Heavy metals
in South African medicinal plants

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Contents

Abstract

Plants are able to take up and accumulate certain environmental contaminants such as heavy metals. When the plants are ingested by man, these contaminants are transferred along the food chain. Due to the poorly regulated medicinal plant trade in South Africa, many opportunities exist for heavy metal contamination of medicinal plants namely contaminated harvest sites as well as poor drying, processing, storage, transport and manufacturing conditions.

The concentrations of five heavy metals (As, Cd, Co, Ni, Pb) and six microelements (B, Cu, Fe, Mn, Mo, Zn) were determined in some commonly used South African medicinal plants obtained from street markets. Elemental content was determined using inductively coupled plasma optical emission spectrophotometry (ICP-OES). Some of the medicinal plant samples investigated contained As and Cd at levels exceeding the World Health Organization limits of 1 and 0.3 mg kg^{-1} respectively. Lead and Ni were detected in all the samples. Elevated Fe and Mn levels were recorded in certain plant species. The results revealed multiple metal contamination in some medicinal plant parts sold in local markets and is thus grounds for concern.

The effects of Cd application on growth parameters of some medicinal plant species belonging to the Hyacinthaceae (*Albuca setosa, Eucomis autumnalis, Eucomis humilis, Merwilla plumbea*) gave insight into heavy metal accumulation and distribution in these species. Application of Cd at 5 mg l^{-1} over a 12 week period reduced growth in *A. setosa*. The medicinally used *A. setosa* bulbs accumulated 37 mg kg^{-1} Cd after 12 weeks. Cadmium application at 2 mg l^{-1} over a six week period had no effect on growth parameters of *E. autumnalis* or *E. humilis.* However, a substantial difference in total Cd accumulation was detected in the plants (40.2 and 15.3 mg kg^{-1} respectively). Cadmium application at 2 mg l^{-1} significantly reduced the fresh weight of leaves, bulbs and roots of *M. plumbea*. Although most of the Cd was stored in the roots, the medicinally used bulbs accumulated up to 11.6 mg kg^{-1} when applied at 10 mg l^{-1}.

The antagonistic effect between Cd and Zn treatments and their effect on micronutrient distribution in *M. plumbea* were investigated. Five treatments were evaluated: (1) Hoagland's nutrient solution (HS) (control) (2) HS + Cd 2 mg l^{-1} (single) (3) HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1} (combination) (4) HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1} (combination) (5) HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1} (combination). Cadmium readily accumulated in leaves, bulbs and roots of *M. plumbea* when supplied at 2 mg l^{-1}. Zinc at 50 mg l^{-1} led to increased Cd accumulation. However, further increases in Zn concentration showed an antagonistic effect of Zn on Cd uptake and accumulation. Thus, increasing Zn levels in soils may be favourable for reducing toxic Cd accumulation in *M. plumbea* plants. Boron was not significantly affected by the addition of Cd to the media. However, with an increase in Zn, leaf B content increased while the B content in the bulbs and roots decreased. Copper and Mo levels were not significantly affected by treatments with Cd or Cd/Zn combinations. Compared to the control, Cd and Cd/Zn applications caused an increase in Mn content in leaves, bulbs and roots. Iron levels of *M. plumbea* were not significantly affected by Cd in the media. However, with an increase of Zn in the Cd-containing media, Fe content in the leaves, bulbs and roots increased.

Tulbaghia violacea is one of the few medicinal plants that is also frequently used as a leafy vegetable. Application of Cd at 2 and 5 mg l^{-1} to *T. violacea* of varying sizes (small 8 - 10 g, medium 16 - 20 g, large 80 – 95 g) elicited a difference in growth response, Cd accumulation and micronutrient distribution. Leaf length and fresh weight of leaves of the medium-size plants decreased with application of Cd at 2 mg l^{-1} whilst 5 mg l^{-1} Cd significantly decreased the number of leaves in small-sized plants. Small plants accumulated more Cd in the leaves than medium- or large-sized plants. Application of Cd at 2 mg l^{-1} and 5 mg l^{-1} lowered the leaf Cu, Fe, Mo and Zn contents in small- and medium-size plants. This study indicated that *T. violacea* has the ability to accumulate Cd. In addition, plant size plays an important role with regards to Cd accumulation and elemental distribution.

The effect of various nutrient applications (10%, 50% and 100% Hoagland's nutrient solutions (HS); and HS deficient in N, P or K) on growth parameters and micronutrient distribution in *Dioscorea dregeana* were investigated. Irrigating plants with 50% HS resulted in better growth performance, whereas a deficiency of either N,

P or K negatively affected seedling growth. Plants grown in 10% HS contained higher total B, Fe and Mo levels compared to seedlings grown in 50% and 100% HS. Compared to the control, P deficiency resulted in a Fe increase in the leaves, tuber and roots while a lack of P and K significantly increased total Mn content in *D. dregeana*.

The effect of excess Zn (100, 200 and 300 mg l^{-1}) on growth performance, chlorophyll content and microelemental distribution on *Dioscorea sylvatica* was investigated. Growth parameters showed a significant decrease when supplied with Zn at 100 mg l^{-1}. Zinc phytotoxicity was evident by the reduction in chlorophyll content. Highest Zn concentrations were detected in the roots. Certain micronutrients appear to be redistributed due to Zn toxicity.

The effect of microelements (Cu, Zn) and heavy metals (Cd, Pb, Hg) on germination and seedling development of *Bowiea volubilis, Eucomis autumnalis* and *Merwilla plumbea* was investigated. Copper and Zn applied at 1 mg l^{-1} significantly reduced the percentage germination of *E. autumnalis*. Low concentrations (≥ 1 mg l^{-1}) of Cu and Zn negatively affected the root growth of all three species. Mercury concentrations of 0.5 and 1 mg l^{-1} significantly decreased the percentage germination of *B. volubilis* and *E. autumnalis* respectively. Cadmium and Hg at 2 mg l^{-1} showed a negative effect on the root growth of *B. volubilis*. Concentrations of 0.5 mg l^{-1} of all heavy metals tested significantly decreased shoot length of *M. plumbea*.

The effect of Cd on biological activity (anti-inflammatory, antibacterial and antifungal) of medicinal plants with previously confirmed activity was evaluated. When supplied with Cd at 2 mg l^{-1}, *Eucomis humilis* bulbous extracts showed lower anti-inflammatory activity than the control for both COX-1 and COX-2 activity. *Eucomis autumnalis* bulbous extracts had greater COX-1 activity compared to the control. However, Cd suppressed the activity of COX-2. Compared with non-Cd-treated *Merwilla plumbea* plants (control), those supplied with Cd at 10 mg l^{-1} showed increased antibacterial activity against *Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus aureus.* However, no change in activity against *Escherichia coli* was observed. Cadmium accumulation in the bulbs had no effect on antifungal activity of *Tulbaghia violacea.*

Thus, optimized agricultural practices are essential for quality control of cultivated medicinal plants.

The studies presented in this thesis collectively answer several questions related to heavy metal involvement in South African medicinal plants. The findings substantiate the need to regulate and monitor the South African medicinal plant trade against heavy metal contamination which will in turn provide a product of safety and quality to the consumer.

Declaration

I hereby declare that this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden, and the co-supervision of Doctor W.A. Stirk and Doctor C. Southway, in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg.

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STREET, R.A., KULKARNI, M.G., STIRK, W.A., SOUTHWAY, C., VAN STADEN, J. (**2007**) Wild garlic (*Tulbaghia violacea*): a heavy metal accumulator? The SA Pharmacology and Toxicology Congress (TOXSA). Buffelspoort, South Africa.

STREET, R.A., KULKARNI, M.G., STIRK, W.A., SOUTHWAY, C., VAN STADEN, J. (**2007**) Effect of heavy metals on germination and seedling development of widely used medicinal plants belonging to the Hyacinthaceae. Thirty third annual conference of the South African Association of Botanists (SAAB). Cape Town, South Africa.

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List of Abbreviations

AA	atomic absorption
ANOVA	analysis of variance
As	arsenic
B	boron
Cd	cadmium
$CdCl_2 \cdot H_2O$	cadmium chloride monohydrate
$(CH_3COO)_2Pb \cdot 3H_2O$	lead acetate trihydrate
Chl	chlorophyll
Co	cobalt
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CRM	certified reference material
Cu	copper
$CuSO_4 \cdot 5H_2O$	copper(II) sulfate pentahydrate
FAO	Food and Agriculture Organization of the United Nations
Fe	iron
HS	Hoagland's nutrient solution
HNO_3	nitric acid
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
Hg	mercury
$HgCl_2$	mercuric chloride
ICP-OES	inductively coupled plasma-optical emission spectrophotometer
K	potassium
$mg\ kg^{-1}$	milligram per kilogram
$mg\ l^{-1}$	milligram per liter
Mn	manganese
Mo	molybdenum
nm	nanometer
Ni	nickel
$NiSO_4 \cdot 6H_2O$	nickel sulfate hexahydrate
P	phosphorous
Pb	lead

S	sulfur
S.D.	standard deviation
S.E.	standard error
WHO	World Health Organization
WRC	Water Research Commission of South Africa
Zn	zinc
$ZnSO_4.7H_2O$	zinc sulfate

1   Introduction

1.1 IMPORTANCE OF SAFETY AND QUALITY CONTROL OF SOUTH AFRICAN MEDICINAL   PLANTS

A common misconception is that medicinal plants are "pure and natural" and that this equates to "harmless". Based on their long history of use, users of traditional medicines deem them safe for human consumption. However, the absence of their regulation provides no such guarantee.

Reliance on plants collected from the wild causes not only a threat to medicinal plant biodiversity but also speculation with regards to safety, as industrial encroachment has led to contamination of water tables and soil. Furthermore, potentially harmful contamination, either as a result of improper cultivation methods, harvesting or storage practices, result in degradation in medicinal plant quality.

1.2   AIMS AND OBJECTIVES

The overall aim of this study was to provide some verification for the need to monitor South African medicinal plants against heavy metal contamination. This was achieved through a number of experiments on different aspects relating to this topic. Thus, the specific objectives of this study were:

- To assess heavy metal contamination in medicinal plants sold at informal street markets;
- To quantify heavy metal uptake of some frequently used bulbous medicinal species and to determine their distribution within the plant;
- To determine the effect of heavy metal stress on microelemental distribution in medicinal plants;
- To assess the effects of heavy metals on germination and seedling establishment on three heavily used medicinal plant species; and

- To evaluate the effect of heavy metals on biological activity in selected medicinal plants.

1.3   GENERAL OVERVIEW

The research presented in this thesis examined a range of different studies relating to the topic of heavy metals in South African medicinal plants. Collectively, they provide insight towards understanding the potential risk of an unmonitored medicinal plant trade, the necessity to investigate potential heavy metal accumulation traits of commonly used medicinal plants and to gauge the effect of these heavy metals on plant growth and development.

