentrations of ammonium nitrate (NH_4NO_3) on the growth and the amount of nickel accumulated by *B. coddii* growing on serpentine soil. Figure 18 shows that there is no marked difference in growth in *B. coddii* with the addition of up to 2 000 mg/l NH_3NO_3. However, a treatment of 4 000 mg/l NH_3NO_3 clearly causes growth to be inhibited. As expected, the absolute nickel uptake (Figure 19) was also inhibited at this level during the course of the experiment. Thus, the addition of nitrogen

up to 2 000 mg/l would be acceptable for plant growth. However, from Figure 19 it can be seen that optimal nickel uptake was achieved at 250 mg/l NH_4NO_3, which also gave the highest mass gain, suggesting that this concentration of nitrogen would be the most appropriate for phytoremediation on serpentine-like soils. Obviously, chemical differences between the serpentine-like RBMR soil would have to be taken into consideration once the phytoremediation on the RBMR sites commenced. However, comparison with the control clearly suggests that some nitrogen treatment would be beneficial to nickel uptake. Thus these findings agree with Brooks (Howes, *pers. comm.*) who has found that nitrogen enhances nickel uptake in some *Alyssum* nickel hyperaccumulators.

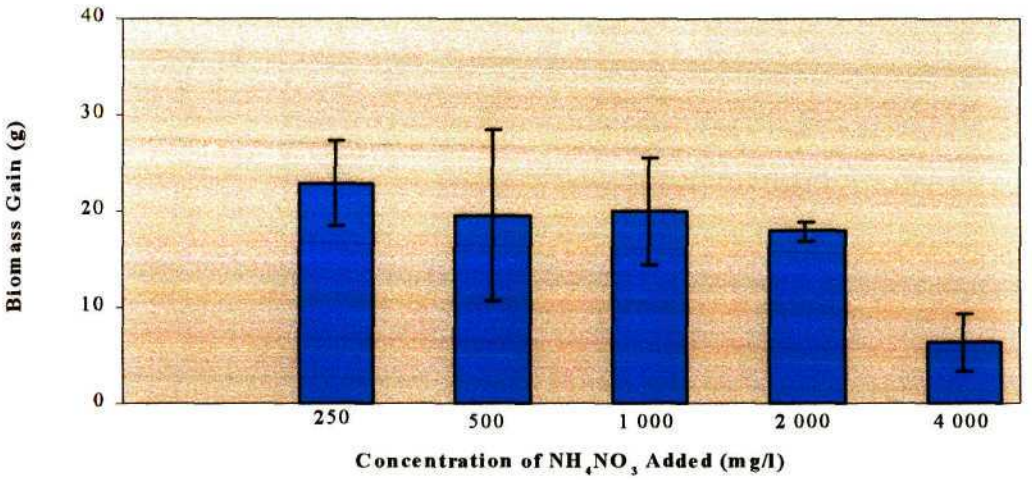


Figure 18: Effect of Ammonium Nitrate on *Berkheya coddii* Growth on Sepentine Soil, (Section 4.2.5.5)

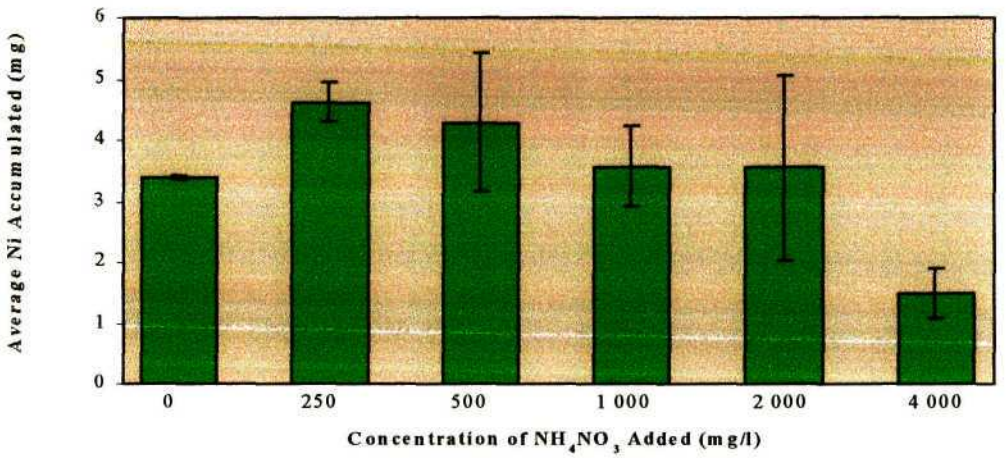


Figure 19: Effect of Ammonium Nitrate on the Average Amount of Nickel Accumulated by each *Berkheya coddii* Plant Growing on Sepentine Soil (Section 4.2.5.5)

Figure 20, Figure 21 and Figure 22 illustrate the effects of calcium phosphate $Ca(H_2PO_4)_2$ on plant growth and nickel uptake. Figure 20 shows that $Ca(H_2PO_4)_2$ treatment caused a massive increase in plant biomass, which was paralleled by corresponding increases in absolute nickel uptake (Figure 21). The decrease in the nickel concentration within *B. coddii* (Figure 22), with an increase in biomass (Figure 20), suggests that *B. coddii* outgrew its ability to accumulated and store nickel in the new tissues, during the course of the experiment. The observed effects from $Ca(H_2PO_4)_2$ were probably due, first and foremost, to the phosphate, since Figure 23 and Figure 24 show an entirely different pattern of biomass production and nickel uptake in response to $CaCl_2$ treatment. Thus, for the purposes of phytoremediation, treatment with 600 mg/l $Ca(H_2PO_4)_2$ would improve nickel uptake by about 0.5 mg, compared with the control, and would maximise biomass production. Further experiments with greater concentrations of $Ca(H_2PO_4)_2$ need to be performed to fully assess the influence of this nutrient on *B. coddii* growth and nickel uptake.

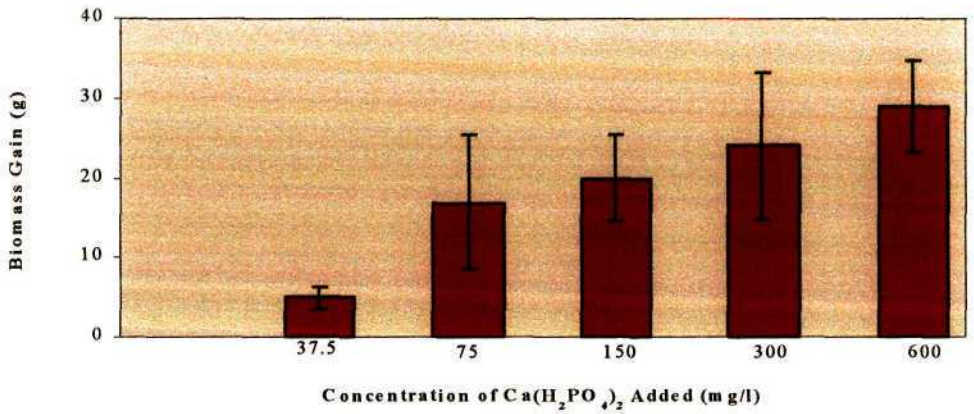


Figure 20: Effect of Various Concentrations of Calcium Phosphate on *Berkheya coddii* Growth on Serpentine Soil (Section 4.2.5.5)

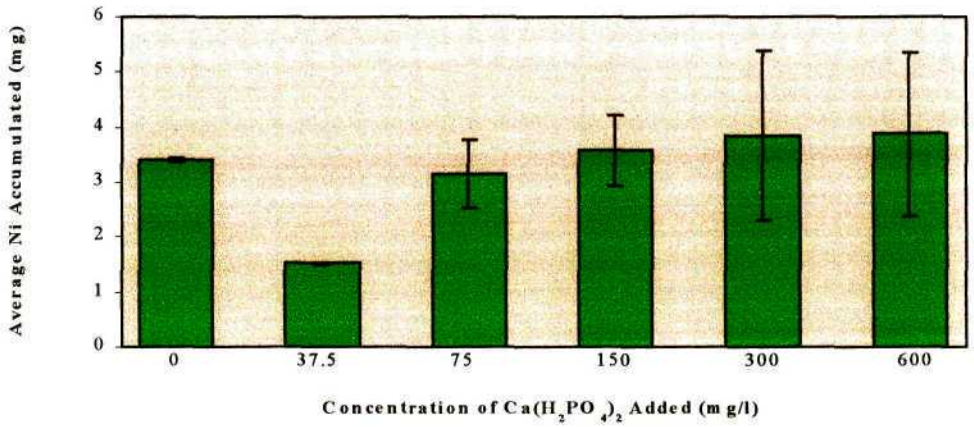


Figure 21: Effect of Calcium Phosphate on the Average Amount of Nickel Accumulated by *Berkheya coddii* Growing on Sepentine Soil (Section 4.2.5.5)

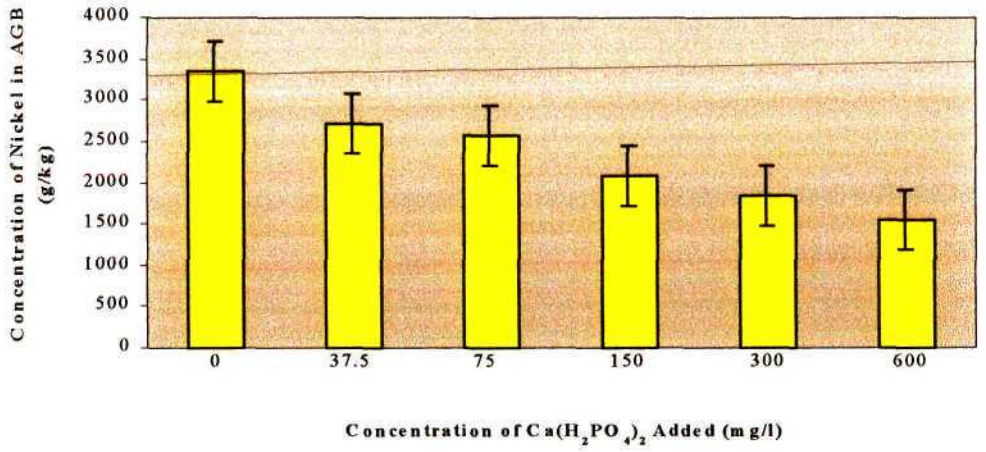


Figure 22: Effect of Calcium Phosphate on the Concentration of Nickel found within *Berkheya coddii* Growing on Sepentine Soil (Section 4.2.5.5)

Figure 23 indicates that calcium chloride ($CaCl_2.2H_2O$) had little or no effect on biomass production, whereas there was a variable effect on nickel uptake (Figure 24). The enhancement of nickel uptake at 500 mg/l and 2 000 mg/l of $CaCl_2.2H_2O$ agrees with the results of Howes (*pers. comm.*) who found that $CaCl_2.2H_2O$ significantly enhanced nickel uptake by mature plants growing on the Queens River site in Barberton. In this regard, it has been speculated (Dutton, *pers. comm.*) that nickel could be actively taken up via the calcium ports in the roots, which would be stimulated by the presence of calcium ions. Other unpublished data (Howes, *pers. comm.*) has shown that lanthanum chloride, a known stimulator of calcium pumps, also stimulated nickel uptake in *B. coddii*. On the other hand, sodium chloride treatment did not

enhance nickel uptake by *B. coddii* suggesting that calcium (and lanthanum) rather than chloride is responsible for the increased nickel accumulation. Obviously, for phytoremediation purposes, 2 000 mg/l $CaCl_2.2H_2O$ should be added to the soil, as this appears to increase the nickel uptake by about 1.8 mg, compared with the control. Again this needs to be tested further and, if need be, the amounts adjusted for the actual RBMR waste site.

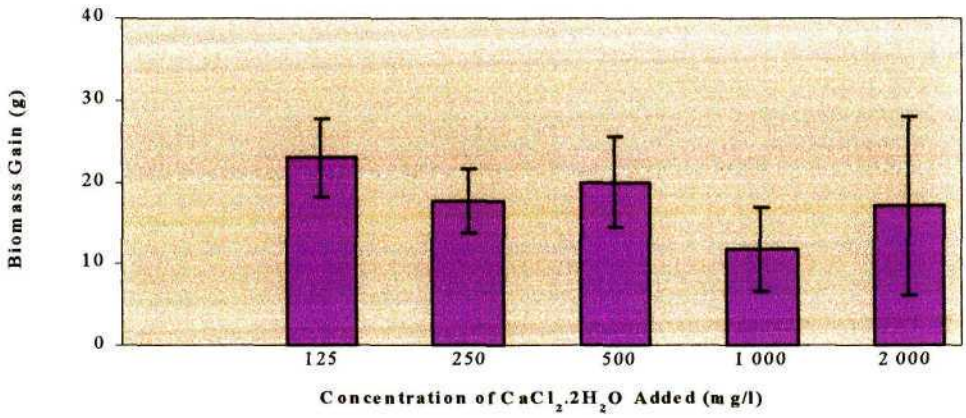


Figure 23: Effect of Calcium Chloride on the *Berkheya coddii* Growth on Sepentine Soil (Section 4.2.5.5)

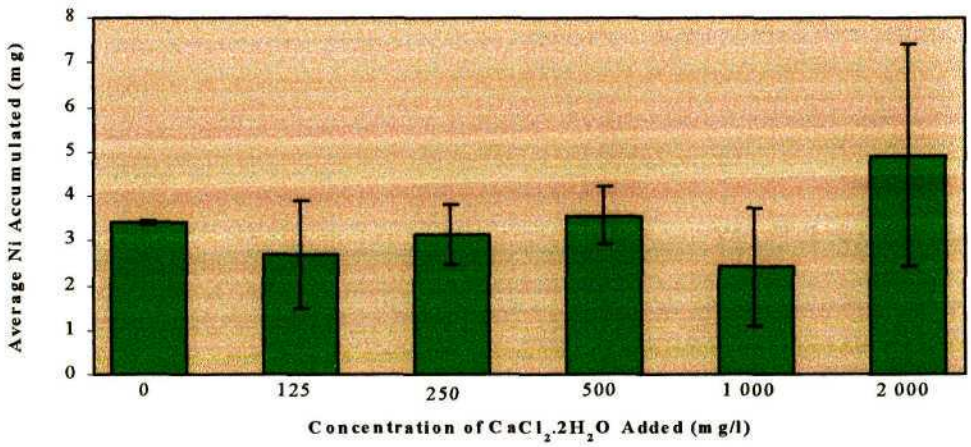


Figure 24: Effect of Calcium Chloride on the Average Amount of Nickel Accumulated by *Berkheya coddii* Growing on Sepentine Soil (Section 4.2.5.5)

Figure 25 shows that potassium chloride (KCl) treatment enhanced biomass production up to a level of 600 mg/l, but that doubling this quantity was possibly toxic to the plant. The nickel uptake data (Figure 26) shows that nickel uptake also increased, above the control, when 300 mg/l or 600 mg/l KCl were added to the plants. Further addition of KCl caused the nickel

uptake to decrease. These results indicate that a concentration of 600 mg/l KCl should be added to the serpentine-like RBMR soil, but again the effects of this would need to be tested.

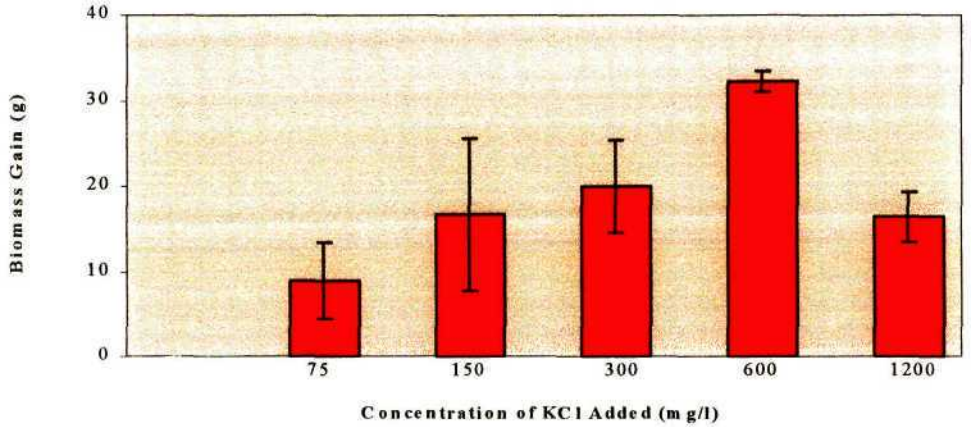


Figure 25: Effect of Potassium Chloride on *Berkheya coddii* Growth on Sepentine Soil (Section 4.2.5.5)

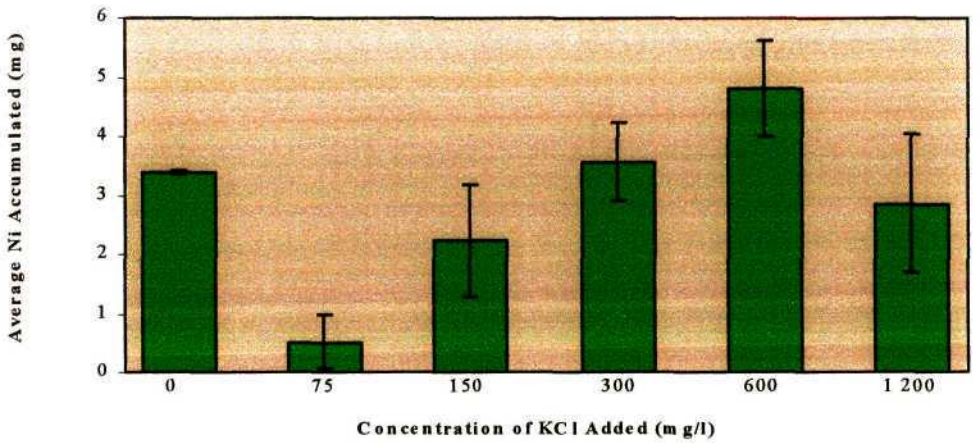


Figure 26: Effect of Potassium Chloride on the Average Amount of Nickel Accumulated by *Berkheya coddii* Growing on Sepentine Soil (Section 4.2.5.5)

Notwithstanding the unexpected mass loss at the 500 mg/l treatment with magnesium sulphate ($MgSO_4.7H_2O$) (Figure 27), addition of this nutrient over the range 125 – 1 000 mg/l improved biomass production in *B. coddii* to a similar extent whereas, below 125 mg/l i.e. 62.5 mg/l, the biomass increase was much lower. Treatment with $MgSO_4.7H_2O$ had a slightly positive effect, compared with the control, only at a concentration of 250 mg/l. Thus, as this concentration of $MgSO_4.7H_2O$ also had a positive effect on biomass it is suggested that

250 mg/l of $MgSO_4.7H_2O$ be added to the plants for the purpose of phytoremediation, although further testing with RBMR soil is required.

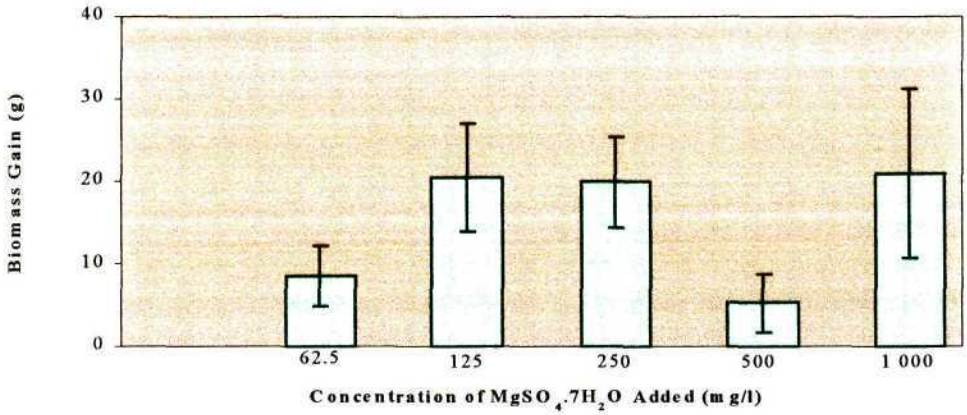


Figure 27: Effect of Magnesium Sulphate on *Berkheya coddii* Growth on Sepentine Soil (Section 4.2.5.5)

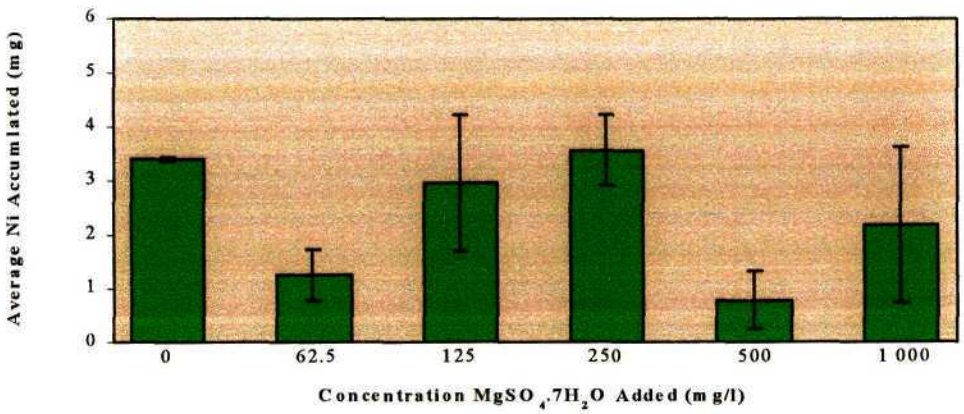


Figure 28: Effect of Magnesium Sulphate on the Average Amount of Nickel Accumulated by *Berkheya coddii* Growing on Sepentine Soil (Section 4.2.5.5)

From the above results it can be seen that the addition of nutrients to serpentine-like soils is important for both the growth of *B. coddii* and its ability to accumulate nickel. In order to fully test the interactive effects of the nutrients on their availability to *B. coddii*, an experiment needs to be performed in which all the nutrient concentrations that gave the greatest biomass production and/ or nickel uptake are combined. By applying all these nutrients to the plants it can be seen whether the effects are additive, or less or greater than the sum of the individual effects. These experiments would be performed on RBMR soil in order to thoroughly test the

nutrients in terms of phytoremediation, and a control plant would be included to see the actual effect of nutrient addition to plants growing in RBMR soil alone. From the above results it can be seen that the lack of a control plant in the biomass production experiments limited the interpretation of the results. Despite this, however, the results did indeed provide useful knowledge about the general nutrient requirements of *B. coddii* and allowed for approximate estimates of the requirements for plants growing on the RBMR sites, to be made.