Chapter 2 provides a comprehensive literature review on the topic of heavy metals in medicinal plants.

Chapter 3 presents the heavy metal content in medicinal plants collected from undisclosed locations and sold at informal street markets. The results emphasize the unsafe consequences of selling plants where their trade is not regulated.

Chapter 4 assesses the effect of Cd on four Hyacinthaceae species that are used medicinally. Results of growth parameters and Cd distribution within the plants are presented.

Chapter 5 presents results from experiments determining the effect of Cd and Zn combinations on uptake, distribution and microelemental content in *Merwilla plumbea* and the effect of Cd uptake on the microelemental distribution in *Tulbaghia violacea,* two heavily used medicinal species.

Chapter 6 presents results evaluating the effect of nutrient levels on microelement uptake and distribution in two medicinal *Dioscorea* species.

Chapter 7 presents results on the effect of essential and non-essential elements on germination and seedling growth of three heavily used Hyacinthaceae species. These

species are frequently proposed for cultivation and extensively used in traditional medicine in South Africa.

Chapter 8 presents results on the effect of Cd on the antibacterial, antifungal and anti-inflammatory activities in extensively used medicinal plants. *Eucomis autumnalis, Eucomis humilis, Merwilla plumbea* and *Tulbaghia violacea* were selected based on reported usage with previously confirmed activity.

Chapter 9 covers general conclusions from the various findings discussed in this thesis.

1.4 RESEARCH OUTLINE

The research outline highlights areas investigated in this thesis

PLANT TO PATIENT	RESEARCH CONDUCTED
Plant growth and development	**Seed** • effect of heavy metals on germination and seedling development (Chapter 7) **Mature plant** • effect of heavy metals on plant growth and development (Chapter 4, 5) • heavy metal accumulation and distribution (Chapter 4, 5) • micronutrient distribution (Chapter 5, 6)
Harvest site, storage and transport	Discussed in Chapter 2
Post harvest preparation	Discussed in Chapter 2
South African medicinal markets	**Plants from markets** • heavy metal screening of medicinal plant parts sold at informal medicinal markets (Chapter 3)
Safety and quality of medicinal plants	**Biological activity** • effect of heavy metals on antibacterial, antifungal and anti-inflammatory activity (Chapter 8) **Toxicology of heavy metals** • discussed in Chapter 2

2   Literature review

2.1 MEDICINAL PLANT TRADE IN SOUTH AFRICA

Traditional medicine currently forms the backbone of rural health care in South Africa. The reliance on traditional medicine can be attributed to a number of factors, namely relatively good accessibility, availability, affordability and existence of extensive knowledge and expertise in the local communities (MANDER, MANDER and BREEN 1996). It is estimated that 27 million South Africans depend on over 1 000 medicinal plant species for their health care needs with approximately 20 000 tons of plant material sold annually in South African traditional markets (MANDER 1998).

There is an extensive network related to the sale of medicinal plants in rural and urban areas. Key components of this network include collectors, transporters, hawkers, wholesalers, retailers and traditional healers (MANDER, MANDER and BREEN 1996). Medicinal plant gatherers collect their materials throughout the year to supply the persistent **demand** for medicinal plants. If mature trees or plants cannot be found, then younger ones suffice, which results in availability of inconsistent plant material of the same species (VON AHLEFELDT, CROUCH, NICHOLS, SYMMONDS, MCKEAN, SIBIYA and CELE 2003). A study carried out between 1995 and 2002 on the size-class prevalence of bulbous and perennial herbs sold in the Johannesburg medicinal plant markets showed a significant **decrease in the bulb size** of certain species (WILLIAMS, BALKWILL and WITKOWSKI 2007). Thus, medicinal plant gatherers are harvesting smaller bulbs, which in turn leads to the necessity to increase the harvest of smaller plants to keep up with the demand.

2.1.1 Traditional medicinal markets of South Africa

South African medicinal plants are commonly sold at **informal street markets** or indoor shops. The outdoor markets are customarily positioned in the hub of the city centre to allow easy access for commuters. The bulk of the medicinal plant trade

involves the sale of unprocessed or semi-processed products. A rudimentary cover may keep direct sunlight or rain off the trader but most of the plants are displayed in the open (**Figure 2.1**). Therefore, plant material may come into contact with various kinds of urban pollution such as industrial and vehicular emissions. Furthermore, the plant material may be susceptible to microbial and pest contamination (STAFFORD, JÄGER and VAN STADEN 2005).

Pests are a common problem for plant traders and **fumigation** does occur in **medicinal plant shops**. Shop owners, however, do not seem to be concerned about the consequences of potentially toxic residues on the plant material being sold to their customers (FENNELL, LIGHT, SPARG, STAFFORD and VAN STADEN 2004).

Figure 2.1: Informal street market, Pietermaritzburg, South Africa.

Throughout southern Africa, herbal material that is dried (roots or bark), or has an extensive **shelf-life** (bulbs, seeds and fruits) dominate traditional medicinal markets (**Figure 2.1**) (CUNNINGHAM 1993). The **packaging** used for plant products includes newspaper and plastic packets. A survey of rural clinic patients (n=100) in South Africa revealed that 84% would prefer more hygienically packaged indigenous medicine. Most consumers indicated that they would also prefer more modernized and hygienic trading sites (MANDER 1998).

2.1.2 Collection and storage practices for South African medicinal plants

In Europe, China and India, medicinal plants are often cultivated on a large scale to meet the demands of the people. However, the most common practice in South Africa is still the collection of medicinal plants from **wild populations** (ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN 2000). This not only threatens population stability but also leads to speculation with regards to safety, as industrial encroachment has led to **contamination** of water tables and soil. According to the World Health Organization (WHO; 2003) medicinal plants collected from the wild may be contaminated by other species or plant parts through misidentification, accidental contamination or deliberate adulteration, all of which may have unsafe consequences. Poisoning from South African traditional medicines is frequently a consequence of misidentification (STEWART, STEENKAMP and ZUCKERMAN 1998).

Inadequate drying of the plant material may result in **mould** and **decay** (WHITTEN 1997). A recent study on South African medicinal plants recommended for the treatment of HIV/AIDS revealed that many plants had high bacterial and fungal numbers due to low environmental sanitation and low processing standards (GOVENDER, DU PLESSIS-STOMAN, DOWNING and VAN DE VENTER 2006).

The lack of **storage** facilities and trading infrastructure results in the spoiling of raw plant material. Thus, undesirable wastage and/or a decrease in product quality is common (MANDER 1998). The effect of harvest procedures and storage duration on quality and efficacy of South African medicinal plants, due to the chemical changes, remains unknown (FENNELL, LIGHT, SPARG, STAFFORD and VAN STADEN 2004). However, phytochemical stability is species-specific and no general assumption can be made with respect to recommended shelf-life (STAFFORD, JÄGER and VAN STADEN 2005). Timeous disposal of medicinal plants with unstable compounds will contribute to the improved quality and efficacy of these plants.

2.1.3 Cultivation of South African medicinal plants

With the realization that some wild species are being over-exploited, sustainable **cultivation** systems have been recommended (WHO, IUCN and WWF 1993). Although cultivated medicinal plants are acceptable as an alternative to wild plants in some African countries, namely Ghana, South Africa and Swaziland (CUNNINGHAM 1993), the more conservative traditional healers (for example in South Africa and Botswana) believe that plants grown under typical agricultural practices (i.e., grown in straight lines and with the use of fertilizers) will not have the same medicinal properties as those harvested from the wild. This is not scientifically unsound as harsh natural environments can affect **secondary metabolite production** which may not be expressed under mono-culture where more favourable conditions prevail (SCHIPPMANN, LEAMAN and CUNNINGHAM 2002). However, due to genetic, ecological and environmental differences, wild harvested plants vary in quality and consistency which seriously compromises financial returns (BOPANA and SAXENA 2007). In addition, it is probable that international traders, interested in obtaining South African medicinal plants, will require some form of certification of safe and sustainable sourcing (MANDER, DIEDERICHS and STEYTLER 2006), as strict environmental requirements are increasingly entering international trade agreements (WHYTE 1995).

2.1.3.1 Good agricultural practices

The WHO (2003) has issued a set of guidelines for good agricultural and collection practices (GACP) for medicinal plants to promote sustainable cultivation and conserve both the medicinal plants and the environment. Good agricultural practice (GAP) is the first step in quality assurance upon which the safety and efficacy of plant-based medicinal products directly depend.

To date, only the European Union and a few other countries, such as China and Japan have developed regional and national guidelines for good agricultural and collection practices for medicinal plants (WHO 2003). Such guidelines are regulated and monitored to ensure that the proper plant material is collected and/or cultivated

and that soil and irrigation water are within the limits, or free from, harmful heavy metals, pesticides, herbicides and toxicologically hazardous substances.

Unfortunately, environmental monitoring in Africa is limited, since restricted resources force African governments to focus on immediate health concerns such as malnutrition and infectious diseases (CAMPBELL, DIXON and HECKY 2003).

2.1.3.2 Negative impacts of poor agricultural practices

One of the main aims of cultivation is to increase plant yield by improving growth conditions through addition of agro-chemicals including fertilizers, pesticides and insecticides (SPRING and DIEDERICHS 2006). Commercial fertilizers are a valuable source of nutrients for plant growth and play a critical role in increasing food production worldwide. In addition, the elemental ratio in soils can directly influence phytochemical yield therefore impact on quality (MCALISTER and VAN STADEN 1995; BRISKIN, LEROY and GAWIENOWSKI 2000). However, **poor nutrient management** and **agro-chemical application** may have undesirable effects on both crop and phytochemical yield and the environment.

All pesticides are toxic by nature and can cause health hazards to humans and animals through exposure or dietary intake (WILSON and OTSUKI 2004). The South African Government has reportedly used pesticides such as DDT, which are banned in other countries, for pest and disease control (WHYTE 1995). Pesticide contamination of African medicinal plants has been reported (DOGHEIM, ASHRAF, ALLA, KHORSHID and FAHMY 2004; ELGORASHI, STAFFORD, MULHOLLAND and VAN STADEN 2004). Health risks associated with incorrect application of pesticides include discharge of poisonous chemicals into the environment and consumption of foods that contain pesticide residues by consumers (WILSON and OTSUKI 2004).

Many agro-chemicals contain As, Cu, Fe, Mn and Zn. Some contaminants such as Cd and Pb enter the soil due to **fertilizer impurities** (HE, YANG and STOFFELLA 2005). Heavy metal contamination in soils is often caused by repeated use of metal-enriched fertilizers (HE, YANG and STOFFELLA 2005). Polluted agricultural soils

can result in land degradation and in turn affect food safety and crop production (MORENO, VÍLLORA, SORIANO, CASTILLA and ROMERO 2005; HEIKENS 2006). Phosphate fertilizers represent a potentially significant source of Cd to soils, with the Cd content in various phosphate fertilizers varying considerably (LUGON-MOULIN, RYAN, DONINI and ROSSI 2006). Despite the cautionary suggestion by LUGON-MOULIN, RYAN, DONINI and ROSSI (2006) that fertilizers should be screened for contaminants, this is not always feasible, especially for developing countries. Agricultural runoff contains pesticides and other agricultural chemicals, but there is very little monitoring of agro-chemical use in South Africa (WHYTE 1995). Unfortunately, the continued use of contaminated fertilizers over an extended period of time may cause accumulation of these **contaminants** to high levels in the soil, thereby increasing the risk to environmental and human health.

2.2 HEAVY METALS

2.2.1 Defining heavy metals

The term "heavy metals" has been used increasingly as a collective name for metals and metalloids associated with contamination and potential toxicity (DUFFUS 2002). The term has never been defined by any authoritative body but has been classified on a number of criteria by numerous researchers i.e. density, atomic weight, atomic number, chemical properties or definitions without a clear basis other than toxicity (DUFFUS 2002). Despite the fact that the term heavy metals has no sound technological or scientific basis, it is used in this work, as it is in much of the scientific literature, to refer to a group of metals or semi-metals, essential and non-essential to both humans and plants.

Despite being essential for plant growth and/or human nutrition, several elements may also be toxic at high concentrations, for example Cu, Mo, Ni or Zn. Other non-essential elements such as As, Cd, Hg and Pb, may also inadvertently enter the food chain through ingestion of contaminated plants and pose health risks to humans and animals (MCLAUGHLIN, PARKER and CLARKE 1999). Unlike organic pollutants, heavy metals cannot be biodegraded and therefore reside in the environment for long periods of time.

2.2.2 Heavy metal pollution in South Africa

South Africa has a high concentration of **industrial** and **mining activity**. Inadequate measures to control and enforced safe disposal of waste means that industrial waste is illegally dumped in urban areas, posing severe health risks to communities and environments (WHYTE 1995). The deposition of processed and unprocessed waste materials has led to continuous, persistent leaching of dissolved metals into soil and water systems (NAICKER, CUKROWSKA and MCCARTHY 2003; ROYCHOUDHURY and STARKE 2006). Numerous reports have indicated heavy metal contamination of South African **rivers** and **soils** (ABBU, PILLAY and MOODLEY 2000; BINNING and BAIRD 2001; OKONKWO and MOTHIBA 2005).

The Witwatersrand region of South Africa is famous for its gold production and a major metropolis, centered on Johannesburg, has developed as a result of mining activity. NAICKER, CUKROWSKA and MCCARTHY (2003) revealed that the ground water within the mining district is **heavily contaminated** and acidified as a result of oxidation of pyrite (FeS_2) contained within mine tailing dumps, which contain elevated concentrations of heavy metals. Similarly, a marked deterioration of groundwater quality in the abandoned Witbank Coalfield in the Middelburg Colliery area has been reported due to the seepage of acid mine drainage from the mine (BELL, BULLOCK, HALBICH and LINDSAY 2001). Effects of contaminated water persist for considerable distances downstream of the pollution source. Many local, often ephemeral, streams in the Middelburg Colliery area eventually drain into the Loskop Reservoir and the Olifants River. The Olifants River eventually flows through the Kruger National Park. The catchment area of the Olifants River is thus potentially **hazardous** for tourism and nature conservation. In addition, the lower and middle sections of the river are areas of intensive agricultural activity (BELL, BULLOCK, HALBICH and LINDSAY 2001). According to VERSTER, DU PLESSIS, SCHLOMS and FUGGLE (1992) some 30 000 ha of land are watered with polluted water and some 150 000 to 250 000 tons per year of dry sewage sludge is being disposed of on South African soils – much of this on agricultural land.

''Dirty fuels'' are major contributors to urban **air pollution** in South Africa (LEIMAN, STANDISH, BOTING and VAN ZYL 2007). Due to the availability and affordability, D-

grade residential coal is widely used as a fuel source for heating and cooking by most of the lower-income urban communities in South Africa (ENGELBRECHT, SWANEPOEL, CHOW, WATSON and EGAMI 2002). Emissions from the use of residential coal are a major cause of elevated air pollution levels in the industrialized areas of South Africa. The negative health effects caused by exposure to residential coal combustion emissions have been a major public concern for several years (ENGELBRECHT, SWANEPOEL, CHOW, WATSON and EGAMI 2002).

Investigations to assess **environmental contamination** are essential to depict the pathways of exposure from environmental media, namely air, water, soil and plants (CAUSSY, GOCHFELD, GURZAU, NEAGU and RUEDEL 2003).

2.3 HEAVY METALS IN SOILS

While essential and non-essential elements are primarily inherited from **parent rock**, their distribution in the soil reflects various pedogenic processes as well as the impact of external factors such as agriculture (KABATA-PENDIAS 2001). Parent materials differ extensively in elemental content which can be affected by a number of factors such as varying modes of deposition and weathering regimes. Therefore heavy metal content, distribution and availability vary widely among soils within and between regions (WHITE and ZASOSKI 1999).

2.3.1 The effect of soil properties on bioavailability of heavy metals

A large proportion of metals is bound to the **solid matrix** of the soil and must be mobilized into the **soil solution** before being taken up by the plant (DE LA FUENTE, CLEMENTE and BERNAL 2008). The **total** or **pseudo-total** concentration consists not only of those metal ions that are readily **exchangeable** between the solid and solution phases, but also those more **strongly bound** within solid phases of the soil, and therefore not available for transport from the soil or for plant uptake (RIEUWERTS, ASHMORE, FARAGO and THORNTON 2006). Critical elemental concentrations are more appropriately expressed as **extractable** or dissolved concentrations rather than total metal levels (RIEUWERTS, ASHMORE, FARAGO and THORNTON 2006). A number of **extraction** methods have been suggested in

recent years for the assessment of trace elements in soils. KABATA-PENDIAS (2001) classified these methods as follows: acids (HCl, aqua regia), chelating agents (ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid-triethanolamine, (DTPA [+TEA])), buffered salt solutions (AAAc buffer), and unbuffered salt solutions ($CaCl_2$, $MgCl_2$, $NaNO_3$, NH_4NO_3).

The transfer of metal ions between the readily available and less-available phases is greatly influenced by competition for surface exchange sites by other cations (especially H^+) and by the occurrence of binding surfaces such as **organic matter, clay** and **hydrous oxides** (RIEUWERTS, ASHMORE, FARAGO and THORNTON 2006). The ability of clays to bind metal ions is correlated with their **cation exchange capacity** (CEC), and usually the greater the CEC, the greater the amount of cation adsorbed (KABATA-PENDIAS 2001). Soils with a high **organic matter** content have a multifaceted influence on the behavior of trace elements. In general, it can be expected that up to 50% of the total trace element content is fixed by organic matter in mineral soils (KABATA-PENDIAS 2001). The most stable compounds in soils are humic substances partitioned by the fractions of humic acid, fulvic acid and humin. Owing to a particular grouping of diverse functional groups (mainly OH and SH), humic substances are able to form complexes with certain cations (KABATA-PENDIAS 2001).

The chemical **speciation** of an element determines its **bioavailability** and **toxicity** and thus controls its transport and fate in soil and water (VAN DER PERK 2006). For example, Cr toxicity to plants depends on its oxidation state: Cr(VI) is acutely toxic and mobile whereas Cr(III) is less toxic (SHANKER, CERVANTES, LOZA-TAVERA and AVUDAINAYAGAM 2005). The soil solution speciation of trace elements is imperative for assessing their bioavailability and potential threat to the environment (SAUVE, MCBRIDE and HENDERSHOT 1997; KIRKHAM 2006).

2.4 HEAVY METALS IN HIGHER PLANTS

2.4.1 Excluders, indicators and accumulators of heavy metals

The sensitivity of plants towards metals is influenced by plant species and genotypes. According to BAKER (1981) plants can be grouped into three categories: excluders, indicators and accumulators. **Excluders** survive through restriction mechanisms that prevent uptake of heavy metals from soils. Avoidance by exclusion is the most common mechanism of plant adaptation to metal toxicity (PATRA, BHOWMIK, BANDOPADHYAY and SHARMA 2004). **Indicators** show poor control over metal uptake and transport processes, and the metal content in plants often reflects the concentration in the soils. As **accumulators** do not prevent metals from entering the roots, they have evolved a specific mechanism for detoxifying high metal content accumulated in the cells. Plants known to accumulate extremely large amounts of heavy metals are called **hyperaccumulators**. Threshold values of metal concentrations have been used to define metal hyperaccumulation, including 10 000 mg kg^{-1} dry weight of shoots for Zn and Mn, 1 000 mg kg^{-1} for As, Co, Cu, Ni and Se, and 100 mg kg^{-1} for Cd (MCGRATH and ZHAO 2003). Hyperaccumulators usually have a low biomass because they use more energy in the mechanisms needed to adapt to the elevated metal concentrations in the tissues (KABATA-PENDIAS 2001). Hyperaccumulators contain a higher concentration of a heavy metal in their shoots than the roots as apposed to non-accumulator species, which concentrate metals in their roots when exposed to high metal concentrations (KIRKHAM 2006).

A comparison of different plant species has confirmed that most **dicotyledonous** plants absorb more heavy metals than **monocotyledonous** ones (SAUERBECK 1991). Phylogenetic variation in heavy metal accumulation in angiosperms was reviewed by BROADLEY, WILLEY, WILKINS, BAKER, MEAD and WHITE (2001). Heavy metal accumulation characteristics in plants differ at the family level (KUBOI, NOGUCHI and YAZAKI 1986; PRASAD 1995). However, further research is needed to evaluate the variation in heavy metal uptake by different **plant species**, especially of those that will eventually enter the food chain (ISLAM, YANG, HE and MAHMOOD 2007).

2.4.2 Heavy metal uptake by plants

Heavy metal absorption can be both **passive** (non-metabolic) and **active** (metabolic) (KABATA-PENDIAS 2001). Plant roots are the main organs involved in elemental uptake and function at the interface with the **rhizosphere** (SHIN and SCHACHTMAN 2004). They are able to synthesize, accumulate and secrete compounds (WALKER, BAIS, GROTEWOLD and VIVANCO 2003). Root secretion of organic acids or protons can acidify the rhizosphere and increase metal dissolution (YANG, FENG, HE and STOFFELLA 2005). For example, a remarkable enhancement of malic, citric, and succinic acids was found in barley plants exposed to Al compared to control plants. The Al-tolerant cultivar showed noticeably higher exudation of these organic acids than the Al-sensitive cultivar. Thereby demonstrating that Al-induced enhancement of these organic acids is very likely to be connected with Al tolerance (GUO, ZHANG, ZHOU, WU and CHEN 2007). In contrast, organic acids can inhibit metal uptake by forming a complex outside the root, thus preventing its uptake (MURPHY, EISENGER, SHAFF, KUCHIAN and TAIZ 1999; PINEROS and KOCHIAN 2001).