The above results show that nitrogen should be added to the plants at a concentration of 250 mg/l for maximum biomass production and nickel uptake. As RBMR soil contains less nitrogen than serpentine soil (Table 18), more than this quantity may need to be added for phytoremediation purposes. The addition of phosphorus at 600 mg/l would enhance phytoremediation in RBMR soil and, as RBMR soil contains the same amount of phosphorus as serpentine soil, it is thought that this quantity, or more, should be added. Less calcium is available to *B. coddii* growing in RBMR soil than in serpentine soil and it is therefore thought that the addition of about 2 000 mg/l calcium would optimise the plants ability to accumulate nickel and to grow. The level of 600 mg/l potassium was seen to maximise nickel uptake and biomass production in the plants growing on serpentine soil. As less potassium is found in RBMR soil than in serpentine soil slightly more potassium may be needed to optimise the growth of the plants on the waste sites. Finally, magnesium at 250 mg/l was thought to be required by the plant. This amount should probably be decreased for plants growing on the RBMR waste sites as this soil contains more magnesium than serpentine soil. Thus, although the results obtained in the nutrient trials reported in this section cannot be extrapolated directly to the situation on the RBMR soil, very useful knowledge was gained that would be used during the optimisation of the phytoremediation at the RBMR site (Chapter 5).

4.3.5.2 Nutrient Trials Performed on Fully-Formed *Berkheya coddii* Explants Growing in Tissue Culture

All ten of the explants grown on MS media containing 2 000 mg/l nickel (section 4.2.5.2) died, suggesting that the nickel was too toxic for the explants, possibly because of the greater availability of plant-available nickel in the medium (2 000 mg/l), compared with serpentine soil (225 mg/l plant-available nickel). Furthermore, it is possible that the *B. coddii* explants lacked the nickel-exclusion mechanism present in whole plants. The loss of the exclusion mechanism

for plants grown in tissue culture has been observed by Taylor and Crowder (1983), in the nickel, copper and iron accumulator, *Typha latifolia*. Another possible reason for plant death in culture is that a symbiotic relationship between *B. coddii* and a soil organism may exist in which the soil organism is responsible for the mechanism conferring nickel tolerance on *B. coddii*, (section 1.8.2). If this were the case, the absence of the organism in sterile culture would lead to an over supply of nickel to the plant, ultimately leading to cell death. Finally, death might also have occurred due to a loss in nickel tolerance as the plants in culture were not germinated on media containing nickel. If during germination genes are only switched on when nickel is present then this is a plausible theory. However, the pot trials performed on plants germinated on cotton wool (section 4.3.5.1) were successful and showed that the plants were able to accumulate nickel once they had been transferred to a medium containing nickel, indicating that a loss of tolerance does not occur. Further studies are, therefore, required to establish the real reason for the loss of the explants, but it is thought that the very high levels of soluble nickel in the media accounted for plant death due to uptake and exclusion mechanisms being overcome.

No further nutrient trials could be performed on the explants growing in tissue culture, due to all the plants being lost as a result of mechanical failure of the Conviron.

4.4 *Conclusions*

From the results reported in this chapter, it can be seen that *Berkheya coddii* is well adapted to the poor soil conditions found in the serpentine soil. As RBMR appears to have fairly similar soil properties it is felt that the plant will be able to grow fairly well in this soil. The soil analyses show that no metal toxicity should be seen in *B. coddii* growing on the RBMR soil as the metals found in RBMR soil are similar in concentration to those found in serpentine soil. The nutrient status of both soils is similar although there is less calcium and more magnesium in the RBMR soil, which may cause plant growth on the RBMR waste sites to be a bit stunted, if the plant is not fertilised with these elements.

From the propagation experiments it was found that although the plants could easily be propagated by tissue culture, once a sterile plant had been produced, it was felt that this method of populating a large area was extremely expensive and time consuming. As *B. coddii*

was easily germinated on cotton wool and could then be successfully transferred to serpentine-like soils without any apparent loss in the ability to accumulate nickel, it was felt that this would be a more feasible way of populating large areas. Howes (*pers. comm.*) (section 5.2.3) also successfully tested other germination media and plants grown on such media have subsequently been used to populate the large contaminated areas at RBMR (section 5.2.4).

The nutrient trials were successful and showed that there is a need to add nutrients to *B. coddii* to maximise both its biomass production and its nickel uptake. Without the comparison of a control plant for the mass gain experiments, it was difficult to determine the absolute effects of the nutrients. However, the trends followed were fairly clear and could be extrapolated, to a certain extent, to include the effect of no nutrient addition. Until the suggested nutrient trials (section 4.3.5.1) have been performed on the RBMR soil, it is felt that a standard fertiliser containing nitrogen, potassium and phosphorus should be used to enhance plant growth on the RBMR soil. Thus the fertiliser 2:3:2 (N:P:K) was used in setting up the RBMR sites (section 5.2.4).

CHAPTER 5

DEVELOPMENT AND APPLICATION OF THE PHYTOREMEDIATION PROCESS

5.1 *Introduction*

Since industry is one of the main contributors of pollution there is the threat of stringent laws being forced upon it to "clean up its act". With this in mind, Amplats embarked on a project to evaluate the feasibility of rehabilitating a nickel-contaminated soil by phytoremediation, using *Berkheya coddii* as a tool. The plant is endemic to the Barberton area where it only occurs on serpentine soil (section 1.6). This restricted growth indicates that *B. coddii*, like many other hyperaccumulators, is a poor competitor (McGrath *et al.*, 1993; Salt *et al.*, 1995) and so it was felt that introducing the plant to the contaminated site should pose no threat to the indigenous vegetation of the area. The other advantage of it being a poor competitor is that once the site has been rehabilitated the natural vegetation of the area should outgrow the *B. coddii* plants and so there would be no need to embark on an expensive project to manually remove the plants. *B. coddii* also complies with the following criteria set out by Salt *et al.* (1995), to assess whether or not a plant can be considered as a phytoremediation tool:

1. It has a large biomass allowing a relatively large concentration of nickel to be removed from the soil each season via leaf harvesting;
2. It is perennial and does not need to be planted each year, saving time and money;
3. It has a well-developed root system allowing a large amount of surface contact with the contaminating nickel. The roots reach a depth of about 0.5 m causing the soil to be well penetrated; and
4. It has a fairly rapid growth rate.

This chapter reports on how the results, experiences and knowledge gained from the experiments detailed in the previous chapters, especially Chapter 3 and Chapter 4, were applied to the phytoremediation of the Rustenburg site with *B. coddii*. In order to institute an efficient phytoremediation programme it was necessary to develop methods and equipment for growing

and collecting the seeds from Barberton, for populating the contaminated site with *B. coddii*, for harvesting the plants at the end of the growing season, and for extracting the accumulated nickel into a metallic form to prove that nickel can be removed from a contaminated site by phytoremediation. These details are also reported in this chapter.

In the interests of clarity a flow diagram (Figure 29) has been prepared to illustrate the key experiments performed when setting *B. coddii* up as a tool for the decontamination of the RBMR sites. All the section numbers have been given in order to facilitate links with the relevant experimental procedures.

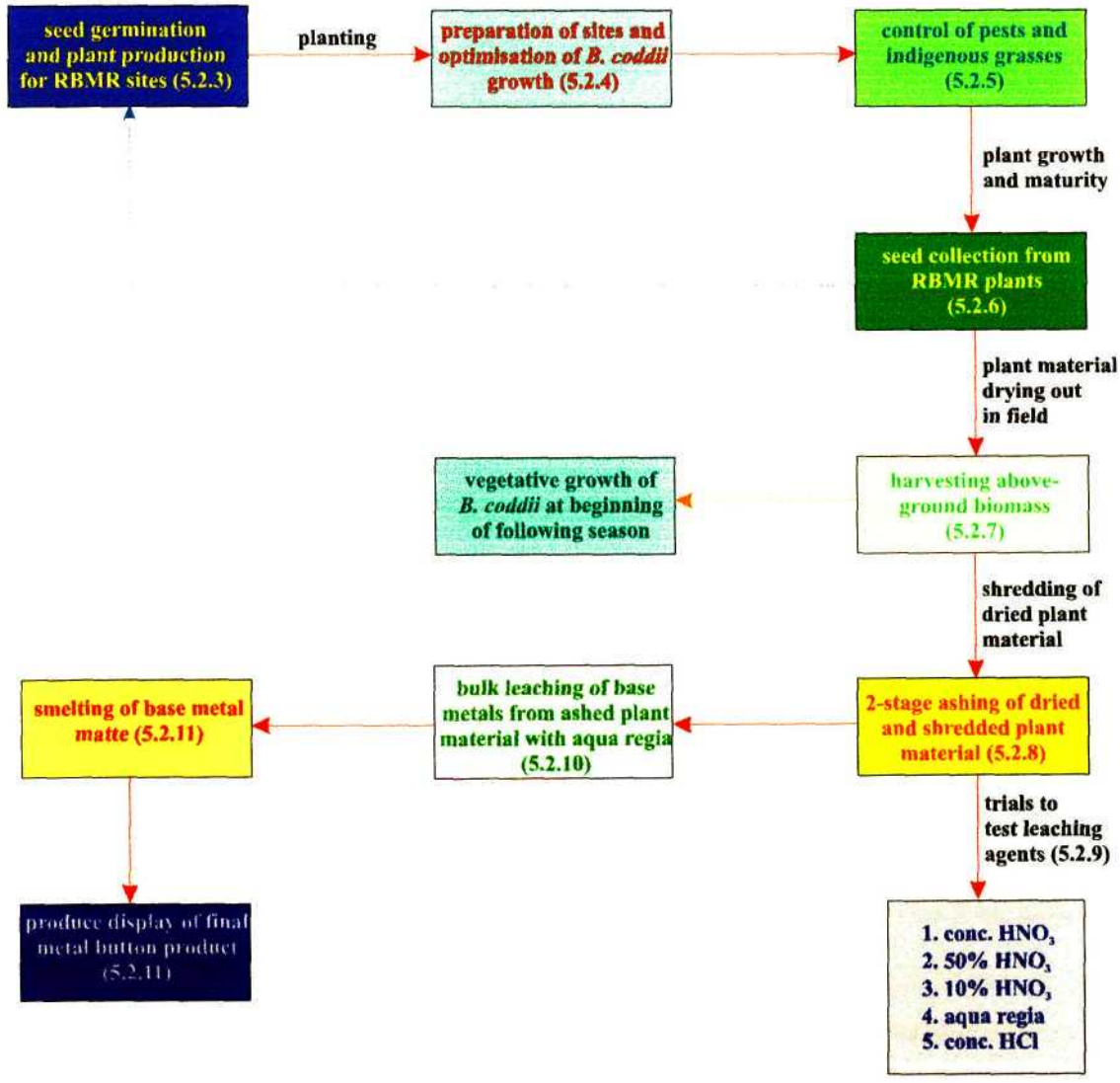


Figure 29: Overview of the Experiments Performed with *B. coddii* in order to Establish it as a Phytoremediation Tool in the Decontamination of the RBMR Sites

5.2 *Materials and Methods*

5.2.1 Analytical Instrumentation

In order to test samples for their base metal content it was necessary to utilise the X-ray fluorescence (XRF) instrument in addition to the AAS (section 2.6.2.1), ICPS and ICPMS (section 2.6.2.2). The XRF was used to qualitatively analyse the samples produced in section 5.2.9 as immediate analyses were required in order that the experiments in section 5.2.10 were not delayed. Although the author did not operate the instrument, an understanding of its operation was gained.

5.2.1.1 X-Ray Fluorescence (XRF)

X-ray fluorescence (XRF) was developed on the principle that the ejection and replacement of electrons from the inner orbitals of an atom results in the emission of X-rays (Carr-Brion and Payne, 1970). Each element emits X-rays at certain characteristic energies that are equal to the energies of the corresponding electron transitions. Thus, by suitably exciting a substance, electrons will be ejected allowing the component elements to be determined. As the X-ray intensity at each characteristic energy level depends on the concentration of the element emitting it, both qualitative and quantitative analysis are possible with X-ray detectors, which convert the incident X-ray into electrical pulses suitable for counting (Carr-Brion and Payne, 1970). XRF is one of the most widely used analytical methods for the qualitative identification of elements having atomic numbers greater than oxygen (Skoog and Leary, 1992).

Samples prepared for XRF were all dried thoroughly overnight at 60°C before being read.

5.2.2 Collection of Seeds from Barberton Site

Dry, ripe seeds were collected from the dying back *B. coddii* plants at the Barberton site, at the end of February 1996 (section 2.3.1). In order to count the seeds a random sample of 200 seeds was weighed and the number of seeds per gram was determined. All the seeds collected from the site were then weighed and the number of seeds per gram used to estimate the total

number of seeds collected. These seeds were used for germination and plant production (section 5.2.3) for the phytoremediation experiments.

5.2.3 Seed Germination and Production of Plants for the RBMR Site

5.2.3.1 Materials

B. coddii seeds collected in section 5.2.2.

Serpentine soil collected from the Barberton site.

Rustenburg Base Metal Refineries (RBMR) soil collected from the contaminated site.

Speedling Trays

The 128-compartment, polystyrene speedling trays were obtained from Starke Ayres Nurseries, Rustenburg.

Dursban

Dursban is an insecticide used for the control of crickets, grasshoppers, worms and white-fly. A solution of 5 ml Dursban per 10 l tap water was used to spray *B. coddii*. Dursban was obtained from a local Rustenburg nursery.

Vydate

A solution of 80 ml Vydate per 10 l tap water was also used to control pests on *B. coddii*. Vydate was purchased at Magalies Graan Korporasie (MGK), Rustenburg.

Potting Bark

Potting bark, also known as potting soil, was used as a rooting medium as it is fairly porous, allowing the roots to be well aerated. It was purchased from a local nursery in Rustenburg.

Vermiculite

Vermiculite was used for water retention, thereby ensuring that the soil medium did not dry out. Vermiculite was purchased from a local nursery in Rustenburg.

Zeolite

Zeolite (Figure 30) is a porous mineral with a high cation exchange capacity (Eberl, 1998). It is able to bind to fertilisers and in this way is used to control the release of nutrients to plants. It

is also able to improve water retention of the soil, as the water tends to bind to zeolite. Zeolite was purchased from Pratley®, Krugersdorp.

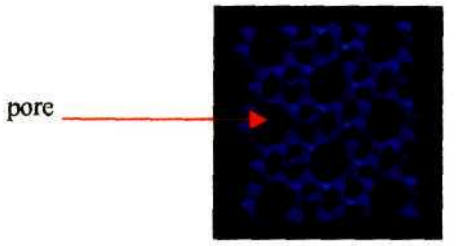


Figure 30: Diagrammatic Structure of Zeolite showing its Pore Formation (Eberl, 1998)

Greenhouse

The greenhouse at the TCRI was used for seedling germination and plant growth. At no stage was the greenhouse heated. The seedlings and plants were watered twice a day with an automated sprinkler system.

5.2.3.2 Procedure

At the beginning of April 1996, some seeds were planted into speedling trays containing a mixture of 1:1, serpentine soil : RBMR soil; other seeds were planted into speedling trays containing a mix of 10% zeolite, 10% serpentine soil, 10% RBMR soil, 40% potting bark and 30% vermiculite (Howes, unpublished). As the germination of *B. coddii* seeds had been found to be low (section 4.3.4.2), 5 seeds (instead of one) were planted within each compartment of the speedling tray. The seeds were germinated in the greenhouse and watered twice a day, for 10 min, at 10h00 and 14h00. The seedlings were grown in the speedling trays for 2 months before being transplanted, in June, to 1 litre black plastic potting bags containing a medium of 50% RBMR soil and 50% potting soil. These plants were grown for at least three months in the greenhouse at the TCRI and watered daily, for 10 minutes, with the automated sprinkler system.

All seedlings and plants were sprayed alternately with Dursban whenever an infestation of crickets, grasshoppers or worms occurred. Spraying was also performed when white fly was seen, and repeated every two days, for six days, until the white fly had been destroyed.

5.2.4 Preparation of the RBMR Site and Optimisation of *Berkheya coddii* Growth and Nickel Uptake

5.2.4.1 Materials

Fertilisers

As suggested from the findings reported in Chapter 4, the fertiliser 2:3:2, containing nitrogen, phosphorus and potassium in the ratio 2:3:2, was employed for the phytoremediation trials.

Multifeed solution, at a concentration of 100 g/ 100 litre, was also used as a fertiliser.

Both fertilisers were obtained from MGK, Rustenburg.

Sites

Three nickel-contaminated sites were identified at RBMR – site A and site B were both approximately one hectare in size, while site C was approximately half a hectare (section 2.2.3).

Irrigation

A drip irrigation system, supplied by WaterMech, Rustenburg, was erected on Site A.

Overhead sprinkler irrigation systems, supplied by WaterMech, Rustenburg, were erected on Sites B and C.

Scarifier

The scarifier used in ploughing was supplied and operated by a local farmer.

Weedeater

A normal garden weedeater was purchased from Game Stores, Rustenburg.

5.2.4.2 Procedure

The planting out of the five-month-old *B. coddii* plants (section 5.2.4.1) on the above mentioned sites, at RBMR, commenced at the beginning of September 1996 and ended at the end of December 1996 and was done using manual labour. A section of Site A (Site A_1), however, was initially set up in November 1995 in order to test the effects of fertiliser on *B. coddii* (Howes, unpublished). In November 1995 a drip irrigation system was installed over the

whole site and then about two thousand *B. coddii* plants were planted over a small section of site A, into holes of 20 cm diameter x 20 cm depth, in January 1996. Half of the plants (1 000) were planted into holes containing 1 g of 2:3:2 (N:P:K); after this initial fertilisation these plants were not fertilised again (Howes, unpublished). The remaining plants (1 000) were placed in unfertilised holes and half of these (500) unfertilised plants were then watered with 5 litres Multifeed, per plant, every month, whilst the other 500 remaining plants were left unfertilised for the duration of the growing season. All the plants were drip irrigated daily for 30 minutes with Rand Water Board (RWB) water until the beginning of April 1996 when they naturally started to go into dormancy.

From the beginning of September 1996 to the end of October 1996, the remaining unplanted area of Site A (Site A_2) was planted with about 4 500 *B. coddii* plants (Howes, unpublished), germinated as described in section 5.2.3. Each plant was planted into a hole 20 cm x 20 cm and, due to the apparent success of fertilising the plants with 2:3:2 (N:P:K) (Chapter 4), each plant had 1 g of this fertiliser added to it. After planting out, the whole of site A contained about 6 000 *B. coddii* plants, giving a plant density of about 0.6 plants per square metre (Howes, *pers. comm.*). The plants were watered daily, in the morning, with RWB water until the end of December 1996 when the rains started.

Site C was heavily contaminated with nickel (actual concentrations are not known but the area had a green tinge to it) and so it was decided that the area should be capped, to a depth of 30 cm, with silt that had built up in the river near the mine (Howes, unpublished). After capping, the site was ploughed with a scarifier and *B. coddii* plants were then manually planted into holes containing 1 g 2:3:2 (N:P:K) fertiliser. Planting commenced in the middle of November 1996 and ended at the end of December 1996. Five thousand plants were planted in site C, giving a plant density of about 1 plant per square metre. An overhead sprinkler irrigation system was used to water the plants daily, with RWB water, for 30 minutes, until the end of December 1996.

Site B was also capped, but with topsoil taken from the building of a dam on the mine. The topsoil was stored dry for six weeks, while the old buildings and large amounts of rubbish were removed from site B, and no nutrients were lost from the soil. After capping, the site was scarified before planting. Again each plant was hand planted with 1g of 2:3:2 (N:P:K) fertiliser

per plant (Howes, unpublished). Fourteen thousand plants were planted from the beginning of October to the middle of December 1996, giving site B a plant density of about 1.4 plants per square metre. An overhead sprinkler irrigation system was again employed on this site and the plants were watered daily, in the morning, with RWB water for 30 minutes, until the end of December 1996.

Samples of leaves from *B. coddii* plants were taken from sites A and C on a monthly basis (Howes, unpublished), from the end of November 1996 to the beginning of April 1997. The plant material was wet ashed with 5:2 (v/v) nitric : perchloric acids (section 2.6) and analysed for nickel uptake by AAS (section 2.6.2.1). So that the pattern of biomass increase versus nickel uptake in *B. coddii* could be investigated, estimates of the average height and mass of the plants growing on each site were also made every month, from the end of November 1996 to the beginning of April 1997 (Howes, *pers. comm.*).

5.2.5 Control of Pests and Indigenous Grasses on the RBMR Sites

As *B. coddii* is not indigenous to the Rustenburg area, and is thought to be a non-competitive plant (section 5.1), insects and indigenous grasses threatened to destroy the plants. Solutions of Vydate and Dursban (section 5.2.3.1) were therefore used to control the grasshoppers, locusts, worms and crickets. A weedeater was used to keep the grass trim and away from the *B. coddii* plants. Mowing was performed on a biweekly basis.

5.2.6 Seed Collection from the Plants Growing on the RBMR Sites

Many of the plants on sites A, B and C produced seeds at the end of January 1997, towards the end of the growing season. These seeds were hand-collected in the middle of February 1997, once they had dried out. Germination trials were then performed on a few of the seeds, using the zeolite germination mix, (section 5.2.3), in order to determine their fertility (Howes, unpublished). It was felt that it would be preferable to populate any further sites requiring rehabilitation with *B. coddii*, with plants germinated from seeds produced from RBMR sites A, B and C, as these plants may have adapted slightly to the Rustenburg conditions.

5.2.7 Harvesting of the RBMR Plants

5.2.7.1 Materials

Gardena secateurs for harvesting *B. coddii* plants, purchased from Pick 'n Pay Hypermarket, Northriding.

The shredder used to shred plant material was a Viking GE 320, with a 2.2 kW capacity. It was manufactured in Germany by Stihl Gruppe.

5.2.7.2 Procedure

Due to the late rains the plants only started to die back at the beginning of April 1997. They were then left to dry out in the ground until the end of May 1997 – before the dead leaves started to fall off the stems. The plants were harvested with secateurs from the end of May 1997 until the middle of July 1997 (Howes, unpublished). Although most of the nickel is found in the leaves (section 3.3.1), with a rather small percentage occurring in the stems, it was felt that, for ease of harvesting, the stems should be cut at the base of the plant, causing all the leaves and stems to be harvested. Any leaves that fell off the stem before or during harvesting were picked up and combined with the harvested material. Material from each site was wet-ashed (section 2.6.2) and analysed for nickel uptake using the ICPS at ARC (section 2.6.2.2).

All the harvested plant material (stems and leaves) from sites A, B and C was combined, placed on tarpaulin sheeting and left to dry in the sun for two weeks. No rain fell in June, July or August 1997 and so the material dried out well. Once dry, the material was shredded, in order to decrease its bulk, and then weighed, bagged and stored. A random sample of the combined material, weighing 500 g, was collected by taking handfuls of material from the top, middle and bottom of each of the two storage bags. This material was then wet-ashed (section 2.6.2) and fully analysed by AAS (section 2.6.2.1), and ICPS and ICPMS at ARC (section 2.6.2.2).

5.2.8 Bulk Ashing the Plant Material

5.2.8.1 Equipment Design and Calibration

Even after shredding, it was felt that the plant material was still too bulky to effect total and efficient nickel extraction. It was, therefore, decided that the material should be ashed to further reduce its bulk. With advice from Mr K Ehlers (ARC), the present author designed an ashing vessel, which was then built by the ARC workshop, Figure 31.