Compared to the bulk soil, the rhizosphere is populated by large numbers of microorganisms which mostly consist of **bacteria** and **mycorrhizal fungi.** Root-colonizing bacteria and mycorrhizae are known to catalyze redox transformations or exude organic compounds which may significantly increase the bioavailability of various heavy metal ions for uptake (YANG, FENG, HE and STOFFELLA 2005). A recent study showed that the plant growth promoting bacteria, *Methylobacterium* and *Burkholderia,* alleviated heavy metal stress induced in *Lycopersicon esculentum* L. (MADHAIYAN, POONGUZHALI and SA 2007).

2.4.3 Transport and translocation of heavy metals

There is now extensive interest in heavy metal transport by plants because of the repercussions for **phytoremediation**. However, current knowledge of the transport processes for heavy metals across plant membranes at the molecular level, for the most part, remains elementary (WILLIAMS, PITTMAN and HALL 2000).

The transport of ions within plants and plant tissues involves several processes including (1) movement in the xylem, (2) movement in the phloem, and (3) storage, accumulation and immobilization (KABATA-PENDIAS 2001). Although **chelating ligands** are important in the control of cation translocation in plants, several other factors such as pH, oxidation-reduction state, competing cations, polymerization and the formation of insoluble salts (e.g. phosphate) preside over metal mobility within plant tissues (KABATA-PENDIAS 2001).

Many **transporters** involved in Cu, Fe and Zn uptake can transport a range of divalent cations but each transporter is generally transcriptionally and/or post-transcriptionally controlled by a particular metal (GROTZ and GUERINOT 2006). Given that intracellular levels of heavy metals must be carefully controlled, transporters denote good candidates for **regulation** (WILLIAMS, PITTMAN and HALL 2000). Until now there are no indications of how heavy metals may be regulated in higher plants but, potentially, this could arise at the transcriptional level (control on initiation rates, mRNA stability, differential mRNA splicing) or at the post-translational stage (targeting, stability) (WILLIAMS, PITTMAN and HALL 2000). Mechanisms for heavy metal transporters in plants have been reviewed by WILLIAMS, PITTMAN and HALL (2000) and COLANGELO and GUERINOT (2006).

2.4.4 Cellular mechanisms of heavy metal tolerance in plants

Tolerance of plant species and cultivars to elevated heavy metal concentrations could be achieved by excluding the uptake mechanisms from the root (KIRKHAM 2006), or by efflux or compartmentalization and detoxification of the metals following uptake (WILLIAMS, PITTMAN and HALL 2000).

Plants directly coordinate a particular element by using the most chemically suitable ligand to form stable non-toxic complexes (e.g. in the case of Cd, Pb and Ni) (SALT, PRINCE and PICKERING 2002). Alternatively, the element may be first chemically reduced to improve its tendency to be strongly coordinated (e.g. As). Chemical reduction can also lower the toxicity of an element (e.g. Cr), or be the starting point for the amalgamation of non-metals (e.g. Se) into organic compounds for detoxification. Thus, plants control both the oxidation state and coordination

environment for either their detoxification, transport, or both (SALT, PRINCE and PICKERING 2002).

Cellular mechanisms for metal tolerance can be classified into two basic strategies according to TONG, KNEER and ZHU (2004). The first strategy is to maintain a low concentration of the metal ions in the **cytoplasm** by preventing the metal from being transported across the **plasma membrane**. This is carried out by increased binding of metal ions to the cell wall, reduced uptake through customized ion channels or by pumping the metal out of the cell with active efflux pumps. The second strategy is to detoxify metal ions entering the cytoplasm through inactivation via **chelation** or alteration of a toxic ion into a less toxic or easier to handle form and/or **compartmentalization**. A common mechanism for detoxification of heavy metals in plants is the allotment of metals to apoplast tissues (e.g. trichomes and cell walls), chelation of the metal ions by a ligand, and finally the sequestration of the metal–ligand compound into the vacuole (YANG, FENG, HE and STOFFELLA 2005). Compartmentalization of metal ions is a key component of metal tolerance (ZHOU and QIU 2005) and compartmentalization of metals in the vacuole is most commonly observed (MCGRATH and ZHAO 2003; TONG, KNEER and ZHU 2004; KIRKHAM 2006).

2.5 HEAVY METALS IN MEDICINAL PLANTS

2.5.1 Sources of contamination

With the unregulated medicinal plant trade in many developing countries, several opportunities for contamination exist. According to CHAN (2003) potentially harmful contaminants in medicinal plants may come from:

- environments where the medicinal plants are grown and conditions where they are collected;
- conditions under which they are dried and processed;
- transport and storage conditions; and/or
- manufacturing processes during the final stage of preparation.

The correct **documentation** and **traceability** of medicinal plants that enter into regional and international trade need to be maintained and monitored. However, in the absence of regulatory controls, the safety and quality of medicinal plants vary considerably.

2.5.2 Effect of heavy metals on humans

Plants are an important link in the transfer of contaminants from the soil to humans (MCLAUGHLIN, PARKER and CLARKE 1999). Nearly half of the mean ingestion of Cd, Pb and Hg is as a result of food of plant origin (ISLAM, YANG, HE and MAHMOOD 2007).

Heavy metals differ extensively in their bioavailability or ability to enter organisms and cause toxicity. According to CAUSSY, GOCHFELD, GURZAU, NEAGU and RUEDEL (2003) the term **bioavailability** has not been used consistently in the literature. External bioavailability, also known as bioaccessibility, is mainly determined by the ability of the metal ions to be solubilized and released from environmental media (e.g. soil and food), whilst internal bioavailability determines the ability of the metal ions to be absorbed and reach the target organ, where it has a toxic effect (CAUSSY, GOCHFELD, GURZAU, NEAGU and RUEDEL 2003).

Despite being essential in human nutrition, sustained high dietary intakes of certain trace elements can lead to abnormal accumulation in tissues, or overloading of normal metabolic or transport pathways (RENWICK, FLYNN, FLETCHER, MÜLLER, TUIJTELAARS and VERHAGEN 2004). For example, Cu and Fe toxicity can result in considerable oxidative stress and subsequent tissue damage (URIU-ADAMS and KEEN 2005; ALIMONTI, BOCCA, LAMAZZA, FORTE, RAHIMI, MATTEI, FIORI, IACOMINO, SCHILLACI, DE MASI and PINO 2008). Considerable levels of trace elements in humans and their correlation with different diseases, including various types of cancer, have motivated extensive research toward quantitative determination of these elements in biological tissues (ALIMONTI, BOCCA, LAMAZZA, FORTE, RAHIMI, MATTEI, FIORI, IACOMINO, SCHILLACI, DE MASI and PINO 2008).

Continual heavy metal ingestion, even at low-levels, has damaging effects on humans and animals as there is no good mechanism for their elimination (ISLAM, YANG, HE and MAHMOOD 2007). Heavy metals exceeding acceptable physiological levels may be highly toxic, with almost all heavy metals being able to generate **free radicals**. A large amount of DNA damage appears to be linked to metal-induced free radicals (DESOIZE 2002).

Cadmium is a potent human **carcinogen** and human exposure has been associated with cancers of the lung, prostate, pancreas and kidneys (WAISBERG, JOSEPH, HALE and BEYERSMANN 2003). A critical mechanism contributing to the genotoxic potential of Cd is the inhibition of DNA repair (HARTWIG and SCHWERDTLE 2002). Similarly, Ni toxicity agitates cellular homeostasis through changes of intracellular Ca levels which results in oxidative stress (DENKHAUS and SALNIKOW 2002). DNA damage, DNA methylation or suppression of histone acetylation, caused by Ni toxicity, allows changes in gene expression to occur (DENKHAUS and SALNIKOW 2002). The correlation between cancer and a range of heavy metals is widely recognized by oncologists (DESOIZE 2002).

Investigations into **heavy metal poisoning** require comprehensive studies taking into consideration distribution and concentration of heavy metals in a range of contaminated media and identifying potential exposure routes (CAUSSY, GOCHFELD, GURZAU, NEAGU and RUEDEL 2003).

2.5.3 International standards regarding heavy metals in medicinal plants

Health, safety and quality assurance are key aspects with respect to regulatory requirements and standards worldwide. However, there are vast discrepancies between countries regarding **regulation requirements** to pledge safety and quality of plant-based products (DIEDERICHS, FEITER and WYNBERG 2006).

The WHO (2004) has issued a set of guidelines for safety monitoring of traditional herbal medicines which urges the development of national and regional guidelines/policies. Certain countries, including Canada, China, Malaysia, Singapore and Thailand, have developed their own national guidelines to ensure acceptable

levels of heavy metals in medicinal plants (**Table 2.1**). The WHO (1998) recommends maximum permissible levels in raw materials for As, Cd and Pb which amount to 1.0, 0.3 and 10 mg kg^{-1}, respectively. Despite the fact that certain essential elements can be toxic at high levels; the WHO limits for these metals have not yet been established.

Table 2.1: National limits for heavy metals in medicinal plants/herbal products (mg kg^{-1}) (WHO 2005).

Country	Raw / finished product	As	Cd	Cr	Cu	Hg	Pb
Canada	raw medicinal plant material	5	0.3	2		0.2	10
China	medicinal plant materials	2	1		20	0.5	10
Malaysia	finished herbal product	5				0.5	10
Singapore	finished herbal product	5			150	0.5	20
Thailand	raw medicinal plant material, finished herbal product	4	0.3				10
WHO recommendations		1	0.3				10

2.5.4 Studies regarding heavy metal contamination in medicinal plants

There have been many reports indicating that medicinal plants or traditional remedies contain significant or even toxic levels of metals (STEENKAMP, VON ARB and STEWART 2000; OBI, AKUNYILI, EKPO and ORISAKWE 2006). It is known that in certain cultural groups, such as Indian communities worldwide, heavy metals (e.g. Hg) are intentionally added to products for alleged medicinal properties (ERNST and COON 2001).

The examples in **Table 2.2** indicate an extensive range of heavy metals in medicinal plants which have been evaluated in various parts of the world. CHIZZOLA, MICHITSCH and FRANZ (2003) concluded that the heavy metal content of Austrian medicinal plants grown under common field conditions is not critical. However, as certain plant species were reported to have a tendency toward Cd accumulation, the study stated that careful choice of cultivation site and the management of soil conditions is imperative to avoid heavy metal contamination of medicinal plants. Results collected on elemental content in 31 kinds of aromatic and medicinal plants

collected from the south of Turkey revealed a considerable range between samples for Al, Fe and Zn (58–2 963, 45–1 800 and 7–48 mg kg^{-1} respectively). In addition, levels of Cd (0.5–1.05 mg kg^{-1}), Cr (2.66–24.7 mg kg^{-1}) and Ni (1.81–28.6 mg kg^{-1}) were detected in all samples (OZCAN and AKBULUT 2007).

Table 2.2: Examples of heavy metal assessment of medicinal plants from various regions/countries.

Region/Country	Elements assessed	Reference
Africa		
Egypt	Cd, Co, Cr, Cu, Pb, Ni, Mn, Fe, Sn, Zn	ABOU-ARAB, SOLIMAN KAWTHER, EL TANTAWY, BADEAA and KHAYRIA (1999)
Egypt	Cd, Co, Cr, Cu, Pb, Ni, Mn, Fe, Sn, Zn	ABOU-ARAB and ABOU DONIA (2000)
Egypt	Cd, Cu, Pb	DOGHEIM, ASHRAF, ALLA KHORSHID and FAHMY (2004)
Egypt	Ca, Cu, Mg, Mn, Fe, K, Na, Se, Zn	SHEDED, PULFORD and HAMED (2006)
Ghana	Al, Br, Ca, Cl, Co, Cu, Cr, K, Mn, Mg, Na, Rb, Sb, Sc, Ta, V, Zn	SERFOR-ARMAY, NYARKO, AKAHO, KYERE, OSAE and OPPONG-BOACHIE (2002)
Mali	Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	MAIGA, DIALLO, BYE and PAULSEN (2005)
Nigeria	Ca, Cu, Mn, Mg, K, Fe, Pb, Zn	AJASA, BELLO, IBRAHIM, OGUNWANDE and OLAWORE (2004)
Nigeria	Cd, Cu, Fe, Ni, Se, Zn, Pb, Hg	OBI, AKUNYILI, EKPO and ORISAKWE (2006)
Nigeria	Ca, Cd, Cr, Fe, K, Mn, Na, Mg, P, Pb, Zn	ABOLAJI, ADEBAYO and ODESANMI (2007)
South Africa	Cu, Pb, Mn, Hg, Se, Zn	STEENKAMP, VON ARB and STEWART (2000)
South Africa	U	STEENKAMP, STEWART, CHIMUKA and CUKROWSKA (2005)
South Africa	unspecified	STEENKAMP, CUKROWSKA and STEWART (2006)
Asia		
India	Cd, Pb	DWIVEDI and DEY (2002)
India	Cu, Cr, Mn, Ni, Cd, Pb, Zn	HAIDER, NAITHANI, BARTHWAL and KAKKAR (2004)
India	Cu, Co, Ni, Mn, Zn, Fe, Na, K, Ca, Mg, P, Al	BHATTACHARJEE, KAR and CHAKRAVARTY (2004)
Malaysia	Hg	ANG and LEE (2006)
Europe		
Austria	Cd, Cu, Fe, Mn, Pb, Zn	CHIZZOLA, MICHITSCH and FRANZ (2003)
Bulgaria	Cd, Cu, Pb, Zn	ANGELOVA, IVANOV and IVANOVA (2006)
Serbia	Cd, Pb, N, Zn	OBRATOV-PETKOVIC, POPOVIC, DELANOVIC and KADOVIC (2006)

Region/Country	Elements assessed	Reference
Croatia	Cd, Cu, Pb, Mn	SEKULIC´, MARTINIS and PEHAREC (2004)
Poland	Al, B, Ba, Bi, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, Se, Si, Ti, V, Zn	LESNIEWICZ, JAWORSKA and ZYRNICKI (2006)
Spain	Ca, Cd, Cu, Fe, Mn, N, Na, P, K, Mg, Mn, Zn	CALA, CASES and WALTER (2005)
Yugoslavia	Mn, Zn, Cu, Pb, Cd	ŠOVLJANSKI, LAZIC, MACKO and OBRADOVIC (1992)
Middle East		
Turkey	As, Ba, Ca, Cd, Co, Cr, Cu, Fe, I, Li, Mg, Mn, Ni, Pb, Se, Sn, Sr, Ti, V, Zn	LOZAK, SOLTYK, OSTAPCZUK and FIJALEK (2002)
Turkey	Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sr and Zn	BASGEL and ERDEMOGLU (2006)
Turkey	Al, B, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, Sr, V, Zn	OZCAN and AKBULUT (2007)
South America		
Argentina	Ca, Cu, K, Li, Mg, Mn, Na, Ni, Zn	GOMEZ, CERUTTI, OLSINA, SILVA and MARTINEZ (2004)
Argentina	Cd, Co, Pb, Al, Cr, Fe, V	GOMEZ, CERUTTI, SOMBRA, SILVA and MARTINEZ (2007)
Brazil	Cd, Hg, Pb	CALDAS and MACHADO (2004)

As African medicinal plants are commonly collected from wild populations (ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN 2000), heavy metal contamination may vary considerably. AJASA, BELLO, IBRAHIM, OGUNWANDE and OLAWORE (2004) revealed that all the heavy metals tested were accumulated to a greater or lesser extent by all 10 Nigerian medicinal plants screened. The highest mean levels of Cu and Zn were 24.4 mg kg^{-1} and 35.1 mg kg^{-1} respectively, while the highest Mn content reached 685 mg kg^{-1}. The highest mean concentration of Pb detected was 0.49 mg kg^{-1}. A further study on Nigerian herbal remedies revealed that 100% of the samples contained elevated amounts of heavy metals, suggesting possible heavy metal toxicity from Nigerian herbal products (OBI, AKUNYILI, EKPO and ORISAKWE 2006). The screening of Egyptian medicinal plants revealed that Pb and Cd were detected in 80% and 43% of the plant samples respectively (DOGHEIM, ASHRAF, ALLA, KHORSHID and FAHMY 2004).

Unfortunately, without **documentation** and **traceability** of medicinal plant source, it is not possible to trace the exact origin of contamination. Nonetheless, regardless of the interest in the screening of African medicinal plants in once off studies and despite certain medicinally used plants containing a high heavy metal content, no regulations have been passed for their monitoring.

2.5.5 Heavy metals in South African traditional medicine

Poisonings by traditional herbal remedies in South Africa are not uncommon, particularly in children (STEWART, MOAR, STEENKAMP and KOKOT 1999; STEENKAMP, STEWART, CUROWSKA and ZUCKERMAN 2002). An analysis of the Johannesburg forensic database over a 5 year period (1991–1995) revealed 206 cases in which a traditional remedy was either stated to be the cause of death or was found to be present in a case of poisoning with an unknown substance (STEWART, MOAR, STEENKAMP and KOKOT 1999). Heavy metals were accountable for 10% of the poisonings.

Senecio coronatus (Thunb.) Harv. (Asteraceae), one of the nine Ni hyperaccumulating plants in Africa (PRZYBYLOWICZ, PINEDA, PROZESKY and MESJASZ-PRZYBYLOWICZ 1995), is a widely used medicinal plant species in South Africa (DOLD and COCKS 2002). Toxicity reports confirm that root decoctions of *S. coronatus,* administered by enema, caused the fatal veno-occulusive disease in infants (SAVAGE and HUTCHINGS 1987). As discussed in **Section 2.5.2**, Ni toxicity has the potential to produce a range of pathologic effects in humans including skin allergies, lung fibrosis and cancer of the respiratory tract (KASPRZAK, SUNDERMAN and SALNIKOW 2003). Medicinally used *Datura metal* L. (Solanaceae) is an accumulator of Co and Ni and it is recommended as a phytomonitor (BHATTACHARJEE, KAR and CHAKRAVARTY 2004). Similarly, *Datura innoxia* Miller, Gard. is a metal tolerant species (KELLY, ANDREWS and DEWITT 2002). Both *Datura* species are widely used in South African traditional medicine even though their toxicity is well established (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996).

Helichrysum candolleanum H. Buek and *Blepharis diversispina* (Nees) C.B.Clarke tolerate high concentrations of metals (NKOANE, SAWULA, WIBETOE and LUND 2005). In South African many *Helichrysum sp.* and *Blepharis sp.* are used in traditional medicine (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). However, evaluation of heavy metal uptake by these species has not yet been considered.

A number of traditional herbal remedies are known to cause severe renal pathology, the mechanism of which is uncertain but it could be associated with heavy metal toxicity (STEENKAMP, VON ARB and STEWART 2000). Nevertheless, few comprehensive studies have been done to asses the heavy metal content in South African medicinal plants. A study by STEENKAMP, VON ARB and STEWART (2000) on heavy metal concentrations in plants and urine from patients treated with traditional remedies concluded that although few plants had high levels of toxic metals, the concentrations in those that did were sufficiently high to cause concern. Mercury levels were high in *Crotalaria agatiflora* Schweinf. seeds (5.4 mg kg^{-1}) and *Senecio latifolius* DC. roots (3.2 mg kg^{-1}) which are the parts of the plant used medicinally. *Datura stramonium* L., associated with fatal poisonings (DIKER, MARKOVITZ, ROTHMAN and SENDOVSKI 2007), showed particularly high levels of Pb (1.5 mg kg^{-1}).

The most recent study on heavy metals in South African medicinal plants by STEENKAMP, CUKROWSKA and STEWART (2006) examined 70 metals in 82 plant remedies and concluded no risk of metal toxicity from plant-based traditional medicines. Unfortunately, the scientific publication did not present a full data set of metal concentrations in individual plants or parts thereof nor did it specify which plants and metals were tested. In addition, the methodology was not thorough. Firstly, there was no indication as to whether these plants were bought from a medicinal market or shop, or collected from the wild. They were "*collected from the Adler Museum of Medicine (University of the Witwatersrand, Johannesburg)*" "*some years previously*". ("*The specimens in the museum were received from various parts of the country, although specific location was not documented*"). Thus, the specimens could have been from botanical gardens and not come into the "potential exposure route" of medicinal plants available to the public. Only examples of the results were given without the 70 metals being named. Secondly, no certified reference material (CRM) was mentioned. Certified reference material gives credibility to the digestion and determination method used (discussed in **Section 3.1.2**). The fact that only one plant sample was used to represent the entire population for that species is unrealistic. *"Each metal concentration was automatically measured in triplicate"* – this however only depicts a "pseudo" replicate without real reproducible replicates and it is unsound to make such assumptions. And lastly, in a previous publication the

author states that the analytical technique could not determine the speciation of Cr since Cr(VI) is highly toxic to humans while Cr(III) is an essential nutrient (STEENKAMP, STEWART, CUROWSKA and ZUCKERMAN 2002). However, in this particular publication using the same technique, it is stated *"chromium is regarded as helpful in the prevention and control of diabetes"*. This statement is too general as plants more readily take up Cr(VI) than Cr(III), so there is no reason to assume that it is safe for human consumption.