The main vessel comprised a modified 44-gallon drum made of mild steel (0.9 mm thick), obtained from the mine at Rustenburg. In order to sparge air into the drum a tube of stainless steel (internal diameter = 1 cm) was bent into a ring having an internal diameter of 50 cm. Eight evenly spaced nozzles were welded to the ring, facing the base of the drum. This ensured that the nozzles did not clog with ash and that the air was evenly distributed across the base of the drum. A separate section of stainless steel tubing was welded to the ring and protruded through a 2 cm hole in the drum, 10 cm from the drum base. The protruding tube was attached to a regulator. A section of reinforced plastic tubing (internal diameter = 1.5 cm) was attached to the regulator, at one end, and to a rotameter, for the regulation of air-flow, at the other. Another section of the reinforced plastic tubing was attached to the top of the rotameter at one end and to a compressor[6] at the other.

A valve, having an internal diameter of 3 cm, was welded to the lid of the drum, to allow free air flow through the drum. A thermocouple, attached to an LED read out, was hung into the plant material in the drum via the valve. This enabled the temperature within the drum to be measured. A hood, attached to an extraction mechanism, was placed over the valve for smoke capture.

[6] Mistral compressor; Model M2A.

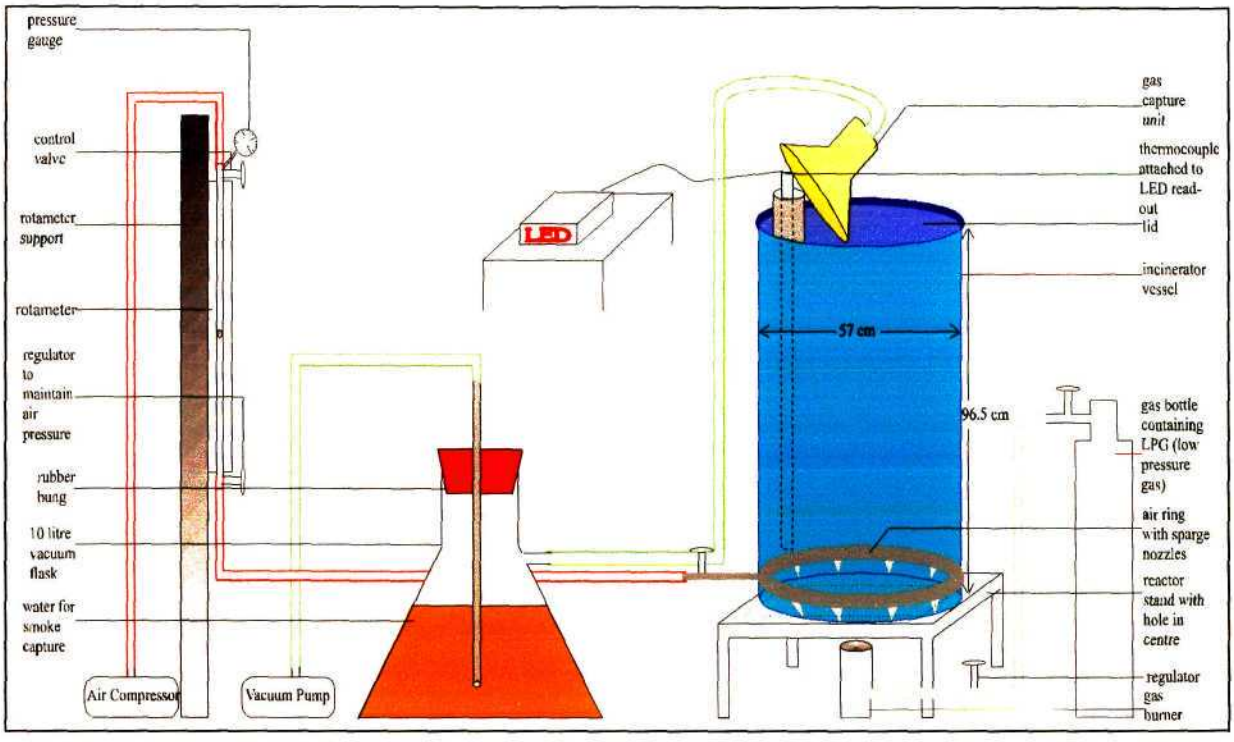


Figure 31: Schematic Diagram Showing the Equipment Used for Ashing Plant Material. The equipment was designed by the present author (with assistance from K. Ehlers, ARC) and built by the ARC workshop.

In order to determine the volume of air flowing through the rotameter at various flow rates (given as a percentage), it was important to calibrate the rotameter at both 1 and 2 bars of pressure. In order to achieve the calibration an air cylinder[7] was placed on a scale, which was then zeroed. Air was released through the rotameter at certain percentage flow rates for set time intervals. The mass loss was read off the scale and recorded. From mass loss and percentage air-flow a calibration curve was drawn, Figure 32.

[7] Commercial air cylinder supplied by Fedgas, Germiston.

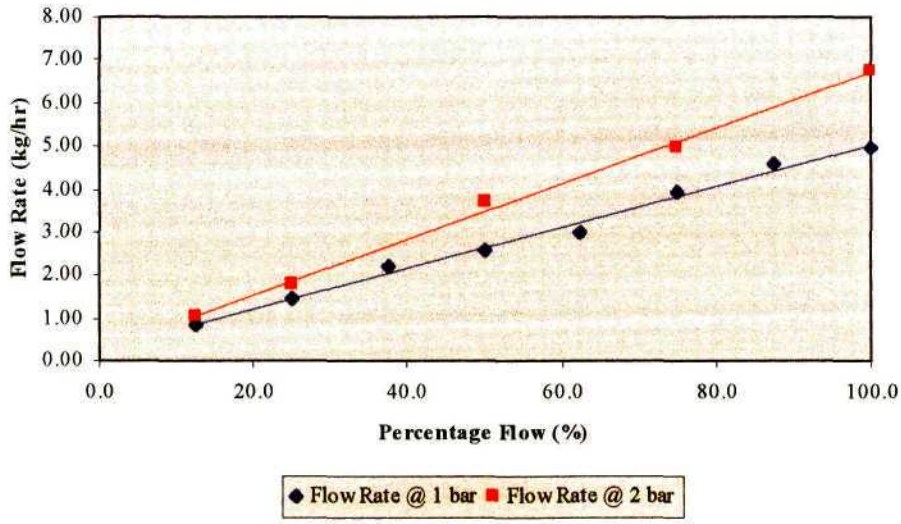


Figure 32: Calibration of Rotameter at 1 and 2 Bars of Pressure

A trial run was performed in which 8.4 kg of dried and shredded plant material was ashed. During this run it was seen that the gas burner was only required to heat the material in the vessel to about 100°C. At this point the material spontaneously combusted and, due to air being fed into the drum, continued to smoulder while the temperature in the drum increased. Once the optimum ashing temperature, between 500°C and 600°C, had been reached the air flow was turned down, using the rotameter, until the correct temperature could be maintained. At no stage was the material allowed to burn with a flame. The ashing temperature never exceeded 700°C as, at this temperature, nickel is oxidised and cannot be acid leached. Some of the smoke from the trial run was channelled, by vacuum, into water contained in a vacuum flask. The water was filtered on Whatman 241 ashless filter paper and both filtrate and filter matte were analysed, by AAS (section 2.6.2.1), for nickel.

In order to ash all the plant material, 5 separate runs were performed. In each run the drum was filled to $^{7}/_{8}$ capacity with a known mass, usually about 24 kg of the dried, shredded plant material. The lid was fitted to the drum and two bricks were placed on the lid to prevent smoke escaping from the sides. The thermocouple[8] was inserted into the plant material with the tip being placed just above the stainless steel air ring – it was found that this was the hottest point

[8] A thermocouple only measures the temperature at the tip of the length of steel cable.

in the drum. At the start of each run the air-flow through the rotameter was set to 100%. The gas burner was lit and the temperature recorded every five minutes. Once the material had spontaneously ignited, at about 100°C, the burner was switched off. The air-flow was maintained at 100% until a temperature of about 500°C was reached. After that the flow rate was decreased until the optimum temperature could be maintained. The air-flow and temperature were recorded every five minutes throughout the duration of the 5 runs. From the air-flow readings the amount of air required to partially ash each kilogram of shredded plant material in each run could be determined, allowing the efficiency of each run to be assessed.

After 2 hours the lid was taken off the drum and the material stirred around. The lid was then replaced and the air-flow increased to 100% until optimum temperature was again achieved. This rabbling process was repeated every hour thereafter or whenever the ashing temperature went over the optimum temperature. Towards the end of each run the lid was removed from the drum to enhance air flow. The lid was only taken off if no flame was produced when sparging air through the drum.

Once all the material had been ashed the air was switched off and the ash left to cool in the drum, overnight, with the lid off. The partially-ashed material was weighed and then ashed to completion in a muffle furnace (section 5.2.8.2).

5.2.8.2 Muffle Furnace

The crude ash from each drum-run was placed in separate stainless steel trays (length = 42 cm; width = 27 cm; height = 15 cm) and ashed to completion in a muffle furnace[9]. The temperature of the muffle furnace was set to 450°C and the material was then left to ash for 36 hours.

The completely ashed material was then cooled for 4 hours – with rabbling – and placed in a pre-weighed plastic bag and weighed. Once all the ashing runs were complete the material was combined and blended and a sample was sent in for full analysis, by ICPS, to the ARC analytical chemistry section to determine the elements present, so that a leaching process could be designed.

5.2.9 Trials for Leaching the Base Metals

5.2.9.1 Introduction

Metallic nickel could not be obtained directly from the ashed plant material due to the presence of carbon in the material, and the exceptionally fine nature of the ashed material (K. Ehlers, *pers. comm.*). Thus the nickel needed to be acid leached out of the ash, causing it and other base metals to go into solution leaving behind insoluble compounds after filtration, including insoluble silicates. As solutions cannot be smelted the acid base-metal solution then needed to be precipitated and filtered.

From the analyses performed as described in section 5.2.8.2, it was seen that 8% silica was contained in the ashed material. Silica is insoluble, except in hydrofluoric acid (HF) and so it was expected that problems would be experienced when filtering the base-metal solution (after initial leaching of the ashed material), as insoluble silica tends to become hydrated and form a gel, which is almost impossible to filter (*pers. comm.* Z. Cruikshank, F. Vosloo, A. Buck and V. Lennard, ARC Analytical Chemistry Division, *pers. comm.*). Since it is known that filtration improves markedly if silica is dehydrated, five methods to dehydrate the silica, using combinations of two strong acids were tested (section 5.2.9.2), in order that the most suitable extraction method be used to leach the bulk of the ashed material. Although silica can be removed from samples with the addition of HF (Skoog *et al.*, 1992), this method was not tested due to the toxicity of HF.

It was decided that the "normal" oxidation method used to liberate nickel from plant material (section 2.6.2) would not be used to leach the ash as perchloric acid tends to be explosive when mixed with large amounts of organic material (Skoog *et al.*, 1992). Thus trials to oxidise the plant material using combinations of hydrochloric and nitric acids, which are not explosive, were performed.

Many workers have used 2M hydrochloric acid (HCl) to liberate the metals from small quantities of ashed plant material (Dakshini *et al.*, 1982 and Brooks *et al.*, 1979). However, it

[9] Muffle furnace name and make unknown due to its age.

was felt that this concentration of acid would not be sufficient to dehydrate the silica and so only leaching with concentrated HCl, which is also able to liberate base-metals, was tested. Nitric acid (HNO_3) was the other acid tested for its base-metal leaching and silica dehydration abilities.

HNO_3 is both a strong oxidising agent and a strong acid (Andrews and Kokes, 1962) and its oxidising nature makes it ideal for leaching material high in organics (e.g. plant ash) as it causes the carbon compounds to be driven off as carbon dioxide (CO_2) and water (H_2O). The only two metals not dissolved by HNO_3 alone are gold and palladium. Thus it was thought that by using HNO_3 to leach the ash, all the metals present should be solubilised. Iron, chromium, aluminium and calcium readily dissolve in 10% HNO_3 but in concentrated HNO_3 no apparent reaction occurs as the metals are passivated by the formation of an oxide skin, caused by the concentrated acid (Andrews and Kokes, 1962). However, it was felt that 10% HNO_3 would be insufficient to dehydrate the silica, while concentrated HNO_3 would not dissolve the metals. Thus it seemed that 50% HNO_3 would probably be the most suitable concentration of HNO_3 to use to leach all the metals whilst dehydrating the silica. Separate trials with concentrated HNO_3, 50% HNO_3 and 10% HNO_3 were performed.

Since aqua regia combines the properties of HNO_3 and HCl, it was considered a good choice for the dehydration of silica and the dissolution of metals. Thus a trial leach was also performed with aqua regia.

In order to fully dehydrate silica a sample should be boiled in the acid of choice and then taken to dryness. However, as the base-metals need to be removed from the insoluble compounds the dry sample needs to be resuspended in an acid. Generally 10% HNO_3 is recommended for general base metal dissolution and so all dry samples were resuspended in 10% HNO_3. To ensure full metal solubility the resuspended samples were boiled for a few minutes before being cooled for filtration. 10% HNO_3 is also the standard acid used to read samples on the ICPS, making sample analysis a bit quicker.

5.2.9.2 Materials and Equipment

Cyberscan 1000 pH meter, made in Singapore.

Trial Acid Solutions

1. Concentrated hydrochloric acid (conc. HCl) (32%). A chemically pure reagent made by SMM Chemicals, RSA.
2. Concentrated nitric acid (conc. HNO_3) (65%). A chemically pure reagent made by Merck, RSA.
3. 50% HNO_3 was made by combining equal volumes of tap water and conc. HNO_3.
4. 10% HNO_3 was made by adding 100 ml conc. HNO_3 to 900 ml tap water.
5. Aqua regia (AR) was made by adding conc. HNO_3 to conc. HCl in a 1 : 3 ratio.

Neutralising Agents

Concentrated ammonium hydroxide (NH_3OH) (32%). Analytical grade reagent (UNIVAR). Made by SAARCHEM, RSA.

Sodium hydroxide (NaOH). Chemically pure reagent (UNILAB). Made by SAARCHEM, RSA.

5.2.9.3 Procedure

In each of the five acid trials, about 50 g of the muffle ashed plant material was weighed into separate 1-litre glass squat beakers. A volume of acid approximately three times that of the mass of the ash (v/g) was then added to the beaker. The beaker contents were then boiled on a hotplate until almost dry (dryness could not be achieved as the samples tended to splutter causing loss of material). The samples were cooled, after which 150 ml 10% (v/v) HNO_3 was added to each beaker. The samples were re-boiled for 5 minutes, cooled and filtered using Whatman GF/A glass fibre paper on a 12.5 cm Buchner funnel. The filtration time for each sample was noted in order to ascertain the effect of each acid on silica. A sample from each leached solution (leachate) was sent in for analysis on the ICPS (section 2.6.2.2), while the remainder of leachates were retained for recovery of the base metals. The leached ash samples were dried overnight at 60°C and weighed.

A sample of each leached ash residue was sent to the Analytical Chemistry section of ARC where it was scanned on a Philips PW 2400 X-ray fluorescence spectrometer. The results of these scans showed how effectively each acid solution had leached out the base metals from

the ashed plant material. The leached residue having the lowest concentration of base metals was the most effectively leached and that acid solution was then used to leach the bulk of the ashed material (section 5.2.10).

In order to recover the base metals from the above leached solutions, sodium hydroxide was added to each solution, until a pH of 8.00 was reached. All base metals precipitate once the hydroxide form (Table 21) is produced (AECI, 1978). The hydroxide forms of platinum, palladium and rhodium also precipitate from solution (Amos, *pers. comm.*, 1997). The base-metal precipitates were then filtered out of solution on a Buchner funnel using Whatman GF/A filter paper. The precipitates were then rinsed in water to remove any soluble impurities, and re-filtered. Samples of the filtrate were taken and sent for analysis on the AAS (section 2.6.2.1) to ensure all the base metals had been precipitated. The filtered base-metal precipitate (matte) was dried overnight at 60°C, weighed and submitted for ICPS analysis of the base metals (section 2.6.2.2).

Table 21: The pH at which certain Base Metal Hydroxides Precipitate out of Solution
(AECI, 1978)

	IRON	NICKEL	COPPER	COBALT
pH of Hydroxide Precipitate	Fe(III): 3 - 4 Fe (II): 6 - 7	7 - 8	4 - 5	7 - 8

5.2.10 Large Scale Recovery of Metals from Ashed Plant Material

All the work was performed in a fume hood due to the dust produced by the ash and the acid fumes produced when adding acids to the ashed material. For each batch of ash to be leached, a weighed amount (not exceeding 1.5 kg) of ashed plant material was placed in a 2-litre glass beaker, and 1.5 litre of aqua regia was made up in a separate 2-litre beaker. 200 ml of this aqua regia were placed in a 5-litre beaker and all the ash from the 2-litre beaker added, while stirring vigorously with a glass rod. The reaction was fairly violent and a lot of heat was generated, so care was taken to ensure all the ash was wet and a thick paste formed. The remaining aqua regia was added slowly by pouring it gently down the sides of the 5-litre beaker **with no further stirring**. The reaction was left to proceed on its own for about 15 minutes.

Once the reaction was complete, the contents of the beaker were stirred thoroughly and the beaker was placed on the hotplate, preheated to a temperature of about 150°C. The mixture was boiled until the material started to splutter (approximately 1½ hours). The beaker was removed from the heat and a volume of 10% HNO_3, equal in volume to that occupied by the mass of ash initially added, was mixed into the partially leached ash. The material was boiled for 30 minutes and then cooled.

Once cool, the material was filtered with Whatman 541 filter paper in a 24 cm Buchner funnel, and the filtrate (leachate) retained. The leached ash was re-pulped in 3 l of water, filtered and the wash water retained. The leached ash was dried overnight in the oven and retained. The procedure was repeated until all the material had been leached.

Samples of leachate were sent in for analysis on the ICPS (section 2.6.2.2) for base-metal content. The leached ash samples were analysed on the AAS (section 2.6.2.1), for residual base-metal content.

The leachates were combined in order to speed up the neutralisation process. Wash water solutions were also combined. These combined solutions were placed in 5 litre beakers and boiled down on the hotplate to reduce the volume to approximately half. The solutions were cooled and a white precipitate that formed was filtered on a large Buchner funnel (24.0 cm), with Whatman 541 filter paper. The precipitate was washed and analysed by XRF (section 5.2.1.1).

The cooled, filtered solutions were adjusted to a pH > 8.0, with weighed amounts of NaOH. Care was taken in neutralising the acidity of the leachates as the reactions were quite violent. Once the solutions had cooled they were filtered using pressure filters requiring 32-cm filter paper[10]. Samples of the filtrate were sent in to the ARC analytical labs for base metal analysis on the ICPS (section 2.6.2.2).

[10] Rundfilter. Supplied by Behn Meyer. Made in Germany by Macherey Nagal.

The filter matte was dried for at least 36 hours at 60°C and weighed. The mattes were then combined and submitted to the ARC labs for full analysis on the ICPS (section 2.6.2.2). A back titration was also performed to determine the concentration of hydroxide present.

5.2.11 Smelting the Base Metal Matte to Produce Metal Buttons

5.2.11.1 Materials and Equipment

A large, high temperature muffle furnace was supplied by Leonard Light Industries.

Clay graphite crucibles were supplied by Salamander, RSA.

5.2.11.2 Procedure

Table 22 shows the conditions used for smelting (Ehlers, 1997). The flux, coke and precipitate were thoroughly mixed in a plastic bag for 10 minutes. This mixture was then placed in a clay-graphite crucible and put into the furnace. This was done fairly slowly in order to allow the crucible to heat up gradually so that it would not suffer a heat shock, caused by a rapid increase in temperature from about 25°C to 1350°C in 30 sec, resulting in a broken crucible. The mixture was allowed to react for one hour in the furnace after which it was removed from the furnace and cast into a pre-heated high carbon steel chill mould. This enabled the molten slag and metal phases to separate. After one hour the mould was removed and the resulting metal button was separated from the slag by chipping. The button was cooled and weighed.

Please Turn to Page 135 for Table 22.

Table 22: Conditions Used in Smelting the Hydroxide Precipitate Produced from Leaching the Plant Ash

Test*	Furnace		Temperature (°C)		Fluxes (g)		Coke (g)	Precipitate (g)
	Induction	Muffle	Start	End	Silica	Borax		
1	6.0 kW	-	20	1400	80	90	42	200
2	5.6 kW	-	20	1380	80	80	36	200
3	5.5 kW	-	20	1350	40	90	36	200
4	5.2 kW	-	20	1340	30	50	36	200
5	5.0 kW	-	40	1330	40	80	36	200
6	5.0 kW	-	50	1330	50	50	36	200
7	5.0 kW	-	60	1330	60	60	36	200
8	-	√	1225	1315	240	240	108	600
9	-	√	1262	1334	240	240	108	600
10	-	√	1242	1334	240	240	108	600
11	-	√	1224	1322	240	240	108	600
12	-	√	1238	1322	240	240	108	600
13	-	√	1250	1334	285	285	128.4	712.4
14	-	√	1299	1341	80	80	36	200

*In tests 1-7 a size A3 crucible was used; in tests 8-14 a size A8 crucible was used.

5.3 *Results and Discussion*

5.3.1 Collection of Seeds from Barberton Site

In order to determine the number of plants that could be germinated it was important to estimate the number of seeds that were collected from Barberton. By estimating the number of plants that could be germinated (bearing in mind that only about 25% of the seeds germinate, section 4.3.4.2) it was possible to work out the maximum plant density that could be achieved on each site.

The mass of seeds collected from the Barberton site was 251.2 g. From Table 23 this equates to about 285 942 seeds. It should be noted that removing this number of seeds from the site appeared to have very little impact on the number of *B. coddii* plants growing in Barberton, as *B. coddii* growth is naturally vegetative. The production of seeds tends to be used as a back-up mechanism in case the established plants are destroyed.

Table 23: Mass Corresponding to Seed Number

NUMBER OF SEEDS	MASS OF SEEDS (g)
200	0.1757
285 942	251.20

The number of seeds collected was considered to be sufficient to establish *B. coddii* plants on the RBMR sites for phytoremediation since, even with a 25% germination rate 71 485 plants could be produced while only 23 500 plants were required (section 5.2.4.2). This allowed some trials and errors to be made in the germination of seeds (section 5.3.2).

5.3.2 Seed Germination and Production of Plants for the RBMR Site

Due to the speed with which plants were required for the phytoremediation project, no time was given to performing germination trials on large amounts of *B. coddii* seeds. Unfortunately, of the 258 000 seeds planted in the 1:1, serpentine soil : RBMR soil mix, only about 11% germinated. In contrast 60% of the seeds planted in the zeolite mix germinated (Howes, unpublished), suggesting that zeolite mix is a better germination medium for *B. coddii* due to slow nutrient release and good drainage. Thus, in total, about 42 500 *B. coddii* seedlings were produced. It should be noted that, if lower numbers of *B. coddii* plants are required, the seeds can be germinated on cotton wool (section 4.3.4.2), which is both less time consuming and more cost effective.