2.5.6 Monitoring heavy metals in South African medicinal plants

Certain countries have developed their own set of guidelines/limits for heavy metals in medicinal plants (**Table 2.1**). However, the South African traditional medicinal plant trade does not carry out risk control in the collection or preparation of remedies. Currently comprehensive safety and efficacy data on South African traditional medicines are lacking (SPRINGFIELD, EAGLES and SCOTT 2005). As environmental conditions are increasingly considered and form part of international trade agreements (WHYTE 1995), commercially produced traditional medicines will need to meet health, safety and quality assurance standards in order to be traded on the international market (DIEDERICHS, FEITER and WYNBERG 2006).

Evaluating and monitoring heavy metal contamination is an essential step in improving the overall safety and quality of widely used medicinal plants which will in turn result in safeguarding the consumer. Eliminating potential health hazards, which includes identifying heavy metal accumulatory species, and focusing on correct farming practices is an essential step towards increasing agricultural productivity and ensuring product safety (WILSON and OTSUKI 2004).

2.6 SOUTH AFRICAN MEDICINAL PLANTS INVESTIGATED IN THIS STUDY

The range of South African medicinal plants chosen for this study represents a variety of plant species from various habitats and plant families. The selection was based on **popularity** in local communities as indicated by the literature, **availability** at the informal street markets, sufficient **stock plants** from the University of KwaZulu-Natal Botanical Gardens and **seed abundance**.

2.6.1 *Acacia caffra* (Thunb.) Willd.

Acacia caffra (Fabaceae) has a wide habitat tolerance and is found from the coast to highveld areas of South Africa (SMIT 1999). It is a small to medium-sized, single stem tree or a small multiple-stemmed shrub, which grows to a height of 5 to 7 m (SMIT 1999).

The leaves, bark and roots of *A. caffra* are used medicinally (BHAT and JACOBS 1995; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). The leaves are boiled in hot water, cooled and filtered and the decoction drunk for fever and colds (BHAT and JACOBS 1995). Alternatively, children chew and swallow the leaves to relieve abdominal pains (DLISANI and BHAT 1999). The bark, which characteristically contains tannins, is made into an infusion and is taken as a blood cleansing emetic (WATT and BREYER-BRANDWYK 1962). The boiled roots are used to treat stomach disorders (DLISANI and BHAT 1999).

2.6.2 *Agathosma betulina* (Berg.) Pillans

Agathosma betulina (Rutaceae) is endemic to South Africa and has a restricted natural distribution in the mountains of the Western Cape Province. It is a small-gland-dotted shrub of up to 2 m in height, with small typically rounded leaves, with tips that curve backwards (VAN WYK and WINK 2004).

The leaves of *A. betulina* are used to treat sprains, pain, arthritis, bladder and kidney ailments, back pain, stomach pain, fever and for the prevention of cancer (THRING and WEITZ 2006). Dried leaves are placed on a cloth, sprinkled with brandy or vinegar and the cloth wrapped around the affected area to relieve pain. Alternatively, a tea is made from the fresh leaves (THRING and WEITZ 2006).

Agathosma betulina contains an essential oil with limonene, isomenthone, diosphenol and terpin-4-ol as the main components (VAN WYK and WINK 2004).

2.6.3 *Albuca setosa* Jacq.

Albuca setosa (Hyacinthaceae) is distributed in summer rainfall areas, on rocky ground up to 2 400 m (POOLEY 1998). The large fleshy underground bulb contains erect white or yellow flowers with a green stripe (VAN WYK and MALAN 1997).

The bulbs, administered to both people and animals, are used in ritual cleansing and as a protective charm against lightning, and to end quarrels between enemies (POOLEY 1998; ARNOLD, PRENTICE, HAWKER, SNYMAN, TOMALIN, CROUCH and POTTAS-BIRCHER 2002). While there is little information in the literature regarding the medicinal usage of *A. setosa*, an unspecified *Albuca* species is reportedly used as a purgative and vermifuge (GERSTNER 1938).

2.6.4 *Bowiea volubilis* Harv. ex Hook. f.

Bowiea volubilis (Hyacinthaceae) is a bulbous geophyte widely distributed in the eastern parts of South Africa (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). It is usually found in colonies in grassland, thickets or forest edges, often amongst rocks. The bulbs are white with green, fleshy bulb scales (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006). The flowers are small, greenish in colour and rather inconspicuous (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).

Bulbs of *B. volubilis* are used to treat a variety of ailments, including bladder problems, sterility in women and oedema (PUJOL 1990). It is also used for cleansing blood, skin complaints, headaches and as a love charm. The fresh juice may be rubbed into the skin of a sick person or a decoction applied for sore eyes. A hot water extraction is also used (WATT and BREYER-BRANDWYK 1962).

Bowiea volubilis contains the cardiac glycoside, bufadienolide (VAN WYK, VAN HEERDEN and VAN OUDTSHOORN 2002). All parts of the plant are extremely poisonous and internal use is potentially lethal (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). Symptoms of poisoning include vomiting, purging, excessive salivation and irregular heart palpitations (WATT and BREYER-BRANDWYK 1962).

2.6.5 *Dioscorea dregeana* (Kunth) Dur. & Shinz.

Dioscorea dregeana (Dioscoreaceae) is limited to the moist, eastern part of South Africa (VAN WYK, VAN HEERDEN and VAN OUDTSHOORN 2002). It is a tuberous geophyte found in coastal and midlands forests. The underground tuber is large, dark on the outside with a covering of fine roots (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006).

The large fleshy tubers and roots of *D. dregeana* are used to treat various convulsions, demential crises and epilepsy. The tubers are hollowed out and the water which is heated in it is used as a lotion on cuts and sores (WATT and BREYER-BRANDWYK 1962). A small piece of the root boiled in water may be used in emergency situations to treat nervous spasms and cramps (PUJOL 1990). The exact dosage is critical as the plant is known to be toxic (WATT and BREYER-BRANDWYK 1962).

2.6.6 *Dioscorea sylvatica* (Kunth) Eckl*.*

Dioscorea sylvatica (Dioscoreaceae), a slender climber of up to 15 m, is found in a wide range of habitats, including scrub, forests, coastal regions and mountains (POOLEY 1998). The leaves are heart shaped (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006) and the underground tubers are characteristically dark brown, corky and flattened, with reticulate markings (POOLEY 1998).

The tuber, containing the alkaloid diosgenin, is used to make a drink for the treatment of rheumatism and fibroids. It is also used in the treatment of cuts and wounds (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006). *Dioscorea sylvatica* is a relatively uncommon species which is threatened in easily reached locations (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006).

2.6.7 *Eucomis autumnalis* (Mill.) Chitt.

Eucomis autumnalis (Hyacinthaceae) is a bulbous geophyte found throughout southern Africa in damp grassland from the coast to 2 450 m above sea level

(CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006). The plant contains white to pale yellow-green flowers and the bulbs grow to about 10 cm in diameter (POOLEY 1998). However, with around 73 tons traded per annum (MANDER 1998), plant populations are declining rapidly and are becoming scarce (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006).

An enema of a bulb decoction is commonly used for low backache, to assist in post-operative recovery and to aid in healing fractures (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). It is also used to treat urinary and pulmonary ailments (POOLEY 1998).

2.6.8 *Eucomis humilis* Baker

Eucomis humilis (Hyacinthaceae) is found predominantly in KwaZulu-Natal (POOLEY 1998). The large bulbs, growing up to around 15 cm in diameter, are commonly found in grasslands and at the base of cliffs (KILLICK 1990). The flowers are white with purple margins and bases (KILLICK 1990).

Although a number of *Eucomis* species are used in traditional medicine (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996), *E. humilis* has not been specifically mentioned in the literature. Nonetheless, bulbous extracts have shown high levels of anti-inflammatory activity, which are comparable to other commonly used *Eucomis* species (TAYLOR and VAN STADEN 2002).

2.6.9 *Helichrysum cymosum* (L.) D. Don

Helichrysum cymosum (Asteraceae) is found in moist places from the South West Cape to KwaZulu-Natal (POOLEY 1998). It is a straggling or sprawling shrublet which grows up to 1 m high and contains yellow flower heads crowded in flat-topped clusters (MANNING 2003).

The young and mature leaves of *H. cymosum* are boiled and filtered and the decoction is taken daily for colds and fever (DLISANI and BHAT 1999).

2.6.10 *Merwilla plumbea* (Lindl.) Speta

Merwilla plumbea (Hyacinthaceae) [*Merwilla natalensis* (Planchon) Speta] is found in damp grassland, cliffs and rocky slopes (POOLEY 1998). The flowers are pale to deep purple or blue (POOLEY 1998). In their natural habitat, the bulbs are often half exposed, revealing papery dark brown bulb scales (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006). *Merwilla plumbea* is ranked as one of the most popular plant species sold at many of the medicinal markets in South Africa, with around 95 tons traded per annum (MANDER 1998).

The bulbs of *M. plumbea* are used for gastro-intestinal ailments including stomachaches, constipation, intestinal worms, diarrhoea, dysentery, nausea and indigestion (HUTCHINGS 1989). The bulbs are also used for the treatment of boils, sores, wounds, sprains and to remove scar tissue. They are also commonly used for the treatment of female infertility and to enhance male libido (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006).

It is thought that *M. plumbea* may contain cardiac glycosides of the bufadienolide type, such as scillaren A. The presence of such glycosides needs to be confirmed (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). The phytochemical screening of *M. plumbea* bulbs revealed the presence of saponins and bufadienolides within the bulbs (SPARG, VAN STADEN and JÄGER 2002).

2.6.11 *Tulbaghia violacea* Harv.

Tulbaghia violacea (Alliaceae) is found in forest margins and stream banks of the eastern part of South Africa. It is an evergreen perennial, 20-30 cm high, with mauve flowers in rounded clusters (MANNING 2003).

The bulbs of *T. violacea* are used for the treatment of fever and colds, asthma, tuberculosis, and gastrointestinal ailments (KUBEC, VELIŠEK and MUSAH 2002). The freshly harvested bulbs are boiled in water and the decoction either taken orally or as an enema (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). However, extensive consumption of this plant has been associated with a variety of undesirable

symptoms, such as abdominal pain, inflammation, and gastroenteritis (KUBEC, VELIŠEK and MUSAH 2002).

2.6.12 *Vernonia neocorymbosa* Hilliard

Vernonia neocorymbosa (Asteraceae) is found on rocky hillsides in mountain grasslands or in scrub forest margins from Eastern Cape to Limpopo Province. It is a shrub, with clusters of mauve to pink flowers, and grows up to 1.5 m high (SCHMIDT, LOTTER and MCCLELAND 2002).