One possible reason for the high seed mortality rate observed in the 1:1 serpentine: RBMR mix might have been due to high water retention, which caused many of the seeds to rot, as there was a lack of gas exchange between the germination medium and the embryo. Only very low oxygen levels are found in water, compared with soil, and *B. coddii* does not have a mechanism like rice (Hartmann *et al.*, 1990), in which shoot development is stimulated and root development suppressed to cope with these low oxygen levels. Although the low germination rate resulted in sufficient plants for this project, it was realised that a more reliable germination method would be required in the future, to populate large waste areas, if the Barberton site was not to be decimated. Thus, on some remaining seeds, A. Howes (*pers. comm.*) performed some germination trials and developed the zeolite medium, given in section

5.2.3, for enhanced seed germination. It is interesting to note that only the medium affected seed germination. Any manipulation of the seeds e.g. with smoke treatment or acid digestion, did not improve their germination rate (Howes, *pers. comm.*). The seed germination rate was also not improved with changes to temperature or light density. Maintaining a damp germination mix was, however, extremely important for seed germination.

The seedlings germinated on the zeolite mix were transferred to a 50 : 50 potting soil : RBMR soil mix to ensure that the plants adapted to the RBMR soil, without suffering too much from a change in medium (Howes, unpublished). No serpentine soil was added and the plants grew well. This result suggested that serpentine soil does not contain any unique combination of minerals essential for the growth of *B. coddii*. The composition of serpentine and RBMR soils is compared in section 4.3.2, and shows that the serpentine and RBMR soils are fairly similar as they are both clay soils, of similar pH, although serpentine soil has more plant available metals than does RBMR soil.

The seedling transfer from the speedling trays to the 1l potting bags, containing 1:1 potting : RBMR soils, was fairly successful. Of the 28 500 seedlings in the soil only trays, 26 400 plants survived (Howes, *pers. comm.*). Of the 14 000 seedlings in the zeolite mix, 13 100 plants survived transplanting. It was observed that the plants tended to suffer from water stress directly after transplanting and so it was essential to closely monitor watering for the month following transplanting. In total about 39 500 plants were produced of which about 23 500 were used for phytoremediation.

Thus, in summary, the germination of *B. coddii* seeds on zeolite, followed by transfer to a 50 : 50 potting : RBMR soil mix proved most successful in producing sufficient numbers of plants for phytoremediation of the RBMR sites (section 5.2.4).

5.3.3 Preparation of the RBMR Site and Optimisation of *Berkheya coddii* Growth

Site A_1, planted at the beginning of January 1996, showed fairly poor growth possibly due the fact that the earth in the site was fairly compacted. Thus it was decided that future sites would be ploughed before being planted and so Sites B and C were scarified which significantly

improved the growth of the plants due to improved root penetration enabling more nutrients to be taken up. The drainage of these sites also improved, causing the roots of the plants not to become waterlogged and oxygen starved.

It was interesting to note the effect of fertiliser on the plants. As mentioned in section 4.3.5.1, the addition of certain nutrients such as potassium and phosphorus, caused an increase in plant growth, while nitrogen appeared to have a detrimental affect on growth. On the basis of these results it was decided that 2:3:2 (N:P:K), a very general purpose fertiliser, should be added as a one off when planting out to allow the plants to adapt to the lower nutrient status found in RBMR soil compared with the germination and transplant mixes.

All the plants fertilised with 2:3:2 (N:P:K) in sites A_2, B, C and half of A_1 grew much faster than did the unfertilised plants in the quarter of site A_1. The unfertilised plants in site A_1, however, remained as healthy as those fertilised with 2:3:2 (N:P:K) suggesting that the increased levels of potassium and phosphorus, found in the soil containing 2:3:2 (N:P:K), caused the plants to grow faster without affecting their health. Consequently, no more fertiliser needed to be added once the plants were growing.

Interestingly, the plants growing on one quarter of Site A_1, responded negatively to the addition of Multifeed. This poor growth pattern was thought to be due to an overloading of magnesium, which is high in RBMR soil, compared with that in serpentine soil (section 4.3.2). Multifeed contains magnesium and, as it is a quick release fertiliser, the extra magnesium was immediately made available to the plant. Thus the nature of the fertiliser added to *B. coddii* is clearly important, and more trials on the effect of various fertilisers on plant growth and nickel uptake should be performed in the future.

Figure 33 and Figure 34 compare the pattern of nickel uptake and biomass gain between the plants growing on sites C and A, respectively. The measurement of both biomass and nickel uptake was based on the dry mass of the plants. It was decided that the growing season should commence with the germination of the seeds and end with the harvesting of the plants. Thus the graphs show the growing season as a twelve-month period. It should be noted that the data given is not very accurate as only one plant was analysed for each data point and that each plant was subjectively chosen as one of average height and mass (A. Howes, *pers. comm.*).

As expected, both figures show that there is a direct relationship between the amount of plant biomass and its capacity to take up and store nickel. Furthermore, by comparing these graphs it can be seen that, on average, the biomass gain in the plants growing on Site A was significantly greater than that for the plants growing on Site C. This can be attributed to the fact that some of the plants growing on Site A (plants on Site A_1) were in their second season whilst all the plants growing on Site C were in their first growing season. This shows that growth in the second season is greater than it is in the first season. This is to be expected as (i) the growing season for plants in their second season is longer because germination occurs at the onset of the first rains; and (ii) in the second season the plants have 3 or 4 stems, whilst in the first season there is only one stem. Another reason for faster growth in the second season is that the plants have become better adapted to the new conditions as compared with those plants in their first season.

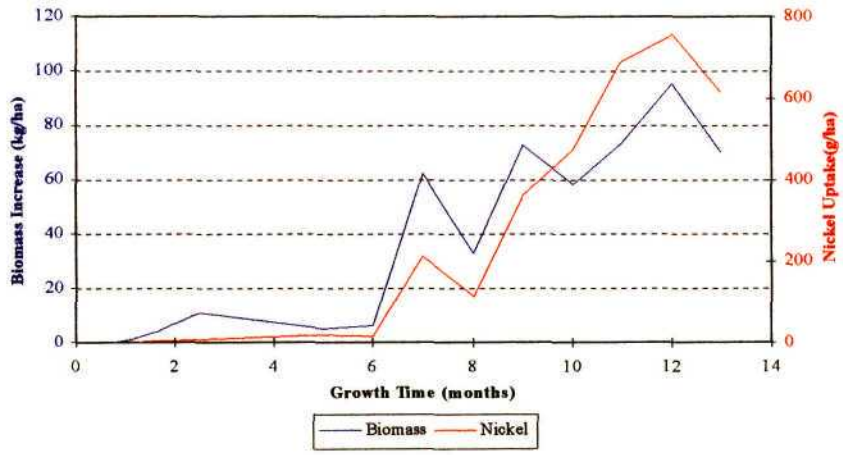


Figure 33: The Increase in Biomass and Nickel Uptake of *Berkheya coddii* Plants Growing on Site C Throughout the '96/ '97 Season (Howes, unpublished).

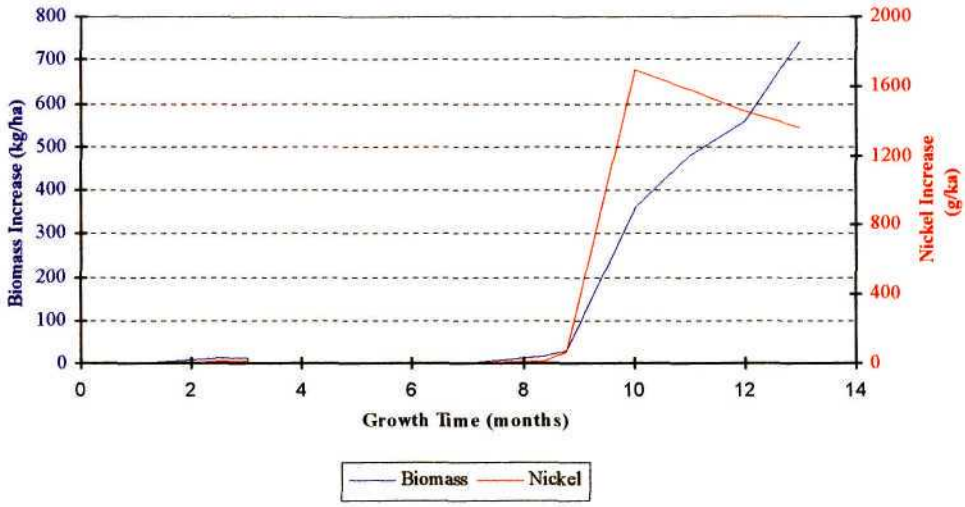


Figure 34: Increase in Biomass and Nickel Uptake of *Berkheya coddii* Plants Growing on Site A Throughout the '96/ '97 Season (Howes, unpublished)

Figure 33 and Figure 34 also show that nickel uptake is greater in the second season than in the first. This shows that the older and larger plants are better able to accumulate nickel, probably because competition between nutrient and nickel uptake decreases as the plants age. The decrease in competition could possibly be attributed to there being more available sites for nickel uptake as the root system grows, and a decrease in nutrient requirement as the season progresses.

Having capped sites B and C it was assumed that their roots did not reach the highly contaminated soil beneath the cap in the first season, as the roots only reached about 20 cm into a cap of soil 30 cm deep. It thus remains to be seen whether or not the plants are able to cope with the toxic effects of the very high levels of nickel found on sites B and C. However, once the roots do penetrate the highly contaminated soil, the growth rate may actually decrease until the plants have adapted to the conditions. Thus for the '97/ '98 season it is expected that fairly low quantities of nickel will be recovered from the sites, but this remains to be verified.

From the above results it can be seen that the plants have adapted well to the conditions found at the RBMR sites and that they appear to be efficiently accumulating nickel from the soil into

their leaves. It is hoped that the growth rate will improve in future seasons as the plants adapt further to the RBMR conditions.

5.3.4 Control of Pests and Indigenous Grasses on the RBMR Site

All the crickets, locusts and grasshoppers were successfully removed by spraying the plants with Vydate and Dursban. This ensured that the plants were not eaten and that the above vectors transferred no diseases to them. The plants remained healthy throughout the growing season.

The indigenous grasses growing on the sites were a problem as they threatened to smother the *B. coddii*. Cutting the grass with a weedeater was fairly difficult due to the uneven ground and the rocks and stones found on the site, as well as the possibility of it damaging *B. coddii* plants. It is, therefore, not recommended that the grass be cut manually. Instead the grass should be removed from the site, before it is planted with *B. coddii*, and then the site should be treated with a grass killer. Once the site has been planted any encroaching grass should be manually dug out.

5.3.5 Seed Collection from the Plants Growing on the RBMR Site

As it was the first growing season, not many plants flowered and only about 5 000 seeds were collected. From the germination trials it was seen that these seeds were not very viable (Howes, *pers. comm.*). The reason for this was thought to be a lack of pollination due to the beetle vector having been destroyed by spraying of the plants. It was, therefore, decided that in future seasons the plants would not be sprayed unless absolutely necessary. If spraying were required it would only be done at the start of the season, in order not to interfere with the life cycle of the vector.

Seed collection can only be performed manually and so people from the local community are generally employed for the month during which seed and plant harvesting occurs. This enables them to earn money for the community and, it is hoped, to learn something about the environment.

5.3.6 Harvesting of the RBMR Plants

The plant material from sites A_1, A_2, B and C was harvested manually and then combined. The dried material shredded fairly easily and 120.8 kg of material was obtained at the end of the first growing season. This equates to an average biomass of approximately 5.1404 g dry weight per plant. Although the harvesting was done manually it was felt that this was the most efficient method of harvesting as mechanical harvesting is very expensive and fairly difficult on the uneven terrain of the contaminated sites. Again, manually harvesting the plants allows people from the local community to be employed. It was felt, however, that the material would not be shredded in future years due to the time and expense associated with the ashing and leaching of the plant material (section 5.3.7 and 5.3.9). In future the fully dry material would be fed directly into the smelter, together with concentrate from the mines. The bulk associated with unshredded plant material will have no effect on the time taken to feed the material into the smelter and thus, the time-consuming shredding procedure will not be required.

From analysis on the ICPS, the average nickel uptake in the plants on each site was determined (Table 24). Due to subjective sampling the concentration of nickel given in Table 24 is merely an approximate estimate of the amount of nickel taken up by the plants on each site. Three dry plants were analysed from each site to give the average nickel concentrations in Table 24.

Table 24: The Average Nickel Uptake of the Plants growing on the Various Sites

	SITE A	SITE B	SITE C
Nickel Uptake (g/ha)	1 362	616	715

Table 24 shows that the plants on site A (A_1 + A_2) accumulated overall more nickel than the plants in sites B and C. This was attributed to the greater biomass of the plants in their second season, allowing better nickel uptake. It should also be noted that the seedlings planted in Site A_2 in the 96'/ 97 season were planted earlier than in the other sites giving a longer growth period and thus more time to accumulate nickel. Therefore, the older the plant the more nickel appears to be accumulated. In section 3.3.1 the theoretical amount of nickel that could be harvested from 15 000 plants growing on a site was 1 457 g. As can be seen in Table 24 site A

matches this theoretical value very closely indicating that, from trial studies, calculations can be made on the amounts of nickel that *B. coddii* can accumulate from a site.

Prior work performed on *B. coddii* growing naturally at the Queens River site (Anderson *et al.*, 1997) shows that comparatively little nickel is accumulated in the roots of the plant compared with the leaves and the stems. The roots of the *B. coddii* plants growing on the contaminated RBMR sites were not harvested, and therefore their nickel content was not determined, for three reasons:

1. The root system forms an intricate network between individual plants and so is extremely difficult to harvest;
2. The root system needs to continue to develop throughout the decontamination period as this will allow the *B. coddii* plants to take up the maximum amount of nickel from the soil due to maximum soil penetration; and
3. If the roots are harvested then *B. coddii* plants will not germinate vegetatively each season and therefore seedlings will need to be sown each season, which is labour intensive, expensive and time consuming.

Presently, 1.3 kg per hectare of nickel can be produced per year, at a value of R26.30. Although this is not a large amount of money the mine will, in the not too distant future, be required by law to remove the nickel, and phytoremediation is by far the cheapest option. The only other means of removing the contaminating nickel is to excavate the site and remove the metal by autoclaving and acid leaching, which causes all the nutrients to be lost, rendering the soil sterile. It is also extremely expensive in terms of excavation and chemicals and so, while the phytoremediation project may not make money for Amplats, it will in the long term, save them a substantial amount.

5.3.7 Ashing the Plant Material

The ashing apparatus designed by the author and K. Ehlers (see details, section 5.2.8) operated extremely well in that it successfully and efficiently reduced the bulk of 120.8 kg of shredded material to approximately 18.25 kg of partially ashed material. This material was then ashed to

completion in a muffle furnace, which further reduced the mass to 14.2 kg, giving a more manageable mass and volume of material to work with.

The smoke collected from the trial ashing run produced a thick, black tar-like substance that floated on the surface of the water. Table 25 shows that the amounts of nickel, copper, cobalt iron collected in the smoke was negligible and thus the smoke did not need to be collected for purposes of metal recovery. Instead it was removed from the air via an extraction system to prevent pollution.

Table 25: Analyses of the Filtrate and Filter Matte Collected from the Smoke Produced During the Ashing of 8.4 kg of Shredded *B. coddii* Material

	Cu (mg/kg)	Ni (mg/kg)	Fe (mg/kg)	Co (mg/kg)
Metal Content in Organics	16.5	17.0	85.5	4.0
Metal Content in Water	0.0	0.0	0.0	0.0

Table 26 gives an overview of the mass of shredded plant material ashed in each run and the corresponding mass of partially ashed material produced. It also shows the amount of air used, and the time taken to partially ash the shredded material in each run. These figures indicate that ashing the material is not a very cost-efficient process as a greater amount of air was required in the process than there was plant material, that is, a ratio of 1.63 : 1, air : plant material was required. The time taken to ash each kilogram of plant material was in the region of 30 min, again showing that the process is not cost-effective as it is very slow. The ashing process was, however, efficient in terms of reducing the bulk and mass of the shredded plant material since, on average (Table 26), each kilogram of shredded plant material was reduced to a mass of 160 g, giving an 84% mass reduction. Thus in theory, the 120.8 kg of shredded material could be reduced to 19.3 kg of partially ashed material; in fact 18.25 kg of partially ashed material was produced from 120.8 kg shredded material.

Table 26: Mass of Air Required and Mass of Ash Produced in Ashing the Dried and Shredded *B. coddii* Material

	Mass Leaves	Total Air Used	Ash Mass	Ash/kg Leaves	Time to Ash 1 kg
	(kg)	(kg)	(kg)	(kg)	(min)
Run 1	22.5	47.77	3.90	0.17	37.87
Run 2	20.8	46.99	3.15	0.15	26.78
Run 3	24.5	37.49	3.85	0.16	30.49
Run 4	23.0	32.40	3.20	0.14	28.17
Run 5	22.8	20.68	4.15	0.18	33.68

As it was felt that the volume and mass of the partially ashed material was still too large, the material was further ashed in a muffle furnace. From the 18.25 kg of partially ashed material, 14.2 kg of fully ashed material was produced. Thus, by ashing and muffle ashing the mass of the shredded *B. coddii* material was reduced by 88.3%, giving a more manageable mass and volume to work with.

It was however, decided that in terms of time, ashing the shredded plant material was not cost-effective and so in future the material would be placed in the smelter, where it would be combined with concentrates from the mines and follow the "normal" Amplats process of metal extraction.

5.3.8 Trials for Leaching the Base Metals

In order to analyse metals in plants the organic material needs to be decomposed first (Skoog, 1992). To achieve this, solutions of strong oxidising agents are generally used, although it has been argued that wet ashing causes losses of elements by volatilisation, but this can be reduced by performing rapid oxidations. An excellent oxidation solution is a perchloric/ nitric acid mix. Due to the explosive nature of perchloric acid, when mixed with organic material, it is essential to commence oxidation with the nitric acid, rather than with perchloric acid and thus the oxidation mixture should have a greater concentration of nitric acid, compared with perchloric acid. The nitric acid then attacks the easily oxidised components in the early stages, converting them to carbon dioxide (CO_2) and water (H_2O). As the solution is heated the water and nitric

acid are lost by evaporation and decomposition causing the solution to become a stronger oxidant as the perchloric concentration increases (Skoog, 1992).

Another means of oxidising the organic material is to dry ash it by igniting it at 500°C in a muffle furnace, before dissolution (Baker and Brooks, 1989). Unfortunately, although this method is simple, there is always uncertainty about the completeness of recovery of supposedly non-volatile elements (Skoog, 1992). Tests are therefore required to determine the applicability of dry ashing to a given type of sample. Baker and Brooks have found that no significant copper, nickel, zinc, manganese, cobalt or chromium losses occur if the plant material is dry ashed. Lead determinations, however, appear to be affected by dry ashing as losses occur and thus this element is always determined after wet ashing.

Blincoe *et al.* feel that the organic material should be oxidised by dry ashing as dry ashing is simple and, because fewer reagents are used, there is less chance of contamination (Blincoe *et al.*, 1987). Due to equipment constraints and a lack of information on the behaviour of certain metals in dry ashing, however, the wet ashing method was used to prepare all plant material for metal determinations, in the phytoremediation trials.

The initial XRF scans showed that concentrated HCl leached nickel from the ash more efficiently than any of the other acids since the HCl residue contained lower nickel concentrations than the other residues. Aqua regia (AR) was also able to remove most of the nickel from the ash, as observed in the XRF scan. The XRF scan also showed that nitric acid is less efficient than conc. HCl and AR at removing the nickel as there is a higher nickel concentration in the residues leached with HNO_3 than in those leached with HCl and AR. The residues leached with 50% HNO_3 contained less nickel than those leached with conc. HNO_3. The residues leached with 10% HNO_3 contained the greatest amount of nickel showing that this acid solution was the least efficient at extracting nickel from the plant ash material. Thus in terms of extraction ability, HCl appeared to extract the nickel from the ashed material more effectively than any of the other acids, although AR was only marginally less efficient. However, other criteria need to be considered before a decision on which acid to use in the bulk leach experiments could be made.

In order that the leachate could be filtered efficiently it was necessary to dehydrate the silica (section 5.2.9.1) and thus the effect of the above acids on silica dehydration was assessed by taking the time required to filter each leachate. Table 27 shows that 10% HNO_3 appears to be the most efficient dehydrating agent, although it is thought that this is misleading and the short time taken to filter the leachate was due to the ashed material not being leached at all, causing no silica to be released into solution. Aqua regia showed an average filtration time indicating that the silica was dehydrated to some extent although not to the extent caused by concentrated HCl. The length of time taken to filter the ash leached with concentrated HNO_3 shows that a lot of silicates were released in the leaching process but that the acid was not concentrated enough to dehydrate them. Thus, conc. HCl was the acid of choice in terms of silica dehydration, although it was felt that the AR leachate would not be difficult to filter as some of the silica had been dehydrated.

Table 27: Filtration Time of the Various Leachates

LEACHING AGENT	FILTRATION TIME (min:sec)
10% HNO_3	2:30
50% HNO_3	5:55
Concentrated HNO_3	7:45
Aqua Regia	5:30
Concentrated HCl	3:45

Figure 35 shows the ICPS analyses of the base metal concentrations in the leachate solutions. From Figure 35 it appears that, in contrast to the XRF scan, conc. HNO_3, is able to leach the ash more efficiently than any of the other agents used. The leaching efficiencies of conc. HCl and AR appear to be lower than that of conc. HNO_3, although the amount of base metals leached are similar, at a 5% significance level. Statistically, at a 5% significance level, it was found that all the acid solutions leached similar amounts of base metals from the ash. Thus any of the five agents could be used to leach the base metals from the ashed plant material.

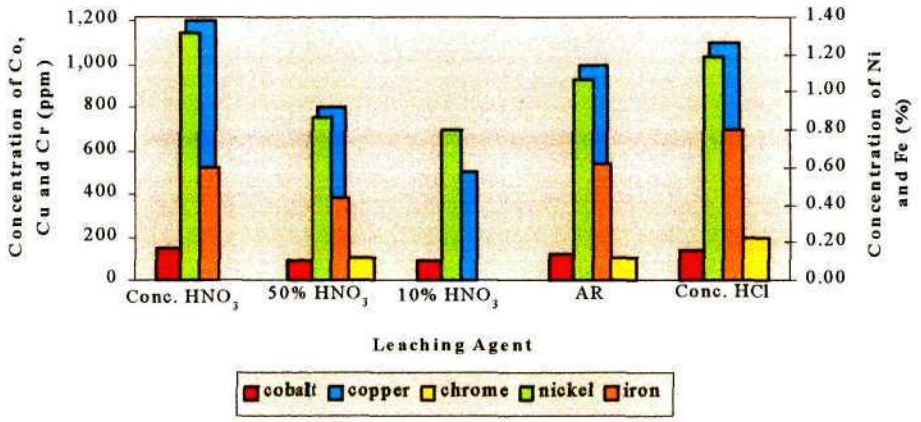


Figure 35: Base Metal Loading in the Various Leachate Solutions

Figure 36 shows the ICPS results of the metal loading on the leached ash. From this graph it is seen that less nickel is found in the ash leached with aqua regia compared with all the other leaching agents. This indicates that the results are slightly inaccurate as, if more nickel was leached into solution by conc. HCl and conc. HNO_3 (Figure 35) then less nickel should be found in these residues. However, statistically all the acids leached the ash to the same extent and thus the slight discrepancies in results are not significant.

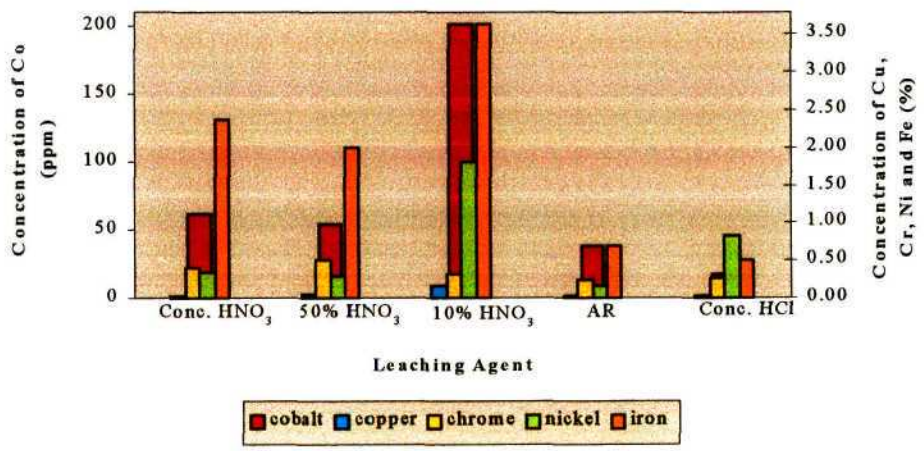


Figure 36: Concentrations of Metals Remaining in the Leached Ash after Solubilisation with the Various Acid Leaching Agents

Figure 37 shows the ICPS results for the base metal concentrations in the hydroxide precipitate. It is interesting to note that there was no significant difference in the concentration of metals in each of the precipitates obtained by neutralising the various acid leachates, except for iron in 10% HNO_3.

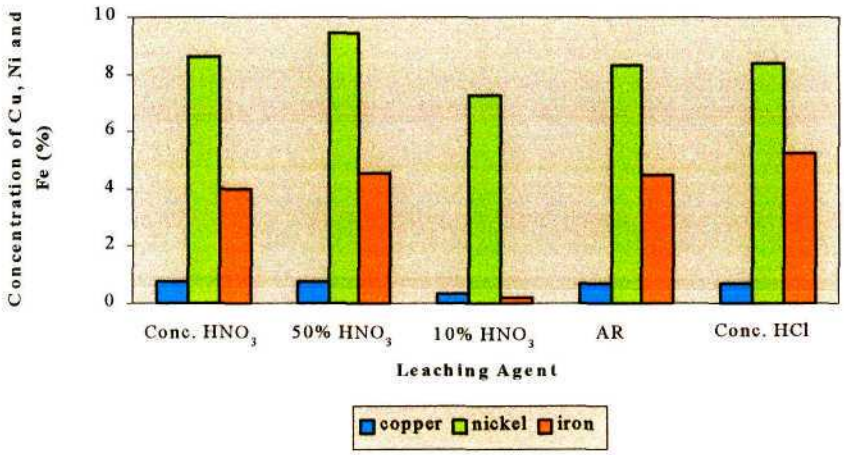


Figure 37: Base Metal Concentrate in the Hydroxide Precipitate Produced after Leaching the Ash

From all the above results it was felt that any of the acids could be used to leach the base metals from the ash, although the results of leaching with 10% HNO_3 were not felt to be reliable enough to use this reagent alone. However, it was decided that aqua regia would be used to extract the base metals from solution as aqua regia is able to quickly oxidise organic material, due to the HNO_3 content, while the strong acidity of the HCl would ensure dehydration of the silica making filtration fairly simple. Concentrated HCl alone could not be used as the extraction system in the laboratory was unable to cope with the large amounts of chlorine gas produced. It was also felt that if only concentrated HNO_3 was used, the amounts of nitrous oxide produced would also be too large for the extraction system to handle. The knowledge gained from the work reported in this section was subsequently applied to the large-scale recovery of metals from the *B. coddii* ash (section 5.3.9).

5.3.9 Large Scale Recovery of Metals from Ashed Plant Material

Using the experimental techniques piloted in the previous section, the base metals were leached out of the ashed plant material. In the trials a 3 : 1 AR : solid ratio was used for leaching (section 5.2.9.3) but in the main leaches it was felt that this amount of acid would take too long to boil down and would put too much pressure on the extraction system. Using this volume of acid would also cause the process to become exceptionally expensive and thus it was decided that a 1 : 1 ratio of AR : solid should be used. This ratio was also used when the leached residue was resuspended and boiled in 10% HNO_3.

The XRF scan of the white precipitate produced when the combined leachates were cooled, after being reduced (section 5.2.10), showed that pure calcium sulphate had been produced. As this substance is fairly insoluble [0.16-0.3 g/100 ml (CRC, 56th Edition)] and easy to filter, most of it was removed. No nickel or other base metals were entrained (glossary) in the precipitate and so it was rinsed and thrown away, allowing a significant amount of the calcium to be removed from the leachate, making the smelting process slightly easier as large amounts of calcium did not have to be removed.

In order that the base-metal-containing hydroxide precipitate, produced by neutralising the acid leachate with sodium hydroxide (section 5.2.10), could be fully smelted to produce metallic nickel, the precipitate was fully analysed by AAS, ICPS and ICPMS (section 2.6). The results of these analyses are given in Table 28. From Table 28 the theoretical mass of nickel metal that could be produced was also calculated. The mass of the hydroxide precipitate, produced from leaching the 14.2 kg of plant ash (generated by ashing 120.8 kg of shredded plant material), was found to be 5402 g. As the precipitate contained 7.25% nickel (Table 28), this meant that 391.6 g of nickel metal could be produced. It is also interesting to note that platinum, palladium and rhodium were contained within the base-metal hydroxide. These metals, as well as copper and zinc were contained in the nickel-button produced from smelting. The iron, aluminium, sodium, potassium, calcium, silicate, chloride, sulphide and hydroxide were lost to the slag phase during the formation of the button; as these are all contaminants of little value the smelting process, and the flux used, is designed to rid the more valuable metal button of these elements.

Table 28: Composition of the Hydroxide Precipitate Formed After Leaching the Plant Ash

ELEMENT	CONCENTRATION
Platinum	17.9 g/t*
Palladium	11.5 g/t
Rhodium	2.0 g/t
Copper	0.65%**
Nickel	7.25%
Iron	4.39%
Sodium	4.86%
Potassium	2.6%
Magnesium	7.12%
Calcium	6.12%
Silicate	0.5%
Aluminium	2.68%
Zinc	0.35%
Chrome	<0.1%
Complexed Hydroxide	13.7%
Chloride	8.57%
Sulphate	15.1%

* g/t = gram of metal per ton hydroxide precipitate.

** % = g metal per 100 g hydroxide precipitate (w/w).

5.3.10 Smelting the Base Metal Matte to Produce Metal Buttons

From the data given in Table 29, section 5.3.9, K Ehlers developed a flux that could be used to smelt the hydroxide precipitate (Ehlers, 1997) thereby forming a nickel button, whilst removing the impurities listed above (section 5.3.9). As the present author knows little about pyrometallurgy and the formation of fluxes to obtain metals from materials, she used the flux developed by Mr K Ehlers without going into the details of why he had used the various quantities of each component. He had sought advice from another party and so was unable to supply all the reasoning for using the flux given in Table 22, section 5.2.11. Initially the smelting tests were performed in a 10 kW induction furnace but due to coil blockage within the furnace, the remaining tests were done in a high temperature muffle furnace.

Once all the hydroxide matte had been smelted, 14 metal buttons were produced, giving a combined mass of 476.01 g, which is greater than the calculated theoretical mass of 391.6 g (section 5.3.9). It is thought that the difference between the theoretical and actual mass can be attributed to the fact that the buttons did not contain pure nickel but were diffused with some impurities such as copper, sulphide, platinum, palladium and zinc. Mr N Plint (ARC) scraped a few grains off the button and analysed them on the Time of Flight Simulated Mass Spectrometer (TOF SIMS), where he confirmed that copper and sulphide were present in the button.

The masses of the buttons produced are given in Table 29. Problems were experienced with the production of buttons 6 and 7 due to the fact that the coil in the induction furnace began to block. This caused the furnace to heat irregularly resulting in some of the base metal being lost to the slag and the lowering of the button masses. The mass of button 1 was proportionately greater than the mass of the other buttons as more iron was incorporated into it. Mr K Ehlers then modified the flux (section 5.2.11, Table 22) in order to reduce the iron content in the buttons. This, therefore, shows that metal buttons can be produced from hyperaccumulator plants, making the phytoremediation process a tangible one as the contaminating metal can be seen by everyone to have been removed from the waste site by *B. coddii*.

The form of the metal buttons is given in Figure 38. Initially, after producing the buttons, the nickel was going to be re-melted and placed in a mould to form two or three ingots, of about 150 g, embossed with the Amplats logo. However, it has since been decided that the buttons will be distributed, to management and various interested parties, in the form given in Figure 39, which explains how the button was formed.

From Figure 38 and Figure 39 it can be seen that phytoremediation on the RBMR sites has been successful as metal buttons were produced allowing the casual observer to have an insight into the power of biological systems. It is with the production of these buttons that Amplats has decided to go ahead with phytoremediation and so is looking at populating other nickel-contaminated sites with *B. coddii*.

Table 29: Masses of the Nickel Buttons Produced after Smelting the Hydroxide Precipitate

TEST	MASS OF BUTTON (g)
1	41.91
2	21.15
3	14.63
4	19.80
5	14.85
6	8.25
7	7.49
8	50.31
9	55.50
10	54.96
11	51.04
12	52.10
13	68.07
14	15.95

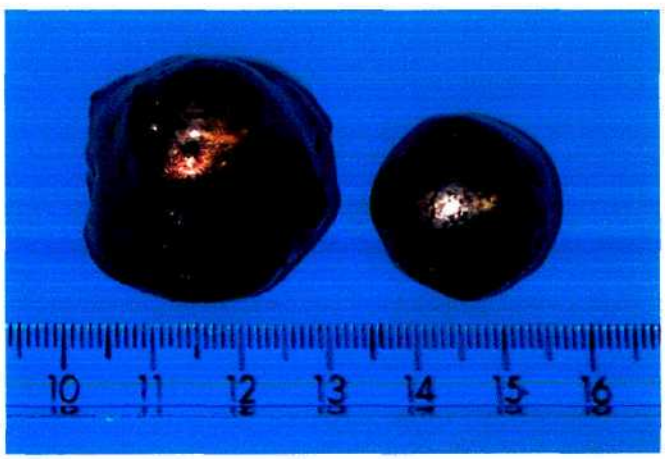


Figure 38: Comparison of the Nickel Buttons Produced - the Larger with the A8 Crucible (left) and the Smaller with the A3 Crucible (right)

Figure 39: Presentation Format for the Nickel Buttons

5.3.11 The Economics of Phytoremediation

The above results show that, with the aid of hyperaccumulators, metals can be removed for commercial gain, allowing some of the costs incurred in setting up the remediation site to be slightly offset. It should, however, be noted that unless the metal has a very high market value, like platinum, the phytoremediation process would generally not be a profitable one. Instead, the process will serve to save a company money and the embarrassment of heavy fines because of pollution of areas. Amplats found that to excavate and remove the contaminated soil from their RBMR site to a landfill would cost in the region of 8 million Rand. However, the landfill site has now been closed and so, in order to remove the metal contamination, Amplats would probably need to remove the soil and chemically leach it to remove the metals. Not only would this process be extremely expensive in terms of excavation and chemicals, but it would also render the soil infertile. This, in turn, would mean that the soil would need to be fertilised and revegetated (adding to the cost of the process), or it would need to again be dumped in a site where eventually, by natural process, it would again become fertile. All this shows that methods, other than biological ones, used in the rehabilitation of contaminated soils are generally vastly expensive, being in the region of millions of Rand. Biological processes, on the other hand, are much less expensive although they tend to take a much longer time.

In terms of using *B. coddii* for the rehabilitation of the Rustenburg waste sites, the greatest expense incurred was/ is labour. Producing and planting the plants is fairly labour intensive but, as this is a "one-off" for each site, the cost can be diluted over the years. Harvesting the seeds and the above-ground biomass is an ongoing labour cost for the duration of the remediation and it is thought that the total labour costs, over a period of 10 years, will be in the region of R1.5 million. Setting up the sites was not all that costly even though irrigation, levelling, ploughing and capping with topsoil was required. The total cost for the preparation of each site was about R200 000.

The final cost associated with phytoremediation is the removal of the metal from the plant material. This project showed that it was not viable to remove the metal from the plant material alone as the labour costs associated with ashing, leaching and smelting are prohibitive, as are the chemical costs incurred with acid leaching and neutralisation. Thus in future, the shredded plant material will be passed directly to the full-scale smelter at RBMR where the plant material will be incorporated into the general process used by Amplats to recover base metals from smelted material. In this way the nickel that is removed from the plants will be incorporated into general metal recovery and so it will not be known exactly how much metal is produced from the plants. For monitoring purposes, however, it will be useful to analyse metal recoveries from the plants on a yearly basis.

The above shows that the costs associated with phytoremediating one-hectare sites with *B. coddii* are in the region of R800 000 per hectare, over a period of about 10 years. With the low nickel price (about US $7.00 per kg in 1998) the monetary gain from phytoremediation is small, but the entire process is economically viable as it is much cheaper than removing the soil and then chemically leaching out the elements. At present it is not known how long it will take to clean up the RBMR sites and so the total costs involved at RBMR cannot be estimated. All that can be said is that the mine is saving a lot of money by embarking on phytoremediation before laws are drawn up that make the removal of metal contamination compulsory.

5.4 *Conclusions*

The results presented in this chapter confirm that *Berkheya coddii* is a highly efficient hyperaccumulator suitable for phytoremediation of waste areas such as those found at RBMR.

Furthermore, the plants were able to adapt to the conditions found in Rustenburg as they grew well throughout the wet season, flowered and produced seeds in their second season and generally followed the growth cycle of the Barberton plants. *B. coddii* was also found to take up significant amounts of nickel from the contaminated site and, as expected, it accumulated more nickel the older it became due to greater adaptation and less competition for nutrients.

In order that the nickel accumulated by the plant could be "seen" it was decided that, for the first season's harvest of above-ground biomass, the nickel should be extracted from the plant and metallic nickel produced. This was done successfully and, from 120.8 kg dry plant material, 476.01 g of metal was produced. However, the ashing and extraction procedures used to remove the base metals from the plant were costly and time consuming and so, in future, it is hoped that a method to feed the shredded plant material into the smelter at Rustenburg can be developed. It is envisaged that the material will need to be pelletised before being fed into the smelter otherwise it will be sucked straight out of the stack. In smelting, the plant material will be combined with concentrates produced from the various Amplats mines and so it will be treated in the "normal" downstream process. From the high value matte produced from the slag the base metals will be removed by solvent extraction and electrowinning.

Even though metals can be obtained from the plants it is not envisaged that phytoremediation will be a profit-making project. Instead, it will be a money-saving project as removing contaminating metals with plants is much more cost-effective than removing and chemically leaching the contaminated soils.

CHAPTER 6

FINAL DISCUSSION

Since the purpose of this project was to determine the feasibility of using *B. coddii* in the phytoremediation of nickel-contaminated sites, all the academic studies reported in this thesis were performed to ensure the successful transferral of *B. coddii* plants from Barberton to the waste sites at RBMR. It was also important to ascertain the ability of *B. coddii* to take-up certain platinum group metals as this would be useful for scavenging such metals from sites in which it is not economical to extract them by conventional processes. It was found that *B. coddii* is indeed able to accumulate platinum, palladium, rhodium and even gold from FIC and, to a certain extent, metallics. As more metals are available to the plants from FIC than from metallics this result is to be expected. The uptake of precious metals by *B. coddii* was not unexpected as it was assumed that the metals were accumulated using the uptake mechanism for nickel. How significant this result is, remains to be seen as no literature could be found on the uptake of precious metals by other nickel hyperaccumulators, but this may be due to the fact that these hyperaccumulators have not been subjected to soils containing precious metals. Kothny (1990) has reported that the concentrations of precious metals within plants tends to be in the parts per billion range, which makes the 3 116 mg/kg platinum, 3 021 mg/kg palladium, 966 mg/kg rhodium and 11 mg/kg gold taken up by *B. coddii* an extremely significant finding, suggesting that more work needs to be done on *B. coddii* to assess its suitability for biomining areas where conventional mining processes are uneconomic.

It would appear that *B. coddii* is able to tolerate and adapt to metals with which it has never been in contact, although this tolerance appears to slightly affect nickel uptake in that, with an increase in uptake of other metals, nickel uptake decreases. This indicates that either a second tolerance mechanism comes into play with the other metals, causing the activity of the nickel uptake mechanism to decrease, or that competition between the other metals and nickel occurs causing less nickel to be accumulated. This theory of competition suggests that there are limited binding sites within the stems and leaves of *B. coddii* and only a set quantity of metal (regardless of what it is) can be accumulated by the plant.

From the results given in section 3.3.1 it could be seen that the uptake of platinum, palladium, rhodium and gold appeared to have very little effect on the growth of *B. coddii* until a certain level of these metals was exceeded. When 60 g FIC was added to *B. coddii* the plant became extremely unhealthy and started to die as a result of the accumulation of toxic levels of platinum, palladium, rhodium and gold. This result, while it is a negative one in terms of phytoremediation, is positive in terms of biomining as in biomining it is not important whether the plant lives or dies, only that the plant accumulates valuable concentrations of metals. Thus this result again shows that further experiments should be performed on *B. coddii* with a view to the biomining of valuable metals that may even be toxic to the plant.

One of the most important aspects in setting up the sites at RBMR was seed germination and seed production as, without seedlings, the sites could not be populated. Analyses of *B. coddii* seeds showed that the plant's reproduction was not affected either by nickel accumulation (regardless of the concentrations of nickel accumulated) or by the uptake of platinum and palladium into the seeds. This means that, regardless of the metals taken up by *B. coddii*, seed production and seed germination are not affected, even though the seeds appear to act as sinks for heavy-metal accumulation.

In order to maximise the recovery of nickel accumulated by *B. coddii* it was important to ascertain where the greatest concentration of the metal occurred and whether the entire plant, or only a portion of the biomass, should be harvested for metal recovery. The results obtained in sections 3.3.1 and 3.3.2, which show that most of the nickel occurs in the leaves of *B. coddii*, agree with those of Morrey *et al.* (1989) and confirm that nickel tends to be stored in the leaves while the stems act only as a transport medium. This result was also in accordance with that of other workers who have found that hyperaccumulators transport the metal from their roots, via the stems, to their leaves where the metal is then stored (Salt *et al.*, 1995). Thus in terms of nickel, as well as platinum, palladium and rhodium recovery, it is essential that the leaves be harvested. Harvesting the stems for metal recovery is a contentious issue. On the one hand it is much cheaper to harvest the entire AGB as it is extremely time-consuming and labour intensive to remove each leaf from the plant. Also, if the entire AGB is harvested more nickel is recovered since 20% of the AGB nickel is in the stems. On the other hand, ashing the stems of *B. coddii* is problematic and also time-consuming, but less nickel is recovered. It is,

therefore, recommended that the decision, on which part of the AGB should be harvested, should be made according to whether facilities are available for ashing the plant material. As no other full-scale phytoremediation process has been reported in the literature, it is not known whether any other process for retrieving accumulated metals from plant biomass has been implemented. Amplats will still harvest the entire AGB as the smelter will be used to ash the plant material in conjunction with the normal smelting of the processed ore. All the smelted material will then be treated in Amplats' downstream processes in which the base metals and precious metals are recovered.

Howes (*pers. comm.*) has proposed that malate might play a role in the detoxification of nickel in *B. coddii*. The results reported in sections 3.3.3 and 3.3.4 constitute further evidence that malate does indeed play a role in the storage of nickel in the plant. The results showed that there is more free L-malic acid in the leaves than in the stems of the plant, and the concentration of free L-malic acid in the leaves appears to be proportional to the concentration of nickel in the leaves. There is no such correlation in the stems suggesting that no nickel storage occurs in the stems. However, the fact that a significant amount of free L-malic acid was found in the stems suggests that this organic acid may not only play a role in nickel storage but also in nickel transport. It should be noted that the above conclusions are based on the assumption that the extraction procedure causes any bound malate to become unbound to form the free L-malic acid isomer.

The concentrations of free L-malic acid found in *B. coddii* appeared to depend on the levels of plant-available nickel found in the soil on which the plant was growing. When little or no plant-available nickel is found in the soil, very little free L-malic occurs in the plant. However, as soon as plant-available nickel is found in the soil the levels of free L-malic acid increase within the plant. This suggests that there may be a response mechanism present within *B. coddii* that is switched on in the presence of plant-available metals, which produces a set amount of L-malic acid. This would explain why, if it is assumed that only free L-malic acid was assayed and that the bound malate did not become unbound in the extraction procedure, the plants in RBMR soil, which has less plant-available nickel than serpentine soil, have greater free L-malic acid levels than the plants growing in serpentine soil. As the above two explanations contradict one another more work needs to be done to establish whether the analyses for L-malic acid

included nickel-bound malate and whether the levels of D-malate were insignificant. It was interesting to note that the free L-malic acid levels observed in the leaves of *B. coddii* plants growing on serpentine soil (4.3 mg/kg dry mass) were significantly higher than those seen in an unknown *Berkheya* species growing on non-serpentine soil (1.95 mg/kg dry mass). These results again suggest that malate is indeed involved in metal storage in *B. coddii*. These findings agree with results obtained by Lee *et al.* (1978) and Pelosi *et al.* (1976) who found that hyperaccumulators that use organic acids in their metal storage mechanisms, tend to contain the particular organic acid in concentrations an order of magnitude higher, compared with non-accumulating plants.

Before *B. coddii* could be planted at the RBMR sites it was important to compare the climatic and soil conditions of Barberton and Rustenburg. From the comparisons (sections 4.2.1 and 4.2.2) it was established that the Rustenburg climate, although drier than Barberton, was similar in terms of temperature and therefore it was felt that the climate would have little effect on the plant. This proved to be true since *B. coddii* adapted very well to the dryness of Rustenburg. Furthermore it was found that hail, while damaging the plants, did not destroy them and they were able to recover completely. Thus *B. coddii* was found to be extremely tough and very adaptable and it appeared that its growth-cycle (section 4.2.3) remained similar to that of the plants found in Barberton. *B. coddii* also easily adapted to the soil conditions found at Rustenburg and very few plants were lost during the trials (Chapter 5). While it may appear that *B. coddii* was able to adapt to the Rustenburg conditions as they are fairly similar to Barberton, it is in fact felt that the plant is fairly insensitive to climatic conditions as *B. coddii* has been established on a serpentine area in New Zealand (Anderson, 1998). As the climate in New Zealand is colder and wetter than Barberton it is felt that the reason for *B. coddii*'s confinement to serpentine areas is purely due to the fact that it is a non-competitive, slow-growing plant and so is unable to invade surrounding non-serpentine areas. Work will continue with *B. coddii*, at Massey University, to establish whether or not it can be used in phytoremediation on a more worldwide scale.