The leaves and stems are used medicinally (MANDER 1998) to treat colds, stomach aches, hysteria, epilepsy and to ensure easy child birth (POOLEY 1998).

3 Variation in heavy metals and microelements in South African medicinal plants obtained from informal street markets

3.1 INTRODUCTION

3.1.1 Monitoring heavy metal contamination of medicinal plants

Rural plant gatherers are anxious to generate a cash income, and will therefore harvest large numbers of medicinal plants for sale to the markets (MANDER 1998). South African medicinal plants are frequently collected from wild populations (ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN 2000) which could cause not only a threat to medicinal plant biodiversity and population stability, but also raises concerns about possible contamination of the plant material due to anthropogenic activities polluting the environment. Furthermore, storage and manufacturing methods are not regulated and contamination through improper practices may occur. According to the World Health Organization (WHO) (1998), heavy metal contamination of medicinal plants should be monitored to ensure their safety.

3.1.2 Heavy metal analysis of plant material

In general, during analysis of metals, one should always aim to use the best and most appropriate methods whenever possible. To begin with, the plant material needs to be properly digested. Digestion of samples is carried out to convert solid samples into aqueous solutions suitable for analysis. Mineralization of plant material may be efficiently performed in two ways: dry oxidation or wet digestion. Dry oxidation is done by heating the sample in a muffle furnace which ensures the decomposition of organic matter at high temperatures (450-550 °C). The ash obtained is commonly dissolved in nitric or hydrochloric acids. With the use of such high temperatures, it may be assumed that volatile elements (e.g. As, Hg and Se) are partly or completely lost. For this reason, wet digestion procedures at lower

temperatures may ensure better elemental recovery (HOENIG, BAETEN, VANHENTENRIJK, VASSILEVA and QUEVAUVILLER 1998).

Wet digestion involves the destruction of organic matter through the use of both heat and acids. Determining the appropriate acids for sample decomposition is dependent on the type of analysis to be carried out. The most commonly used acids are nitric, sulfuric, hydrochloric, hydrofluoric and/or perchloric acids. Perchloric acid is a very effective destructive agent, but it has the risk of explosion (PLANK 1992; HOENIG 1995). Hydrogen peroxide is often added to an acid combination to enhance digestion of the organic matter, but this procedure is not powerful enough to digest siliceous material (SHTANGEEVA 2005). The elementary difference between plant and animal inorganic matrices is the content of silicon, which is one of the main components of soil and may be directly assimilated by plants (HOENIG, BAETEN, VANHENTENRIJK, VASSILEVA and QUEVAUVILLER 1998). The relatively abundant silica can however be volatilized during a hydrofluoric acid (HF) attack (HOENIG 1995).

The WHO (2005) recommends microwave digestion for plant digestion. The use of microwave ovens to aid acid digestion is well-documented (SMITH and ARSENAULT 1996; HOENIG, BAETEN, VANHENTENRIJK, VASSILEVA and QUEVAUVILLER 1998) and can be used as an alternative to open air hot plate digestion procedures. The microwave process offers reduced risk of contamination, reduces the amount of acid used and decreases digestion time compared with traditional methods (MARGUÍ, QUERALT, CARVALHO and HIDALGO 2005). However, a report by TÜZEN (2003) comparing dry, wet and microwave digestion methods showed no statistically significant differences in elemental recovery; with similar findings from RODUSHKIN, RUTH and HUHTASAARI (1999). Except for the fact that wet digestion is more time-consuming and complicated than microwave digestion, there are no advantages in terms of efficiency (MESTER, ANGELONE, BRUNORI, CREMISINI, MUNTAU and MORABITO 1999). The main disadvantage of the microwave method is its greater expense and this will compel many scientists, especially from developing countries, to resort to wet digestion.

Irrespective of the technique used, it is crucial to ensure that all methods are fully validated. A certified reference material (CRM) forms an important part of any analysis. Certified reference materials contain the analyte(s) in an authentic matrix (e.g. olive leaves) and have undergone testing in a number of laboratories. In this way an unbiased estimate of the analyte concentration is obtained, which is certified by the issuing body. The International Organization for Standardization (ISO) defines certified reference materials as follows: *A reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by uncertainty at a stated level of confidence.* Certified reference materials contain a range of different elements at different certified concentrations and can be used to verify that the entire analytical method from sample preparation to the results obtained is accurate. This is important as it ensures the digestion procedure is complete, the instrument is optimized and the analytical method provides accurate results.

Currently there are no regulatory safety standards for South African medicinal plants and heavy metal monitoring is uncommon. The aim of this study was to assess the variation of heavy metals and microelements found in medicinal plant species sold at informal street markets.

3.2 MATERIALS AND METHODS

3.2.1 Sample collection

In this study, eight medicinal plants were investigated (**Table 3.1**). A selection of similar sized plant parts (n=5, unless otherwise specified) were purchased from traders at an informal outdoor market in Pietermaritzburg, while *Agathosma betulina*, indigenous to the Western Cape Province, was purchased from a Cape Town market.

Table 3.1: Plant species investigated for heavy metal contamination.

Botanical name	Family	Plant parts collected
Acacia caffra (Thunb.) Willd.	Fabaceae	Root
Agathosma betulina (Berg.) Pillans	Rutaceae	Leaves + stem
Bowiea volubilis Harv. ex Hook. f.	Hyacinthaceae	Bulb
Dioscorea dregeana (Kunth) Dur. & Shinz	Dioscoreaceae	Tuber
Eucomis autumnalis (Mill.) Chitt.	Hyacinthaceae	Bulb
Helichrysum cymosum (L.) D. Don	Asteraceae	Leaves + stem
Merwilla plumbea (Lindl.) Speta	Hyacinthaceae	Bulb
Vernonia neocorymbosa Hilliard	Asteraceae	Root

3.2.2 Preparation of samples

Plant parts were rinsed under tap water followed by distilled water before being cut into small pieces and dried at 50 °C for approximately 72 h. Once dry, the individual plant parts were ground into a fine powder (< 0.5 mm) using an IKA A11 analytical laboratory grinder (IKA Works, Inc.), placed in air-tight containers, and stored in the dark at room temperature until analysis. To minimize the risk of contamination, all glassware used was immersed in 10% HNO_3 for 24 h, washed with distilled water and rinsed with ultra-pure water (UP) before use.

3.2.3 Elemental analysis

3.2.3.1 Plant digestion

A method was developed based on the WHO (2005) recommendation and modified to suit our working conditions. Borosilicate glass digestion tubes, containing 0.5 g of homogenized plant material and 10 ml HNO_3-HCl-H_2O_2 (8:1:1, v/v/v) were placed on a heating block with the temperature increasing to 120 °C over 3 h. After digestion was complete, the clear, colourless solution was transferred to a 50 ml volumetric flask. Each digestion tube was rinsed with ultra-pure water to collect any possible residue, and added to the volumetric flask which was made up to volume with UP water. The dilute samples were stored in 100 ml plastic bottles (high density polyethylene) until analysis. Each plant sample was digested and analyzed in

triplicate. The blank solution was a mixture of 8 ml HNO_3, 1 ml HCl and 1 ml H_2O_2. All reagents (55% HNO_3, 32% HCl, 30% H_2O_2), supplied by Merck (Germany), were of analytical grade. Methods and calibrations were fine-tuned until the results were within the acceptable range of the CRM.

3.2.3.2 Standard solutions

Standard solutions were prepared from 1 000 mg l^{-1} stock solutions. High purity stocks were purchased (BDH Spectrosol®, Fluka Chemika®). Concentration range of calibration standards were 0.00125 – 5 mg l^{-1} for As, B, Cd, Co, Cu, Mn, Mo, Ni, Pb and Zn, and 0.0125 – 50 mg l^{-1} for Fe. The standards were made up with UP water. All standards and blanks were made up with the appropriate amount of HNO_3 to ensure the matrix effects were minimal. For each set of analyses, a calibration was carried out.

3.2.3.3 Analytical instrumentation

Inductively coupled plasma-optical emission spectrophotometry (ICP-OES) was preferred because it provides a multi-elemental analysis and supports a broad linear calibration range. Elemental analysis was performed using ICP-OES (Varian 720-ES, Varian, Palo Alto, CA, USA). The operating conditions are presented in **Table 3.2**.

Table 3.2: ICP-OES operating conditions for determination of heavy metals and microelements in plant samples.

Parameter	Level
Power	1.00 kW
Plasma flow	15.0 l min^{-1}
Auxiliary flow	1.50 l min^{-1}
Nebulizer flow	0.75 l min^{-1}
Replicate read time	1 s
Instrument stabilizer delay	15 s
Sample uptake delay	50 s
Pump rate	15 rpm

3.2.3.4 Wavelength

It is necessary to choose the appropriate wavelength where there is minimum interference from other elements. Selected wavelengths are presented in **Table 3.3**.

3.2.3.5 Nebuliser

Two different nebulisers were used in this study. A standard glass concentric nebuliser (with cyclonic glass spray chamber) was used for the elements with higher concentrations (B, Fe, Mn, Zn). Because of its ability to achieve lower detection limits, an ultrasonic nebuliser (Cetac U-5000 AT$^+$, Cetac Technologies Inc. Omaha, Nebraska, USA) was used for determination of elements present in very low concentrations (As, Cd, Co, Cu, Mo, Ni, Pb).

3.2.4 Data analysis

Variations in element concentrations amongst plant parts of each species were analyzed using one-way analysis of variance (ANOVA) and Tukey's confidence level ($p < 0.05$) was tested for pair-wise comparison. SPSS$^®$ (SPSS Inc., IL 60606-6412, USA) release 15 statistical software was used.

3.3   RESULTS AND DISCUSSION

3.3.1 Certified reference material

Certified reference material (NCS DC 73349 bush branches and leaves) was purchased from China National Analysis Centre for Iron and Steel supplied by Industrial Analytical (Pty) Ltd. (Johannesburg, South Africa). The CRM was carried through the same analytical procedure as the samples. The results indicate that the digestion procedure was complete (**Table 3.3**).

Table 3.3: Results of determination of elements by ICP-OES in certified reference material (mg kg^{-1}).