The results in section 4.3.4.1 showed that, once in tissue culture, fully formed *B. coddii* explants could be generated with little difficulty by direct organogenesis, and that the plant population could be doubled every three weeks. Root and shoot initiation also occurred in the

plants without the addition of auxin and cytokinin, respectively, although, as root formation was fairly slow. In order to speed up root production, explants could be grown on a nutrient deficient medium. The major problems experienced with putting *B. coddii* into culture were the low seed germination rate (section 4.3.4.2) and the high levels of fungal and bacterial contamination of the few seedlings produced. Since the parachute-like structure of the seeds may have caused the contamination and sterilisation problems, removal of the "parachute" may resolve this issue in the future.

Although successful, it was felt that producing large numbers of plants, for phytoremediation, by direct organogenesis would be both costly and time consuming. Thus other methods of producing plants were examined. In this regard it was found that seedlings could be produced successfully by germinating seeds on moist cotton wool, in a warm environment (about 25°C during the day). However, for phytoremediation purposes this process was both tedious and time-consuming, as the tiny seedlings needed to be transplanted into soil about two weeks after germination. The most efficient means of producing the quantities of plants required for the test sites at RBMR was to germinate the *B. coddii* seeds in a 10% zeolite, 10% serpentine soil, 10% RBMR soil, 40% potting bark and 30% vermiculite mix (section 5.3.2). The germination rate of the seeds was enhanced when only the large seeds were sown into this germination mix, as these seeds contained more viable embryos than the smaller seeds produced by *B. coddii*. As no literature can be found for the propagation of large numbers of hyperaccumulators it is thought that the zeolite mix, developed by A. Howes, should continue to be used for the germination of the large *B. coddii* seeds.

In order to ensure maximum biomass gain and nickel uptake in the *B. coddii* plants growing on the remediation sites at RBMR, it was important to establish the effect of the addition of various nutrients to the plants. The results given in section 4.3.5.1 were not conclusive as the trials were performed on serpentine soil and not the RBMR soil, and there was no control plant with which to compare the nutrient effects. However, the results did indicate that the addition of 250 mg/l nitrogen enhanced both biomass production and nickel uptake in serpentine and, to achieve similar amounts of nitrogen in RBMR soil (from Table 18), this would equate to an addition of 1 210 mg/l nitrogen. The results given in section 4.3.5.1 also show that the addition of phosphorus at 600 mg/l enhances growth and nickel uptake in *B. coddii* growing in

serpentine soils and, as RBMR soil contains the same amount of phosphorus (from Table 18), it is assumed that the addition of 600 mg/l phosphorus to RBMR soil would enhance phytoremediation. The addition of 2 000 mg/l calcium was also found to have a positive effect on *B. coddii* plants growing in serpentine soil and, as approximately the same amount of calcium is found in RBMR soil (Table 19), it is felt that the addition of 2 000 mg/l calcium would enhance the growth and nickel uptake in the plants growing on the RBMR sites. Potassium at a level of 600 mg/l should also be added to the plants growing at the RBMR site as these levels were observed to optimise plant growth in serpentine soils, which contain similar potassium levels as RBMR soil (Table 19). Lastly it was found that magnesium, at 250 mg/l was required by plants growing on serpentine soils. This amount could be decreased by 15 mg/l for plants growing on the RBMR waste sites as this soil contains more magnesium than serpentine soil (Table 19). These findings, however, do not take into account the interactive effects of all the nutrients and thus further experiments to determine the additive effects on *B. coddii* plants growing in RBMR soil need to be established. The findings also do not show the effect of nutrients on the biomass of *B. coddii* plants and thus, in future nutrient experiments, control plants, to which no nutrients are added, need to be grown.

Finally, from all the results given in Chapter 5 it can be seen that *B. coddii* is a very useful tool in the phytoremediation of nickel-contaminated soils at RBMR. No problems were experienced in establishing the plants on the Rustenburg sites although the plants grew more vigorously in scarified soil, compared with compacted soil, as the roots were better able to penetrate the soil, allowing for better nutrient acquisition. The addition of a multi-purpose fertiliser like 2:3:2 (N:P:K) enhanced initial plant growth and allowed the plants to establish themselves in the new soils. The climate, as mentioned, had no negative effect on the plants and their growth-cycle followed that observed in the plants endemic to the serpentine soil of Barberton. Seeds were collected from the plants that flowered and these seeds were then used to populate other areas at RBMR the following season. Initially the germination rate of these seeds was found to be low and this was due to the fact that the vectors used in pollination had been destroyed by spraying (section 5.3.4) for apparent pests to *B. coddii*. Thus, in the second season it was decided that no insecticides should be used and instead all the grass surrounding the *B. coddii* plants and harbouring the pests, was removed. The vectors were therefore not destroyed and

the germination rate from the seeds collected in the second season improved dramatically, to be in line with that observed for the plants growing in Barberton.

Like the *B. coddii* plants growing in serpentine soil, the RBMR plants also accumulated the plant-available nickel present in the soil although, as there was less plant-available nickel compared with serpentine, less nickel was accumulated in these plants. As expected, the greater the biomass of the plants the more nickel was accumulated within the leaves of the plants and so, as the plants were well established in the second season, more nickel was accumulated in second-season plants than in first-season plants.

At the end of the growing season after the seeds had been collected (section 5.3.5), the plants were allowed to dry out and then the AGB was harvested by hand (section 5.3.6). This material was sun-dried and then ashed (section 5.3.7) in order that the accumulated nickel could be recovered. After completely ashing the material the accumulated metals were acid-leached (section 5.3.9) and then precipitated out of solution by neutralisation. This precipitate was then smelted with a flux (section 5.3.10), and nickel metal was obtained. An economic study was performed to determine the feasibility of recovering the nickel metal from the plants and it was conclusively found that the only way in which the nickel could feasibly be recovered was to smelt the dry AGB in the smelter at Amplats and recover the nickel using the downstream processes set up by Amplats to recover the base metals contained in the mined sulphide ores.

From the above it can be seen that *B. coddii* has been extremely successful in the remediation of nickel-contaminated soils at RBMR. As mentioned, economically it is not feasible to separately recover the nickel accumulated by the plant but rather to combine recovery with that already designed to produce base metals. However, even if the metal cannot be recovered for economic purposes, its removal from the soil is enough to stop a large amount of money being spent on the removal of the metal by more conventional methods. Once in the plant biomass the metal can be contained in a waste site or removed to a facility where it can easily be recovered.

At present it appears that this is the first commercial phytoremediation project in operation and thus it is impossible to compare the success of this project with others. It does, however, seem

that this phytoremediation project, which uses *B. coddii* as a tool, is extremely successful and that the actual levels of nickel recovery (1 362 g Ni per hectare) are comparable with the theoretical ones (1 457 g Ni per hectare), calculated before the project commenced. Perhaps, by increasing the density of the plants on the RBMR sites in future years the nickel recovery can be increased and the time taken to complete remediation of the sites decreased from about 13 years to ten years. Thus, in conclusion, the success of this project has convinced Amplats as to the value of continued research into phytoremediation and biomining, processes that show great potential for cleaning up heavy metal waste areas, both locally and in mining areas occurring world-wide.

REFERENCE LIST

AECI. (1978). *Useful Technical Data* pp: 27. Chemicals Division, Jhb, RSA.

Allen, E. (1991). "Plant Cell Culture". *Plant Cell and Tissue Culture* (Eds: Stafford, A and Warren, G) pp: 1-24. Open University Press, Redwood Press, Wiltshire, UK.

Anderson, CWN. (1998). *Personal Communication*. PhD student, Massey University, New Zealand.

Anderson, TR; Howes, AW; Slatter, KA and Dutton, MF. (1997). "Studies on the Nickel Hyperaccumulator, *Berkheya coddii*". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 261-266. Orstom, Nouvelle-Calédonie.

Andrews, DH and Kokes, RJ. (1962). "Fundamental Chemistry". John Wiley and Sons, USA.

Anhaeusser, CR. (1985). "Archaen Layered Ultramafic Complexes in the Barberton Mountain Land, South Africa". *Evolution of Archaen Supracrustal Sequences* (Eds. Ayres, LD; Thurston, PC; Card, KD and Weber, W) pp:281-301. Geological Association of Canada.

Antonovics, J; Bradshaw, AD and Turner, RG. (1971). "Heavy Metal Tolerance in Plants". *Advances in Ecological Research (7)* (Ed: Cragg, JB): 1-85. Academic Press, London.

Anonymous. (1997). "The Wildlife Habitats of IBM Almaden Research Centre". Taken from an Internet search of "serpentine soil" in Altavista.

Baker, A; Brooks, R and Reeves, R. (1988). "Growing for Gold....and Copper....and Zinc". *New Scientist*, **10 March 1988**: 44 - 48.

Baker, AJM and Brooks, RR. (1989). "Terrestrial Higher Plants which Hyperaccumulate Metallic Elements - A Review of their Distribution, Ecology and Phytochemistry". *Biorecovery* **1:** 81 - 126.

Baker, AJM. (1981). "Accumulators and Excluders – Strategies in the Response of Plants to Heavy Metals". *Journal of Plant Nutrition* **3:** 643-654.

Baker, AJM. (1987). "Metal Tolerance". *New Phytol.* **106:** 93-111.

Baker, AJM; McGrath, SP; Sidoli, CMD and Reeves, RD. (1995). "The Potential for Heavy Metal Decontamination". *Mining Environmental Management* **3 (3):** 12-14.

Baker, AJM; Reeves, RD and Hajar, SM. (1994). "Heavy Metal Accumulation and Tolerance in British Populations of the Metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). *New Phytol.* **127:** 61 - 68.

Balkwill, K; Williamson, SD; Kidger, CL; Robinson, ER; Stalmans, M and Balkwill, J.-M. (1997). "Diversity and Conservation of Serpentine Sites in Southern Mpumalanga (Eastern Transvaal), South Africa". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 133-138. Orstom, Nouvelle-Calédonie.

Barnard, RO; Buys, AJ; Coetzee, JGK; du Preez, CC; Meyer, JH; van der Merwe, AJ; van Vuuren, JAJ and Volschenk, JE. (1990). Handbook of Standard Soil testing Methods for Advisory Purposes. Compiled by "The Non-Affiliated Soil Analysis Work Committee". Soil Science Society of South Africa, Pretoria.

Blincoe, C; Theisen, MO and Stoddard-Gilbert, K. (1987). "Sample Oxidation Procedures for the Determination of Chromium and Nickel in Biological Material". *Commun. In Soil Sci. Plant Anal.* **18(6):** 687-697.

Boehringer Mannheim. (1992). "L-Malic acid". Biochemical Analysis. Cat. No. 139 068.

Booth, RG; Cox, ML and Madge, RB. (1990). "3. Coleoptera". *IIE Guides to Insects of Importance to Man* pp: 71-72. The University Press, Cambridge.

Boyd, RS and Martens, SN. (1991). "The Raison d'être for Metal Hyperaccumulation by Plants" *The Vegetation of Ultramafic (Serpentine) Soils'* (Eds: Baker, AJM; Proctor, J and Reeves, RD) pp: 279-299. Intercept, Andover, UK.

Bradshaw, AD. (1997). "Restoration After Mining for Metals – An Ecological View". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 239-248. Orstom, Nouvelle-Calédonie.

Brooks, RR. (1987). "Serpentine and its Vegetation - A Multidisciplinary Approach". Discordies Press, 9999 SW Wilshire, Portland, Oregon 97225.

Brooks, RR; Morrison, RS; Reeves, RD; Dudley, TR and Akman, Y. (1979). "Hyperaccumulation of Nickel by *Alyssum* Linnaeus (Cruciferae)". Proc. R. Soc. Lond. B. **203 :** 387 – 304.

Brooks, RR; Reeves, RD; Baker, AJM; Rizzo, JA and Ferreira, HD. (1990). "The Brazilian Serpentine Plant Expedition (BRASPEX), 1988". *National Geographic Research* **6(2):** 205-219.

Brown, PH; Welch, RM and Cary, EE. (1987). "Nickel: A Micronutrient Essential for Higher Plants". *Plant Physiol.* **85:** 801-803.

Carr-Brion, KG and Payne, KW. (1970). "X-ray Fluorescence Analysis". *Analyst* **95(1137):** 977-989.

Chaney, R; Brown, S; Li, Y-M; Angle, JS; Homer, F and Green, C. (1995). "Potential Use of Metal Hyperaccumulators". *Mining Environmental Management* **September:** 9-11.

Chapin, FS (III). (1980). "The Mineral Nutrition of Wild Plants". *Ann. Rev. Ecol. Syst.* **11:** 233-260.

CRC Handbook of Chemistry and Physics, 56th Edition. (1975 – 1976). Chemical Rubber Publishing Company, USA. © 1922 – 1956 and 1957 – 1962.

Crowley, DE; Wang, YC; Reid, CPP and Szaniszlo, PJ. (1991). "Mechanisms of Iron Acquisition from Siderophores by Microorganisms and Plants". *Plant and Soil* **130:** 179-198.

Dakshini, KMM; Roonwal, GS and Gupta, SK. (1982). "Heavy Metal Accumulation by *Vernonia cinerea* (L.) LESS. Asteraceae". J. Geochemical Exploration **16 :** 235 - 238.

De Plaen, G; Malaisse, F and Brooks, RR. (1982). "The 'Copper Flowers' of Central Africa and their Significance for Prospecting and Archaeology". *Endeavour, New Series* **6(2):** 72-77.

Dighton, J; Mason, PA and Poskitt, JM. (1990). "Field Use of ^{32}P Tracer to Measure Phosphate Uptake by Birch Mycorrhizas". Hard copy of paper submitted to *New Phytologist*, 1990.

Dixon, RA. (1985). "Isolation and Maintenance of Callus and Cell Suspension Cultures". *Plant Cell Culture: A Practical Approach* (Ed. Dixon, RA) pp: 1-21. IRL Press Ltd, England.

Dyer, A. (1974). *An Introduction to Liquid Scintillation Counting* pp: 1-24 and 43-84. Heyden and Son Ltd, London.

Eberl, DD. (1998). "Controlled-Release Fertilisers Using Zeolite". 3215 Marine Street, Boulder, Colorado, 80303. http://www.usgs.gov?tech-transfer/factsheets/94-066b.html

Ehlers, K. (1997). "Biomass Smelting Tests". *ARC Memorandum 3 December, 1997.*

Ernst, W. (1972). "Ecophysiological Studies on Heavy Metal Plants in South Central Africa". *Kirkia* **8:** 125 - 145.

Ernst, WHO; Verkleij, JAC and Schat, H. (1992). "Metal Tolerance in Plants". *Acta. Bot. Neerl.* **41:** 229-248.

Eskew, D; Welch, RM and Norvell, WA. (1984). "Nickel in Higher Plants: Further Evidence for an Essential Role". *Plant Physiol.* **76:** 691-693.

Gabbrielli, R and Pandolfini, T. (1984). "Effect of Mg^{2+} and Ca^{2+} on the Response to Nickel Toxicity in a Serpentine Endemic and Nickel Accumulating Species". *Physiol. Plant* **62:** 540-544.

Gabbrielli, R; Gremigni, P; Bonzi Morassi, L; Pandolfini, T and Medeghini Bonatti, P. (1997). "Some Aspects of Ni Tolerance in *Alyssum bertolonii* Desv.: Strategies of metal Distribution and Accumulation". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 225-227. Orstom, Nouvelle-Calédonie.

Gabbrielli, R; Mattioni, C and Vergano, O. (1991). "Accumulation Mechanisms and Heavy Metal Tolerance of a Nickel Hyperaccumulator". *Journal of Plant Nutrition* **14(10):** 1081-1090.

Gabbrielli, R; Pandolfini, T; Vergnano, O and Palandri, MR. (1990). "Comparison of Two Serpentine Species with Different Nickel Tolerance Strategies". *Plant and Soil* **122:** 271-277.

Gill, RJ. (1990). "The Morphology of Whiteflies". *Whiteflies: Their Bionomics, Pest Status and Management* (Ed: Gerling, D) pp: 14-27. Intercept Ltd, UK.

Gonçalves, SC; Gonçalves, MT; Freitas, H and Martins-Loução, MA. (1997). "Mycorrhizae in a Portugese Serpentine Community". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 87-89. Orstom, Nouvelle-Calédonie.

Green, R; Govindaraju, RS; Erikson, LE and Roig, L. (1996). "Effect of Vegetation on Contaminant Transport in Surface Flows". Abstract from the *HSRC/ WERC Joint Conference on the Environment, 1996.*

Grill, E; Löffler, S; Winnacker, E-L and Zenk, MH. (1989). "Phytochelatins, the Heavy-Metal-Binding Peptides of Plants, are Synthesized from Glutathione by a Specific γ-Glutamylcysteine Dipeptidyl Transpeptidase (Phytochelatin Synthase)". *Proc. Natl. Acad. Sci. USA* **86:** 6838-6842.

Grill, E; Winnacker, EL and Zenck, MH. (1985). "Phytochelatins: The Principal Heavy-Metal Complexing Agents of Higher Plants". *Science* **230:** 674-675.

Haque, I; Aduayi, EA and Sibanda, S. (1993). "Copper in Soils, Plants and Ruminant Animal Nutrition with Special Reference to Sub-Saharan Africa". *J. Plant Nutrition* **16 (11):** 2149-2212.

Hartmann, HT; Kester, DA and Davies, FT (Jr.). (1990). "Plant Propagation – Principals and Practices" (5th Edition) pp: 230-231 and 459-495. Prentice-Hall Internataional Inc; USA.

Hausinger, PR. (1993). "Plant Nickel Metabolism". *Biochemistry of Nickel* (Ed: E Frieden) pp:203-219. Plenum Press, New York.

Homer, FA; Reeves, RD; Brooks, RR and Baker, AJM. (1991). "Characterisation of the Nickel-Rich Extract from the Nickel Hyperaccumulator *Dichapetalum geloniodes*". *Phytochemistry* **30** (7): 2141-2145.

Howes, AH. (1990). "Investigations into the Nickel Hyperaccumulating Species *Berkheya coddii*". Masters thesis, Natal University, Pietermaritzburg, RSA.

Joughin, J. (1992). "Report on the Uptake of Nickel by *Berkheya coddii* from Nickeliferous Substrates other than Serpentine Soil". *SRK Report to JCI*. Report Number 186456/3. August 1992.

Kinnersley, AM. (1993). "The Role of Phytochelates in Plant Growth and Productivity". *Plant Growth Regulation* **12:** 207-218.

Knoll, C. (1997). "Rehabilitation with Vetiver". *African Mining* **2(2):** 43.

Kothny, EL. (1990). "The Environmental Geochemistry and Biogeochemistry of Palladium". *Nobel Metals and Biological Systems* pp 129 – 157.

Krieg, NR and Holt, JG (Eds). (1984). *Bergey's Manual of Systemic Bacteriology Vol. 1* pp: 244-254. Williams and Wilkins, Baltimore/ London.

Kruckeberg, AR. (1954). "The Ecology of Serpentine Soils. III Plant Species in Relation to Serpentine Soils". *Ecology* **35:** 267 - 274.

Kuyucak, N and Volesky, B. (1988). "A Method of Metal Removal". *Water Pollution Res. J. Canada* **23 (3):** 424-433.

Lancini, G and Parenti, F. (1982). "Antibiotics: An Integrated View". Springer-Verlag, New York.

Lee, J; Reeves, RD; Brooks, RR and Jaffré, T. (1978). "The Relationship Between Nickel and Citric Acid in Some Nickel Accumulating Plants". *Phytochemistry* **17:** 1033-1035.

Levinson, AA. (1974). "Vegetation Surveys". *Introduction to Exploration Geochemistry*: 383-408. Photopress Inc. Broadview Illinois.

Mabberley, DJ. (1987). "The Plant Book". Cambridge University Press, Avon, UK.

Macnair, MR. (1993). "Tansley Review No. 49: The Genetics of Metal Tolerance in Vascular Plants". *New Phytol.* **124:** 541-559.

Marschner, H. (1986). "Mineral Nutrition of Higher Plants". Academic Press, Harcourt Brace Jovanovich, Publishers, London.

McGrath, SP; Sidoli, CM; Baker, AJM and Reeves, RD. (1993). "Plants Clean Up Soils". Paper presented at the Baas "Science for Life" Meeting.

Medina, E; Cuevas, E; Figueroa, J and Lugo, AE. (1994). "Mineral Content of Leaves from Trees Growing on Serpentine Soils under Contrasting Rainfall Regimes in Puerto Rico". *Plant and Soil*: 13-21. Kluwer Academic Publishers, Netherlands.

Meharg, AA. (1993). "The Role of the Plasmalemma in Metal Tolerance in Angiosperms". *Physiologia Plantarum* **88:** 191-198.

Meney, K. (1997). Regeneration Technology Pty Ltd. Perth, Australia.

Mercky, S; Dulieu, D; Pineau, R; Rinaudo, G and Sarrailh, J-M. (1997). "Study of Diazotrophic Bacteria in the Rhizosphere of Poaceae, Leguminoseae and Cyperaceae for the Purpose of Rehabilitating Mining Sites (New Caledonia). *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 91-92. Orstom, Nouvelle-Calédonie.

Mesjasz-Przybylowicz, J; Przybylowicz, WJ and Prozesky, VM. (1997). "Nuclear Microprobe Investigation of Nickel Distribution in Organs and cells of Hyperaccumulating Plants". *The Ecology of Ultramafic and Metalliferous Areas. Proceedings of the Second International Conference on Serpentine Ecology* (Eds: Jaffré, T; Reeves, RD and Becquer, T) pp 223-224. Orstom, Nouvelle-Calédonie.

Modak, JN and Natajaran, KA. (1996). "Biosorption of Metals Using Non-Living Biomass – A Review". *Minerals and Metallurgical Processing* **November:** 189-196.

Morrey, DR; Balkwill, K and Balkwill, M-J. (1989). "Studies on Serpentine Flora: Preliminary Analyses of Soils and Vegetation Associated with Serpentinite Rock Formations in the South-Eastern Transvaal". *S. Afr. J. Bot.* **55 (2):** 171-177.

Neumann, D; Lichtenberger, O; Günther, D; Tschiersch, K and Nover, L. (1994). "Heat-Shock Proteins Induce Heavy Metal Tolerance in Higher Plants". *Plant* **194:** 360-367.

Neumann, PM and Chamel, A. (1986). "Comparative Phloem Mobility of Nickel in Nonsenescent Plants". *Plant Physiol.* **81:** 689-691.

Nicks, LJ and Chambers, MF. (1995). "Farming for Metals?". *Mining Environmental Management* **September:** 15-18.

Northington, DK and Goodin, JR. (1984). "The Botanical World". Times Mirror/ Mosby College Publishing, St Louis, Missouri, USA.

Ogoleva, VP and Cherdakova, LN. (1981). "Patterns of Distribution of Nickel in Plants of Volgorad Oblast". Translated from *Agrokhimiya* **12:** 90-92.

Pelosi, P; Fiorentini, R and Galoppini, C. (1976). "The Nature of Nickel Compounds in *Alyssum bertolonii* Desv. II". *Agric. Biol. Chem.* **40:** 1641-1642.

Peterson, PJ. (1979). "Geochemistry and Ecology". *Phil. Trans. R. Soc. Lond.* B **288:** 169-177.

Philips Atlas of the World and Southern Africa. (1986) Published by Jonathan Ball Publishers, London.

Proctor, J and Woodell, SRJ. (1975). "The Ecology of Serpentine Soils". *Advances in Ecological Research (9)* (Ed: MacFadyen, A): 255 - 359. Academic Press, London.

Proctor, J. (1971a). "The Plant Ecology of Serpentine. II Plant Response to Serpentine Soils". *J. Ecol.* **59:** 397-410.

Raskin, I; Kumar, NPBA; Dushenkov, S and Salt, DE. (1994). "Bioconcentration of Heavy Metals by Plants". *Current Opinion in Biotechnology* **5:** 285-290.

Rauser, WE. (1990). "Phytochelatins". *Annu. Rev. Biochem.* **59:** 61-86.

Reeves, RD and Baker, AJM. (1984). "Studies on Metal Uptake by Plants from Serpentine and Non-Serpentine Populations of *Thlaspi goesingense* Halacsy (Cruciferae)". New Phytol. **98:** 191-204.

Reeves, RD; Baker, AJM and Brooks, RR. (1995). "Abnormal Accumulation of Trace Metals by Plants". *Mining Environmental Management* **3 (3):** 4-8.

Reinecke, P. (1998). Chief Metallurgist at Amplats Research Centre.

Roessler, H. (1959). "*Berkheya coddii* Roessler, spec. nova". *Mitt. Bot. Staats. Mün.* **3:** 231-232.

Salt, DE; Blaylock, M; Kumar, NPBA; Dushenkov, V; Ensley, BD; Chet, I and Raskin, I. (1995). "Phytoremediation: A Novel Strategy for the Removal of Toxic Metals from the Environment Using Plants". *Bio/Technology* **13:** 468-474.

Schlegal, HG. (1988). "General Microbiology" pp. 44. University Press, Cambridge.

Schlegal, HG; Cosson, J-P and Baker, AJM. (1991). "Nickel-Hyperaccumulating Plants Provide a Niche for Nickel Resistant Bacteria". *Bot. Acta* **104:** 18-25.

Schneider, IAH; Rubio, J; Misra, M and Smith, RW. (1995). "*Eichhornia crassipes* as Biosorbent for Heavy Metal Ions". *Minerals Engineering* **8(9):** 979-988.

Skoog, DA and Leary, JJ. (1992). "Principles of Instrumental Analysis" (4th Edition). Harcourt Brace College Publishers, USA.

Skoog, DA; West, DM and Holler, FJ. (1992). "Fundamentals of Analytical Chemistry". (6th Edition). Saunders College Publishing, Fort Worth, USA.

Steffens, JC. (1990). "The Heavy Metal Binding Peptides of Plants". *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41:** 553-575.

Steyn, CE; Lazenby, E; Smith, HJC and Loock, AH. (1995). "Biomass Project Pot Trial: The Influence of Soil Cu, Ni, Fe, Na, P, K, SO_4, Co and Pb Uptake of *Berkheya coddii*". Report: Amplats 1995-06-29. Institute of Soil, Climate and Water, Private Bag X79, Pretoria, 0001, RSA.

Taylor, GJ and Crowder, AA. (1983). "Uptake and Accumulation of Copper, Nickel and Iron by *Typha latifola* Grown in Solution Culture". *Can. J. Bot.* **61:** 1825-1830.

Taylor, GJ. (1987). "Exclusion of Metals from the Symplasm: A Possible Mechanism of Metal Tolerance in Higher Plants". *Journal of Plant Nutrition* **10 (9-16):** 1213-1222.

Taylor, RW; Ibeabuchi, IO; Sistani, KR and Shuford, JW. (1993). "Heavy Metal Concentration in Forage Grasses and Extractability from Some Acid mine Spoils". *Water, Air and Soil Pollution* **68:** 363-372.

Thompson, M and Walsh, JN. (1983). A Handbook of Inductively Coupled Plasma Spectrometry. Blackie, Glasgow and London.

Thurman, D and Hardwick, K. (1988). "How Plants Survive an Overdose of Metal". *New Scientist*, **10 March 1988:** 44 - 45.

Thurman, DA and Collins, JC. (1983). "Metal Tolerance Mechanisms in Higher Plants". *Proc. Int. Conf. Heavy Metals in the Environment*. Heidelburg; 298.

Tomsett, AB and Thurman, DA. (1988). "Molecular Biology of Metal Tolerances of Plants". *Plant, Cell and Environment* **11:** 383-394.

Uren, NC. (1992). "Forms, Reactions, and Availability of Nickel in Soils". *Advances in Agronomy* **48:** 141-203.

Vaquez, MD; Barcelo, J; Poschenrieder, Ch; Madico, J; Hatton, P; Baker, AJM and Cope, GH. (1992). "Localisation of Zinc and Cadmium in *Thlaspi caerulescens* (Brassicaceae), a Metallophyte that can Hyperaccumulate both Metals. *J. Plant Physiol.* **140:** 350 -355.

VarianTechtron. (1979). Analytical Methods for Flame Photometry pp: 35. Varian Techtron, Springvale.

VG PlasmaQuad. (1989). System Manual Version 2b pp: 7. VG Elemental, Cheshire, England.

Walker, CD and Welch, RM. (1987). "Low Molecular Weight Complexes of Zinc and Other Trace Metals in Lettuce Leaf". *J. Agric. Food Chem.* **35:** 721-727.

Walker, RB. (1954). "The Ecology of Serpentine Soils". *Ecology* **35(2):**259-266.

Warren, G. (1991). "The Regeneration of Plants from Cultured Cells and Tissues". *Plant Cell and Tissue Culture* (Eds: Stafford, A and Warren, G) pp: 83-100. Open University Press, Redwood Press, Wiltshire, UK.

Wild, H and Bradshaw, AD. (1977). "The Evolutionary Effects of Metalliferous and Other Anomalous Soils in South Central Africa". *Evolution* **31:** 282 - 293.

Wild, H. (1975). "A Survey of the Effects of Heavy Metals and Other Abnormally Mineralised Soils on Vegetation in Rhodesia". *The Rhodesian Science News* **9 (3):** 74 – 79.

Woolard, C. (1997). *Personal communication*. Environmental Manager – Nickel. Western Mining Company, Western Australia.

Woolhouse, HW. (1983). "Toxicity and Tolerance in the Responses of Plants to Metals". *Encyclopaedia of Plant Physiology*, New Series Vol. 12C (Ed. OL Lange; PS Nobel; CB Osmond and H Ziegler) pp. 245-300. Springer-Verlag, Berlin.

Wuth, MG and Barnes, J. (1986). "Onverwacht Lithologies and Gold Occurences of the Oorschot-Weltevreden Schist Belt". *Geocongress '86* (Ed. Pearton, TN) pp: 66-68. Geological Society of South Africa, Johannesburg.

Yang, XH; Brooks, RR; Jaffre, T and Lee, J. (1985). "Elemental Levels and Relationships in the Flacourtiaceae of New Caledonia and their Significance for the Evaluation of the 'Serpentine Problem'". *Plant and Soil* **87:** 281 – 291.

APPENDIX 1

Species of Plant Hyperaccumulators

Table 30: Hyperaccumulators of Copper and Cobalt (> 1 000 µg/g)

(Baker and Brooks, 1989)

SPECIES	CONCENTRATION (µg/g)		SPECIES	CONCENTRATION (µg/g)	
	Copper	Cobalt		Copper	Cobalt
Aeollanthus biformifolus (LABIATEAE)	3 920	2 820	*Haumaniastrum katangense*	8 356	2 240
Aeollanthus saxitilis	-	1 000	*Haumaniastrum robertii*	2 070	10 200
Alectra sessiliflora var. *sessiliflora*	-	2 782	*Hibiscus rhodanthus*	-	1 527
Alectra sessiliflora var. *senegalensis*	1 590	-	*Icomum tuberculatum*	-	1 429
Alectra welwitschii	-	1 561	*Ipomoea alpina* (CONVOLVULACEAE)	12 300	-
Anisoppapus davyi	2 889	2 650	*Lindernia damblonii*	-	1 113
Anisoppapus hoffmanianus	1 065	-	*Lindernia perennis*	9 322	2 300
Ascolepis metallorum	1 200	-	*Monadenium cupricola*	-	1 234
Becium aureoviride subsp. *lupotoense*	1 135	-	*Pandiaka metallorum* (AMARANTHACEAE)	6 260	2 139
Buchnera henriquesii	3 520	2 435	*Rendlia cupricola*	1 560	-
Bulbostylus mucronata	7 783	2 130	*Silene cobalticola* (CARYOPHILLACEAE)	1 660	-
Celosia trigyna L	2 051	-	*Sopubia dregeana*	-	1 767
Commelina zigzag	1 210	-	*Sopubia metallorum*	-	1 742
Crassula alba	-	1 712	*Sopubia neptunii*	-	2 476
Crassula vaginata	-	1 405	*Striga hermontheca*	1 105	-
Crotularia cobalticola (FABIACEAE)	-	3 010	*Triumfetta digitata*	1 060	-
Cyanotis longifolia (CYPERACEAE)	-	4 200	*Triumfetta welwitschii*	-	2 201
Eragrostis boehmii	2 800	-	*Vernonia petersii*	1 555	-
Gutenbergia cupricola	5 095	2 309	*Vigna dolmitica*	3 000	-
Haumaniastrum homblei (LAMIACEAE)	-	2 633	*Xerophyta retinervis* Bak. var. *retinervis*	-	1 520
Haumaniastrum rosulatum	1 089	-			

Table 31: Hyperaccumulators of Lead (> 1 000 µg/g dry weight)

(Baker and Brooks, 1989)

SPECIES	LOCATION	LEAD CONTENT (µg/g)
Armeria maritima var. *halleri*	Harz Mountains	1 600
Polycarpaea synandra	Australia	1 044
Thlaspi alpestre	Derbyshire	2 740
Thlaspi rotundiflora subsp. *Cepaeifloium*	Central Europe	8 200

Table 32: Hyperaccumulators of Manganese (> 10 000 µg/g dry weight)

(Baker and Brooks, 1989)

SPECIES	MANGANESE CONCENTRATION (%)
Alyxia rubricaulis	1.15
Beaupreopsis paniculata	1.20
Eugenia clusiodes	1.09
Garcinia amplexicaulis	1.05
Macadamia angustifolia	1.16
Macadamia neurophylla	5.18
Maytenus bureauvianus	3.38
Maytenus serbertiana	2.25

Table 33: Zinc Hyperaccumulators (> 1% dry weight)

(Baker *et al*, 1994)

SPECIES	ZINC CONCENTRATION (µg/g)
Thlaspi caerulescens	> 10 000

Table 34: Nickel Hyperaccumulators (> 1000 μg/g dry weight)

(Baker and Brooks, 1989)

N°	GENUS	SPECIES	LOCATION	MAX. Ni (μg/g)
1	*Agatea* (Violac.)	*deplanchei* Brongn. Et Gris.	New Caledonia	2 500
2	*Alyssum* (Brassicaceae)	*akamasicum* Burtt	Cyprus	9 090
3		*alpestre* L.	S. Europe	4 480
4		*anatolicum* Hausskn. ex Nyar	Anatolia	8 170
5		*argenteum* All.	Italy	29 400
6*		*bertolonii* Desv.	Italy	13 400
7		*bertolonii* subsp. *scutarinum* Nyar.	Italy	10 200
8		*callichroum* Boiss. et Bühse	Anatolia	10 900
9		*caricum* TR Dudley et Huber-Morath	Anatolia	16 500
10		*cassium* Boiss	Anatolia	20 000
11		*chondrogynum* Burtt	Cyprus	16 300
12		*cicilicum* Boiss et Balansa	Anatolia	13 500
13		*condensatum* Boiss. Et Hausskn.	Syria, Iraq	4 990
14		*constellatum* Boiss.	Anatolia	18 100
15		*corsicum* Duby	Anatolia, Corsica	13 500
16		*crenulatum* Boiss.	Anatolia	10 400
17		*cypricum* Nyar	Cyprus	23 600
18		*davisianum* TR Dudley	Anatolia	19 600
19		*discolor* TR Dudley et Huber-Morath	Anatolia	11 700
20		*dubertretii* Gombault	Anatolia	16 500
21		*eriophyllum* Boiss. et Hausskn.	Anatolia	11 500
22		*euboeum* Halácsy	Euboea	4 550
23		*fallcinum* Hausskn.	Crete	3 960
24		*floribundum* Boiss. et Balsana	Anatolia	7 700
25		*giosnanum* Nyar.	Anatolia	7 390
26		*heldreichii* Hausskn.	Greece	12 500
27		*hubermorathi* TR Dudley	Anatolia	13 500
28		*janchenii* Nyar.	Albania	9 610
29		*lesbiacum* (Candargy) Rech. f.	Lesvos	22 400
30		*malacitanum* TR Dudley	Spain	10 000
31		*markgrafii* OE Schulz	Albania	13 700
32		*masmenaeum* Boiss.	Anatolia	24 300
33		*murale* Waldst. et Kit.	Balkans	7 080

N°	GENUS	SPECIES	LOCATION	MAX. Ni (µg/g)
34	*Alyssum* (Brassicaceae)	*obovatum* (CA Meyer)	Russia	4 590
35		*oxycarpum* Boiss. et Balsana	Anatolia	7 290
36		*peltarioides* Boiss. subsp. *virgatiforme*	Anatolia	7 600
37		*penjwinensis* TR Dudley	Iraq	7 860
38		*pinifolium* (Nyar.) TR Dudley	Anatolia	21 100
39		*pintodasilvae* TR Dudley	Portugal	9 000
40		*pterocarpum* TR Dudley	Anatolia	22 200
41		*robertianum* Bernard ex Gren. et Godr.	Corsica	12 500
42		*samarifernum* Boiss. et Hausskn.	Samar	18 900
43		*singarense* Boiss. et Hausskn.	Iraq	1 280
44		*smolikanum* Nyar.	Greece	6 600
45		*syriacum* Nyar.	Syria	10 200
46		*tenium* Halą̨esy	Tinos	3 420
47		*trapeziforme* Waldst. et Kit.	Anatolia	11 900
48		*Troodii* Boiss.	Cyprus	17 100
49		*virgatum* Nyar.	Anatolia	6 230
50	*Argophyllum* (Escaloniac.)	*grunowii* Zahlbr.	New Caledonia	1 375
51		*laxum* Schlecht.		1 900
52	*Blepharis* (Acanthaceae)	*acumuinata* Oberm.	Zimbabwe	2 000
53	*Bornmuellera* (Brassicaceae)	*baldacci* (Degen) Heywood subsp. *baldacci*	Greece	21 300
54		subsp. *markgrafi* (Schulz) TR Dudley	Albania	27 300
55		subsp. *rechingeri* Greuter	Greece	12 000
56		*glabrescens* (Boiss. et Bal.) Cullen et Dudley	Anatolia	19200
57		*tymphaea* (Hausskn.) Hausskn.	Greece	31 200
58		X *petri*	Greece	11 400
59	*Buxus* (Buxaceae)	*flaviramea* (Britton) Howard	Cuba	4 500
60	*Cardamine* (Brassicaceae)	*resedifolia* L.	Italy	1 050
61	*Casearia* (Flacourtiaceae)	*silvana* Schlecht	New Caledonia	1 490
62	*Chrysanthemum* (Asteraceae)	*alpimon* L.	Italy	3 200

N°	GENUS	SPECIES	LOCATION	MAX. Ni (µg/g)
63	*Cleidion* (Euphorbiac.)	*lasiophyllum* Pax et Hoffman	New Caledonia	9 900
64	*Dicoma*	*niccolifera* Wild	Zimbabwe	1 000
65	*Geissois* (Cunoniaceae)	*hirsuta* Bongn. et Gris.	New Caledonia	4 000
66		*intermedia* Vieill. ex Pampan		22 900
67		*magnifica* Bak. f.		3 250
68		*montana* Vieill. ex Brongn. et Gris.		5 740
69		*pruinosa* Brongn. et Gris.		34 000
70		*racemosa* Labill.		1 000
71		*trifoliolata* Guill.		6 250
72	*Homalium* (Flacourtiac.)	*austrocaledonicum* Seemann	New Caledonia	1 805
73		*deplanchi* (Vieill.) Warburg		1 850
74		*francii* Guill.		14 500
75		*guillainii* Vieill. Briq.		11 700
76		*kanaliense* Vieill. Briq.		9 420
77		*mathieuanum Vieill. Briq.*		1 694
78		*rubrocostatum* Sleumer		1 157
79	*Hybanthus* (Violaceae)	*austrocaledonicus* Schinz et Guill.		25 500
80		*caledonicus* (Turez.) Cretz.; *floribundus* (Lindl.) F. Muell.	W. Australia	17 500
81		subsp. *adpressus* Bennett		3 000
82		subsp. *curvifolius* Bennett		9 000
83		subsp. *floribundus*		9 800
84	*Lasiochlamys* (Flacourtiaceae)	*peltata* Sleumer	New Caledonia	1 000
85	*Leucocroton* (Euphorbiaceae)	*flavicans* Meull.	Cuba	7 700
86	*Linaria* (Scrophulariaceae)	*alpina* L.	Italy	1 990
87	*Luzula* (Juncaceae)	*lutea* (All.) DC.	Italy	2 050
88	*Merremia* (Asclepiadaceae)	*xanthophylla* Hall. f.	Zimbabwe	1 400
89	*Minuartia* (Caryophyllac.)	*laricifolia* Schinz et. Thell.	Italy	1 910

N°	GENUS	SPECIES	LOCATION	MAX. Ni (µg/g)
90	*Myristica* (Myristicaceae)	*laurifolia* Spruce ex DC. var. *bifurcata*	Obi (Indon.)	1 100
91	*Noccaea* (Brassicaceae)	*aptera* (Velen.) FK Meyer	Yugoslavia	13 600
92	*Noccaea* (Brassicaceae)	*boetica* FK Meyer	Greece	23 400
93		*firmiensis* FK Meyer	Greece	16 200
94		*tymphaea* FK Meyer	Greece	11 800
95	*Oncotheca* (Oncothecac.)	*balansae* Baill.	New Caledonia	2 500
96	*Pancheria* (Cunoniaceae)	*engleriana* Schlecht.	New Caledonia	6 300
97	*Pearsonia* (Fabaceae)	*metallifera* Wild	Zimbabwe	10 000
98	*Peltaria* (Brassicaceae)	*emarginata* Boiss. Hausskn.	Greece	34 400
99		*dumulosa* Post	Asia Minor	18 900
100	*Phyllanthus* (Euphorbiac.)	*aeneus* Baill.	New Caledonia	2 100
101		*balansaeanus* Guill.		1 820
102		*cataractarum* Muell.		1 450
103		*chrysanthus* Baill.		1 180
104		*induratus* S Moore		1 480
105		*kanalensis* Baill.		1 090
106		*maytenifolius* S Moore		1 420
107		*ngoyensis* Schlecht		9 550
108		*peltatus* Guill.		2 830
109		*serpentinus* S Moore		38 100
110	*Planchonella* (Sapotaceae)	*oxyedra* Dubard	Indonesia	19 600
111	*Psychotria* (Rubiaceae)	*douarrei* (Beauv.) Dăn.	New Caledonia	47 500
112	*Rhus* (Anacardiacae)	*wildii* R & A Fernandez	Zimbabwe	1 600
113	*Rinorea* (Violaceae)	*bengalensis*(Wall.) OK	SE Asia	17 500
114		*javanica* (Bl.) OK	Borneo	2 170
115	*Saxifraga* (Saxifragac.)	*aizoon* Jacq.	Italy	3 840

N°	GENUS	SPECIES	LOCATION	MAX. Ni (µg/g)
116	*Saxifraga* (Saxifragac.)	*exarata* Vill.	Italy	2 970
117	*Serbertia* (Sapotaceae)	*acuminata* Pierre ex Baill.	New Caledonia	17 750
118	*Stachys* (Lamiaceae)	*recta* L.	Italy	2 600
119	*Streptanthus* (Brassicaceae)	*polygaloides* Gray	West USA	14 800
120	*Thlaspi* (Brassicaceae)	*alpestre* L.	Central Europe	4 000
121		*alpinum* Crantz subsp. *sylvium* (Gaud.) Clapham	Central Europe	31 000
122		*brachypetalum* Jordan	France	20 000
123		*bulbosum* Spruner ex Boiss.	Greece	2 000
124		*calaminare* (Lej.) Lej. et Court.	Germany	39 600
125		*caerulescens* J et C Presl	Germany, Belgium	27 300
126		*cepaeifolium* (Wulfen) Koch	Italy	21 000
127		subsp. *virens* (Jord.) Hook. f.	France	4 100
128		*epirotum* Halácsy	Greece	3 000
129		*goesingense* Halácsy	Austria	12 000
130		*graecum* Jordan	Greece	12 000
131		*limosellifolium* Reuter	France	11 000
132		*montanum* L. var. *californicum*	West USA	7 940
133		*montanum* L. var. *montanum*	West USA	5 530
134		*montanum* L. var. *siskiyouense*	West USA	11 200
135		*ochroleueum* Boiss. ex Heldr.	Greece	4 000
136		*praecox* Wulfen	Bulgaria	21 000
137		*rotundifolium* (L.) Gaudin	Central Europe	18 300
138		*stenopterum* Boiss. et Reuter	Spain	16 000
139		*tatraense* Zapal.	Czech Republic	27 000
140		var. corymbosum (Gay) Gaudin	Central Europe	2 000
141	*Trichospermum* (Tiliaceae)	*kjellbergii* Burret	Celebes	1 600
142	*Trifolium* (Fabaceae)	*pallescens* Schreber	Italy	2 000
143	*Xylosma* (Flacourtiac.)	*boulindae* Sleumer	New Caledonia	1 930
144		*confusum* Guill.		1 630

N°	GENUS	SPECIES	LOCATION	MAX. Ni (µg/g)
145	*Xylosma* (Flacourtiac.)	*dothioense* Guill.		1 780
146		*kaalense* Sleumer		1 900
147		*molestrum* Sleumer		1 140
148		*pancheri* Guill.		1 130
149		*peltatum* (Sleumer) Lescot		1 000
150		*pininsulare* Guill.		1 280
151		*serpentinum* Sleumer		1 490
152		*tuberculatum* Sleumer		1 600
153		*vincentii* Guill.		3 750

- *Alyssum bertolonii* was the first nickel hyperaccumulator to have been discovered and documented in any genus.

Table 35: A Taxonomic Classification of Nickel Hyperaccumulators

(Baker and Brooks, 1989)

CLASS	SUBCLASS	ORDER	FAMILY	GENUS	N° OF SPECIES
Magnoliopsida	Magnoliidae	Magnoliales	Myristicaceae	*Myristica*	1
	Caryophyllidae	Caryophyllales	Caryophyllaceae	*Minuartia*	1
	Dilleniidae	Theales	Oncothecaceae	*Oncotheca*	1
		Malvales	Tiliaceae	*Trichospermum*	1
		Violales	Flacourtiaceae	*Casearia*	1
				Homalium	7
				Lasiochlamys	1
				Xylosma	11
			Violaceae	*Agatea*	1
				Hybanthus	5
				Rinorea	2
		Capparales	Brassicaceae	*Alyssum*	48
				Bornmuellera	6
				Cardamine	1
				Noccaea	4
				Peltaria	2
				Streptanthus	1
				Thlaspi	13
		Ebenales	Sapotaceae	*Planchonella*	1
				Serbertia	1

CLASS	SUBCLASS	ORDER	FAMILY	GENUS	N° OF SPECIES
Magnoliopsida	Rosidae	Rosales	Cunoniaceae	*Geissois*	7
				Pancheria	1
			Grossulariaceae	*Argophyllum*	2
			Saxifragaceae	*Saxifraga*	2
		Fabales	Fabaceae	*Pearsonia*	1
				Trifolium	1
		Euphorbiales	Buxaceae	*Buxus*	1
			Euphorbiaceae	*Cleidion*	1
				Leucocroton	1
				Phyllanthus	10
		Sapindales	Anacardiaceae	*Rhus*	1
	Asteridae	Gentianales	Asclepiadaceae	*Merremia*	1
		Lamiales	Lamiaceae	*Stachys*	1
		Scrophulariales	Scrophulariaceae	*Linaria*	1
			Acanthaceae	*Blepharis*	1
		Rubiales	Rubiaceae	*Psychotria*	1
		Asterales	Asteraceae	*Chrysanthemum*	1
				Dicoma	1
Liliopsida	Commelinidae	Juncales	Juncaceae	*Luzula*	1

APPENDIX 2

Table 36: Average Concentrations of Essential Mineral Nutrients in Plant Shoot Dry Matter that are Sufficient for Adequate Growth

(Marschner, 1986; Howes, 1990)

ELEMENT	SYMBOL	CONCENTRATION (ppm – dry weight)	FUNCTION
Macronutrients			
Nitrogen	N	15000 (1.5%)	Influences rate of plant growth.
Phosphorus	P	2000 (0.2%)	Exact role unknown.
Potassium	K	10000 (1.0%)	Involved in cell division, the synthesis of carbohydrates and proteins and nitrate reduction.
Calcium	Ca	5000 (0.5%0	Integral part of cell wall where it plays a role in permeability.
Magnesium	Mg	2000 (0.2%)	Central element in chlorophyll molecules and important in enzyme activation.
Sulphur	S	1000 (0.1%)	Present in some amino acids. Promotes root development.
Micronutrients			
Molybdenum	Mo	0.1	Involved in nitrogen fixation and normal growth.
Copper	Cu	6.0	Involved in electron transport and redox reactions. Copper proteins used in lignification of cell wall and nitrogen and carbohydrate metabolism (Haque *et al*, 1993).
Zinc	Zn	20.0	Involved in chlorophyll formation and in the production of the auxin, indolacetic acid (IAA). Carbohydrate metabolism and protein formation (Baker and Brooks, 1989).
Manganese	Mn	50.0	Essential in the oxygen evolving complex in chloroplast membranes. Acts as a structural constituent, an active binding site or a redox system (Baker and Brooks, 1989).
Iron	Fe	100.0	Involved in chlorophyll synthesis and energy conservation in photosynthesis and respiration.
Boron	B	20.0	Involved with sugar movement.
Chlorine	Cl	100.0	Stimulates photosynthesis.

APPENDIX 3

Studies on the nickel hyperaccumulator, *Berkheya coddii*

T.R. ANDERSON[1], A.W. HOWES[1], K. SLATTER[1] and M.F. DUTTON[2]

1 Dept of Biochemistry, University of Natal, P/Bag X01, Scottsville 3209, South Africa

2 Dept of Physiology, University of Natal Medical School, Congella 4013, South Africa

Abstract

Berkheya coddii is a herbaceous perennial of the Compositae family which is endemic to the serpentine soils of the Mpumalanga Province in South Africa, where it has the ability to hyperaccumulate and tolerate nickel.

B. coddii germinates from seed, or vegetatively, in September at the beginning of the summer rains. Seeds collected in January take 4-10 days to germinate, but lose up to 75% of their viability over the subsequent 9 months. We have successfully progagated *B. coddii* in tissue culture and find that the growth of intact plants (especially roots) is inhibited by the presence of auxins and cytokinins in the medium.

Plants growing in the Queens River Valley, hyperaccumulate up to 36 650 mg nickel/kg dry matter, which is approximately double that of plants growing in the Agnes Mine area, despite similar soil contents of available nickel in these two areas.

Uptake studies with ^{63}Ni have shown that metal absorbed by the roots, passes via the xylem to the apical leaves and then redistributes itself via the phloem to the lower leaves.

Calcium chloride, as well as its antagonist lanthanum chloride, significantly enhances ^{63}Ni uptake into the leaves. The enhancement is due to the Ca^{2+} and La^{3+} ions, rather than Cl^- ions, since sodium chloride shows no effect on nickel uptake.

A major nickel-binding ligand has been purified from *B. coddii* leaves and identified to be malate, which coordinates with nickel in a 1:1 molar ratio. Various preliminary evidence suggests that malate might function as a complexing agent in the *in vivo* mechanism of nickel detoxification, although this possibility remains to be confirmed by cellular localisation studies.

B. coddii shows great potential for the cleanup of nickel waste, and future studies will involve testing its performance in field experiments and elucidating its mechanism of nickel tolerance, with a view to optimising its hyperaccumulating ability.

Introduction

Berkheya coddii is a herbaceous perennial that is endemic to the serpentine soils of the Mpumalanga Province (South Africa), particularly near the Agnes Gold Mine and the Queen's River valley in Barberton. Morrey *et al.* (1989), reported that *B. coddii*, growing at the former site, was able to hyperaccumulate nickel up to 13 900 mg per kg dry leaf material, a finding that encouraged us to further investigate this plant with respect to its mechanism of nickel accumulation, and its suitability for waste cleanup and possibly biomining. In this paper, we present a brief overview of our work on *B. coddii*, done at the University of Natal, paying particular attention to its taxonomy, occurrence, germination and path and rate of nickel uptake. We also present our results for the purification and identification of a nickel-ligand complex from the leaf and discuss the potential role of this ligand in the *in vivo* mechanism of nickel storage in *B. coddii*. Finally, we discuss the implications of employing this plant for the detoxification of industrial waste, the mining of metals and the improvement of the environment.

Taxonomy and occurrence of *B. coddii*

Berkheya coddii was first described by Roessler (1959) and classified into the subfamily *Arctotideae* which is a member of the Compositae. It is an upright, herbaceous perennial, about 1.5 metres tall at maturity with one or more evenly-leafed stems branching from a basal root stock. The leaves are alternate, sessile, membranous, ovate-lanceolate and pointed at the end. They are approximately 10-15 cm long and 3-4.5 cm wide with approximately 1 mm long teeth on the margin. The head on top of the

stem consists of a flower with an almost corymbose inflorescence shape approximately 4-6 cm in diameter. It has a lobed corolla with many flowers in a disc on the thickened apex (Roessler 1959). The plant undergoes cross-pollination, probably via butterflies since the flowers have no smell, have a sturdy structure and are bright yellow in colour. The seeds are parachute-like and dispersed by wind (Mabberley 1987).

B. coddii is endemic to the subtropical serpentine regions of the Mpumalanga Province in South Africa where it thrives by virtue of its ability to tolerate high levels of nickel via a mechanism of extraction and hyperaccumulation of the metal, mainly in the leaves. The plants are particularly prevalent near the Agnes gold mine and the Queen's River valley regions near Barberton where they grow alongside only a few other species of nickel-resistant grasses.

B. coddii germinates from seed, or vegetatively, in September, at the beginning of the summer rainfall, is mature and flowers by December and commences seed dispersal in January. At the end of April, the plants begin to die off and have the appearance of dead sticks throughout the winter months (June through to August).

The germination of *B. coddii* seeds

We have found that *B. coddii* seeds, collected in January, show optimal germination if planted in rows on wet cotton wool in plastic (Tupperware) containers, which have been covered with clingwrap to permit the entry of light but prevent the loss of moisture. The containers are kept in a glass house at 30 - 34°C during the day-time and at 25°C during night-time. Under these conditions we find that the seeds take between 4 and 10 days to germinate with most of the seeds germinating on day 7. Seed viability studies have however shown that *B. coddii* seeds rapidly lose viability over a period of about 9 months after collection. Results have shown that seeds collected in January are only 45% viable in February and that this value reduces to 40% by March, and then remains more or less constant on 35% from April to June and 25% from July to September. Examination of seeds under a dissecting microscope, in August, showed that only one in four seeds still contained an embryo. It appeared that the non-viable seeds had lost their embryos through dehydration. We are thus investigating ways of improving the viability of seeds through improved methods of seed storage.

We have also successfully grown *B. coddii* in tissue culture employing Murashige-Skoog media with 3% added sucrose. Because *B. coddii* is extremely sensitive to a lack of water, the plants are cultured on totally liquid media with no added Phytagel or agar. To enhance plant shooting a low auxin (100 ml napthalaneacetic acid per litre media) to high cytokinin (1 ml/l 6-benzylaminopurine) concentration ratio can be added to the medium. However, since these hormones inhibit root growth, we no longer use them, in which case shooting and rooting occurs more slowly but intact plants result.

The extent of nickel uptake and its distribution in *B. coddii*

Our results (Table 1) for the nickel content of *B. coddii*, growing on serpentine soil in the Agnes Mine and Queens River sites have yielded evidence of significant nickel hyperaccumulation by the plant relative to the typical EDTA-available nickel levels (Hughes & Noble 1991 ; Noble & Hughes 1991). Furthermore, the results for the Agnes Mine area also compare very favourably with those of Morrey *et al.* (1989). The results recorded for plants in the Queens River area were, however, at least twice as high as those in the Agnes Mine area, with one leaf sample containing as much as 36 650 mg Ni per kg.

Uptake studies with ^{63}Ni have shown that the majority of the nickel absorbed by the roots, first travels via the xylem up to the apical leaves and, thereafter, some of the metal is transported via the phloem down to the lower leaves where it accumulates to a larger extent than in the upper leaves. The transport of nickel down the phloem of the plant has been confirmed by the presence of ^{63}Ni in aphids that feed exclusively on phloem tissue.

The calcium-inducing effect on nickel uptake by *B. coddii*

Radioactive nickel dosage studies with *B. coddii* in the presence of Ca^{2+} ions has shown that calcium (33 g of $CaCl_2.2H_2O$ per plant) is able to significantly enhance the rate of ^{63}Ni uptake

Table 1 Nickel content of *B. coddii* plants growing in the Agnes Mine and Queens River Valley areas near Barberton, South Africa.

Study Area	Organ	Ni Content mg/kg dry matter	Ni Content* mg/kg dry matter
Agnes Mine[1]	Leaf	13 130	11 637
	Stem	2 302	4 344
	Flower	2 277	2 127
	Root	3 000	nd
Queens River Valley[2]	Leaf	27 811	nd
	Stem	7 606	nd
	Flower	18 179	nd
	Root	3 000	nd

* Morrey *et al.* 1989

[1] 491 mg of EDTA-available nickel per kg of soil

[2] 385 mg of EDTA-available nickel per kg of soil

nd = analyses not done

into the leaves of *B. coddii* (Fig. 1). A possible explanation for this observation could be that the calcium either «turns on», or stimulates the production of, more calcium ports which are then able to simultaneously take up nickel at a faster rate. Indeed, Antonovics *et al.* (1971) have presented evidence that nickel is able to use the calcium transport proteins to facilitate its uptake into the plant. Since calcium is limiting in serpentine soils, it would not be unreasonable to expect a response of this nature from the plant to the sudden availability of calcium. The fact that the enhancing effects are due to calcium rather than chloride was confirmed by the lack of any detectable effects on nickel uptake following inoculation with sodium chloride (Fig. 1).

Further evidence for the calcium enhancement of ^{63}Ni uptake by the plant was found in that lanthanum showed a similar stimulatory effect to that of calcium (Fig. 1). Since lanthanum is a known calcium antagonist, it is conceivable that lanthanum will enhance nickel uptake by the same mechanism as that used by calcium. Whether the enhanced uptake rates will also equate to higher concentrations in the leaves at the end of the plant's growing season, remains to be confirmed by field trials.

The mechanism of uptake and storage/ detoxification of nickel in *B. coddii*

The mode of storage, and therefore detoxification, of nickel in *B. coddii* leaves is currently unknown, although our following inconclusive evidence suggests that the nickel might be stored in the form of a non-toxic complex, whose location in the leaf also remains to be elucidated.

We have isolated and purified to homogeneity a major nickel-binding ligand occurring in the leaves of *B. coddii* and identified it to be the well-known Krebs' Cycle intermediate, malic acid (or malate, its natural anionic form in the cell).

Briefly, the purification protocol involves in sequence : homogenisation of tissue, centrifugation, and 4 chromatography steps : anion exchange on Dowex 2-X8-100, molecular

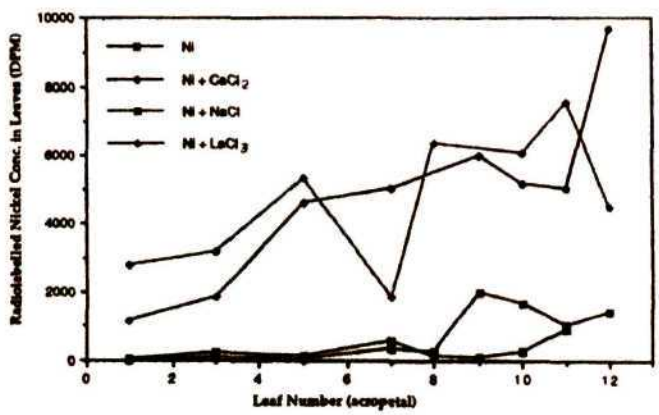


Fig. 1 The effect of calcium chloride, sodium chloride and lanthanum chloride (equimolar amounts) treatments of *B. coddii* on ^{63}Ni uptake by the leaves, 184 hours after treatment.

exclusion on Sephadex G-10, cation exchange on Dowex 50W-X8-200 and molecular exclusion on Biogel P-2, to yield two green peaks, a large-molecular weight peak of nickel-malate complex and a small-molecular weight peak of free nickel (Fig. 2). Purity was confirmed by chromatography on a reverse-phase column using a Waters high performance liquid chromatograph. Electrospray mass spectroscopy, a gentle technique which does not dissociate metal-ligand complexes, confirmed that the Ni-ligand isolated from the leaves is identical in molecular weight to that of a standard nickel-malate complex. Subsequently, the identity of the purified ligand was also confirmed to be malate by means of a Boehringer Mannheim test kit for malic acid, employing malate dehydrogenase.

To establish the nickel-binding potential of malate *in vivo*, we determined the stoichiometry of the nickel-malate complex. This was achieved by making up a purified fraction of the nickel-malate complex to a known volume, removing several aliquots for replicate analysis and applying each aliquot to a cation exchange gel in order to dissociate the complex and release the free nickel and malate. The free nickel was analysed by atomic absorption spectroscopy, while the free malate was quantified by means of a Boehringer Mannheim enzyme assay, employing malate dehydrogenase (uninhibited by nickel ions). The molar ratio of nickel to malate was calculated to be 1 : 1.04.

The following additional results constitute further evidence that malate might function *in vivo* as a ligand for the storage and detoxification of nickel in the leaves of *B. coddii*.

1. If *B. coddii* leaves are homogenised, centrifuged and the supernatant is passed directly through a Sephadex G-10 molecular exclusion column (instead of an ion exchange column), the endogenous nickel and the endogenous malate co-elute from the column, showing that they are associated with each other (Fig. 3). Free endogenous nickel elutes later from the column. This suggests that, either the nickel-malate complex exists naturally *in vivo* or, the homogenisation process has brought them together i.e. the complex is an artefact.

2. Following the ion exchange chromatography stage of the purification process (see above), the eluted endogenous nickel is not bound to anything else in the eluate, and when the retained fraction is incubated with this nickel, it binds to malate only,with no signs of the presence of any other ligand.

3. We have been unable to detect any other ligand in the leaves, besides malate, existing in sufficient concentration to bind all the endogenous soluble nickel found in the plant.

4. Electrospray Mass Spectroscopy, a gentle technique which does not dissociate metal ligand complexes, confirmed that the Ni-ligand isolated from the leaves is identical in molecular weight to that of a standard nickel-malate complex.

5. Various other workers (e.g. Kersten *et al.* 1980 ;

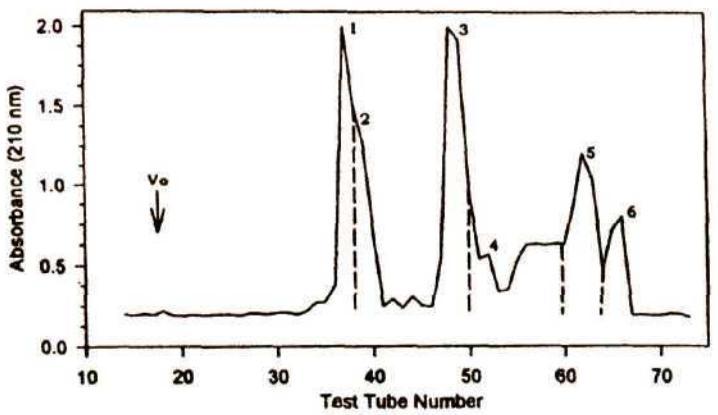


Fig. 2 Elution profile for the purification of malate ligand following molecular exclusion chromatography on Biogel P-2 MEC. Malate was identified as peak 3 (fractions 46-50).

Lee *et al.* 1977) have implicated the involvement of nickel-malate and nickel-citrate complexes in the tolerance mechanisms of other hyperaccumulating plants.

Conclusive evidence for the role of malate in the detoxification mechanism for nickel in *B. coddii* will, however, depend on the results of cellular localisation studies currently being performed on plant protoplasts of the tissue.

The mechanism *B. coddii* employs to extract and absorb the nickel from highly insoluble nickel silicates also remains to be elucidated. However, towards this end, we have recently isolated, purified and identified a major nickel-binding ligand from the roots of *B. coddii* which is novel and could well be involved in the uptake mechanism. The properties of this ligand remain to be fully established. We also believe that the intra-membrane calcium pump (see above) may be involved in the extraction and uptake of nickel by the roots.

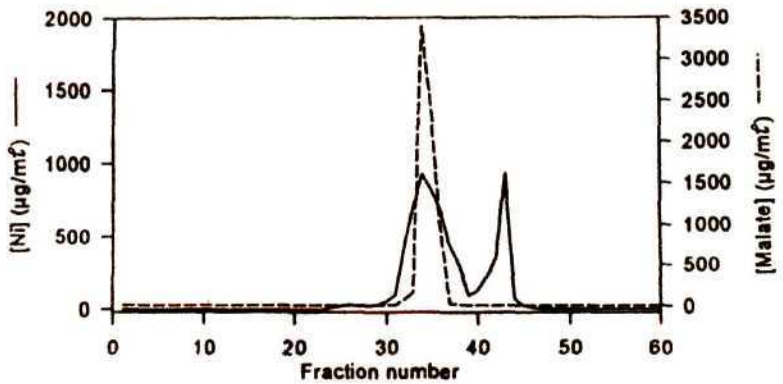


Fig. 3 Sephadex G-10 separation on a cell-free preparation of *B. coddii* leaves, showing the co-elution of the nickel and malate.

Discussion

In view of the ability of *B. coddii* to hyperaccumulate significant amounts of nickel and possibly other metals, this plant shows potential for the cleanup of toxic metal waste, for improving the aesthetic appearance of waste areas and possibly for low intensity biomining as a means of recovering some of the financial outlay. Towards this end, we have successfully mastered the propagation of the plant and are currently performing extensive field experiments to test the performance of the plant. We also intend testing the feasibility of harvesting the nickel twice per growth season by removal of the entire above-ground biomass (which promotes vegetative growth) and are considering various alternative methods of extraction of the nickel from the plant material.

We are also performing field experiments to test the ability of calcium and other nutrients to enhance the uptake of nickel by *B. coddii* as well as the health of the plant. Furthermore, should we succeed in elucidating the mechanism of nickel extraction, uptake and storage, this could lead to further studies on methods for improving the hyperaccumulating ability of the plant and possibly the development of a bioprocess for the *in situ* extraction of heavy metals.

Acknowledgements

The authors are indebted to Mr Martin Watson and colleagues (Department of Chemistry, University of Natal, Pietermaritzburg) and Mr. Ron Berry (Department of Biochemistry) for their invaluable technical assistance, and Prof. Kirsten van der Merwe and Dr. Schalk de Kock (University of Stellenbosch) for their assistance with the electrospray mass spectrometer.

References

Antonovics, J., Bradshaw, A.D. & Turner, R.G. (1971) Heavy metal tolerance in plants. *Advances in Ecological Research*, Vol. **7** (ed. J.B. Cragg), pp. 1-85. Academic Press, London.

Hughes, J.C. & Noble, A.D. (1991) Extraction of chromium, nickel and iron and the availability of chromium and nickel to plants from some serpentinite-derived soils from the Eastern Transvaal as revealed by various single and sequential extraction techniques. *Communications in Soil Science and Plant Analysis*, **22**, 1753-1766.

Kersten, W.J., Brooks, R.R., Reeves, R.D. & Jaffré, T. (1980) Nature of nickel complexes in *Psychotria douarrei*, and other nickel accumulating plants. *Phytochemistry*, **19**, 1963-5.

Lee, J., Reeves, R.D., Brooks, R.R. & Jaffré, T. (1977) Isolation and identification of a citrato-complex of nickel from nickel accumulating plants. *Phytochemistry*, **16**, 1503-1505.

Mabberley, D.J. (1987) *The Plant Book - a Portable Dictionary of the Higher Plants*, Cambridge University Press, Cambridge, U.K.

Morrey, D.R., Balkwill, K. & Balkwill, M. J. (1989) Studies on serpentine flora : preliminary analyses of soils and vegetation associated with serpentinite rock formations in the south-eastern Transvaal. *South African Journal of Botany*, **155**, 171-177.

Noble, A.D. & Hughes, J.C. (1991) Sequential fractionation of chromium and nickel from some serpentinite-derived soils from the Eastern Transvaal. *Communications in Soil Science and Plant Analysis*, **22**, 1963-1973.

Roessler, H. (1959) *Mitteilungen der Botanischen Staatssammlung München*, **3**, 231-232.