Element	Wavelength (nm)	Certified value	Determined value (mean ± S.D.; n=4)
Arsenic (As)	197.198	0.95 ± 0.12	1.04 ± 0.33
Boron (B)	249.772	34.0 ± 7	34.9 ± 1.14
Cadmium (Cd)	228.802	0.14 ± 0.06	0.14 ± 0.01
Cobalt (Co)	228.615	0.39 ± 0.05	0.34 ± 0.01
Copper (Cu)	327.395	5.2 ± 0.5	4.8 ± 0.14
Iron (Fe)	238.204	1020 ± 67	959 ± 11
Manganese (Mn)	257.610	58.0 ± 6	57.1 ± 4.6
Molybdenum (Mo)	202.032	0.26 ± 0.04	0.26 ± 0.06
Nickel (Ni)	231.604	1.7 ± 0.4	1.9 ± 0.47
Lead (Pb)	217.000	7.1 ± 1.1	6.17 ± 0.32
Zinc (Zn)	202.548	20.6 ± 2.2	21.1 ± 0.73

3.3.2 Heavy metals

It is clear from the results obtained from the plant samples (**Figures 3.1** and **3.2**) that heavy metal content varied widely between samples. Samples of South Africa's top selling medicinal bulbs, *B. volubilis*, *E. autumnalis* and *M. plumbea*, exceeded the WHO (1998) limits for As and Cd (1 and 0.3 mg kg^{-1} respectively) (**Figure 3.1**). Arsenic concentrations in plant parts varied from 0.35 to 2.3 mg kg^{-1} (**Figures 3.1** and **3.2**) with the highest content found in *B. volubilis.* Of the *A. caffra* roots and *M. plumbea* bulbs investigated, 60% contained Cd (detection limit - 0.02 mg kg^{-1}). All other plants parts analyzed contained varying degrees of Cd with *B. volubilis* bulbs containing the highest level (0.88 mg kg^{-1}) (**Figure 3.1**).

Although Co is known to be released into the atmosphere from anthropological activities such as coal and fuel oil burning, data concerning Co pollution in plants is scarce (KABATA-PENDIAS 2001). Cobalt, is essential to both plants and humans (HE, YANG and STOFFELLA 2005) and was detected in 60% and 80% of the *M. plumbea* bulbs and *Dioscorea dregeana* tubers respectively (detection limit - 0.03 mg kg^{-1}). However, Co was not detected in samples of *Acacia caffra* roots or leaves and stems of *Agathosma betulina* (**Figure 3.2**). Cobalt in *E. autumnalis* ranged from 0.312 to 1.65 mg kg^{-1}. All plant samples contained Ni which ranged from 2.1 to 8.3

FIG 3.1

FIG 3.2

mg kg^{-1} (**Figures 3.1** and **3.2**) with the maximum recorded in *A. caffra* roots. Lead was found in all plant samples, however, none were over the WHO limit of 10 mg kg^{-1} (**Figures 3.1** and **3.2**). *Eucomis autumnalis* bulbs showed a 10-fold statistically significant Pb variation (0.4 - 4.7 mg kg^{-1}) between the samples analyzed.

Contamination of soil by a single metal is a rare phenomenon. Usually where one metal is highly concentrated, there are others. Compared to other *B. volubilis* samples, sample 1 showed significantly higher As (2.3 mg kg^{-1}), Co (0.74 mg kg^{-1}), Pb (2.9 mg kg^{-1}) and Ni (4.9 mg kg^{-1}) concentrations as well as high Cd (0.88 mg kg^{-1}) (**Figure 3.1**). Similarly, sample 4 of *E. autumnalis* contained significantly higher levels of Co (1.7 mg kg^{-1}), Ni (4.9 mg kg^{-1}) and Pb (4.7 mg kg^{-1}), with high As content (1.2 mg kg^{-1}) indicating a mixed metal contamination. This study shows considerably higher levels of Pb and Ni contamination of medicinal plants compared to medicinal plants from markets in Egypt (ABOU-ARAB, SOLIMAN KAWTHER, EL TANTAWY, BADEAA and KHAYRIA 1999) and Turkey (BASGEL and ERDEMOGLU 2006). With medicinal plant material displayed in the open due to the poor infrastructure of South African informal markets, additional contamination caused by exposure to vehicular and urban emissions may be responsible for these higher levels.

Toxicity symptoms following the ingestion of certain South African medicinal plants include abdominal pain, vomiting, and renal failure (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). Poisoning due to heavy metal levels in medicinal plants cannot be ruled out. Failure to establish the true cause of toxicity could compromise the accurate diagnosis of the patient complaints and diseases.

3.3.3 Microelements

In all the samples tested, B was detected in the range of 6 to 51.3 mg kg^{-1} with the highest level found in the leaves and stems of *A. betulina* (**Table 3.4**). Copper concentrations were between 0.3 and 11.8 mg kg^{-1}. Although *Helichrysum candolleanum,* has been recommended as a Cu/Ni indicator plant due to its ability to accumulate metals (NKOANE, SAWULA, WIBETOE and LUND 2005), the species

TABLE 3.4

TABLE 3.5

evaluated in this study (*H. cymosum*) showed a low Cu content (0.4 to 1.2 mg kg^{-1}) with statistically significant variation. The highest Cu content of 11.8 mg kg^{-1} was recorded in sample 1 of *B. volubilis* (**Table 3.5**). Manganese concentrations in various plant parts ranged from 7.3 to 2 462 mg kg^{-1}. The leaves and stems of *Agathosma betulina* contained the highest Mn content (2 462 mg kg^{-1}). Manganese hyperaccumulators are determined by the ability to accumulate < 10 000 mg kg^{-1} dry weight in the shoots (MCGRATH and ZHAO 2003). Thus, although *A. betulina* cannot be considered a hyperaccumulator, the accumulatory traits need further investigation. In this study, low levels of Mo (0.07 to 0.36 mg kg^{-1}) were found in all plant samples. Zinc levels varied from 4.2 to 53.6 mg kg^{-1} with the highest level detected in *D. dregeana*. Iron levels varied significantly amongst individual plant samples (**Tables 3.4** and **3.5**). For example, the Fe content in sample 1 and 4 of *E. autumnalis* bulbs contained 146 and 2 669 mg kg^{-1} respectively (**Table 3.4**). These results imply Fe contamination in plants varies depending on sight of harvest/collection.

The presence of microelements in medicinal plants may be correlated with therapeutic properties against various health disorders. The levels of B, Cu, Mo and Zn found in this study were comparable to microelemental content in a variety of foods (KABATA-PENDIAS 2001). Although certain elements are vital for the well-being of humans, increased levels have been known to cause various health disorders (TOYOKUNI 1996; THEOPHANIDES and ANASTASSOPOULOU 2002; URIU-ADAMS and KEEN 2005). Elevated Mn levels have been found in the urine of patients admitted to hospital following treatment with traditional remedies (STEENKAMP, VON ARB and STEWART 2000). Oral ingestion of *A. betulina* may result in gastro-intestinal irritation and therefore usage is discouraged for patients suffering from kidney infections (SCOTT and SPRINGFIELD 2004). Although this study reveals high Mn levels in *A. betulina,* bioavailability of this potentially harmful metal needs to be established before toxicity can be assessed. Nevertheless, as Mn toxicity is more prevalent than Mn deficiency in humans (ERIKSON, THOMPSON, ASCHNER and ASCHNER 2007), caution should be exercised. An increase in body Fe stores may be associated with abdominal pain, vomiting, and renal failure (HEIMBACH, RIETH, MOHAMEDSHAH, SLESINSKI, SAMUEL-FERNANDO, SHEEHAN, DICKMANN and BORZELLECA 2000). Similar pathological symptoms

have been reported after the ingestion of *B. volubilis* and *E. autumnalis* (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). Although many Hyacinthaceae species, including *B. volubilis*, contain potentially toxic cardiac glycosides (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996; MARX, PRETORIUS, ESPAG and BESTER 2005), poisoning following the ingestion of high Fe containing bulbs should be thoroughly investigated.

Due to the undisclosed harvest locations of South African medicinal plants, potential exposure to a variety of environmental pollutants is grounds for concern. As shown in the results presented in this chapter, variable concentrations of heavy metals and essential elements are found in plant materials used by consumers of the South African traditional health care system. These findings highlight a potential health threat with the safety of patients being compromised due to the lack of monitoring and regulation. Recognizing toxicity symptoms due to metal ingestion is imperative so that immediate medical action be taken.

The cultivation of South African medicinal plants would allow for good agricultural practice (GAP) thus allowing monitoring of harvest sites and collection procedures.

3.4 SUMMARY

- The variation in five heavy metals (As, Cd, Co, Ni, Pb) and six microelements (B, Cu, Fe, Mn, Mo, Zn) was determined in commonly used South African medicinal plants obtained from informal street markets.
- Heavy metals and microelements varied markedly.
- Samples of *B. volubilis*, *E. autumnalis* and *M. plumbea* contained As and Cd at levels exceeding the WHO limits of 1 and 0.3 mg kg^{-1} respectively.
- Pb and Ni were detected in all samples.
- The levels of B, Cu, Mo and Zn were comparable to microelemental content in a variety of foods.
- Elevated Mn content (2 462 mg kg^{-1}) was recorded in leaves and stems of *A. betulina.*
- Multiple metal contamination of medicinal plant parts gives grounds for concern.

- These findings indicate that the safety of patients is compromised due to the lack of monitoring and regulation.

4 Uptake and distribution of Cd in Hyacinthaceae species

4.1 INTRODUCTION

4.1.1 Cultivation of medicinal Hyacinthaceae species

A number of studies on metal elements in selected rivers and dams in South Africa have shown a high concentration of Cd, with most of the measured concentrations exceeding the South African water quality guidelines (FATOKI and AWOFOLU 2003; OKONKWO and MOTHIBA 2005). Plant species vary considerably in their tolerance to Cd in a growth medium (DE LA ROSA, PERALTA-VIDEA, MONTES, PARSONS, CANO-AGUILERA and GARDEA-TORRESDEY 2004). Although Cd is considered non-essential to plants (VAN DER PERK 2006), it is readily taken up (KABATA-PENDIAS and PENDIAS 1984). Certain heavy metals have the tendency to accumulate in the roots (e.g. As). However, Cd has a higher affinity to accumulate in above ground plant parts (CLEMENS 2006). This poses a potential threat to human health as accumulation in edible plant parts represents the primary route of toxic metal entry into the human food-chain (MCLAUGHLIN, PARKER and CLARKE 1999). Findings in **Chapter 3** showed Cd levels in certain Hyacinthaceae bulbs, being above the acceptable norms. Thus highlighting the problem of heavy metal contamination of medicinally used bulbs and emphasizing the importance of monitoring collection sites.

One of the tasks of modern day agriculture is to safeguard the production of high quality food, in a sustainable natural environment under the prerequisite of pollution not exceeding accepted norms (DACH and STARMANS 2005). As specified by the European Union 'farm to table' policy (CEC 2000), farmers are identified as the link in the food chain having primary responsibility for food safety (DACH and STARMANS 2005). According to the WHO guidelines on Good Agricultural and Collection Practices for Medicinal Plants (2003), risks of contamination caused by environmental pollutants should be avoided. It is imperative that agricultural soils comply with maximum permissible limits as stipulated by local, regional and/or national regulatory authorities. The Water Research Commission of South Africa has

issued a set of maximum permissible heavy metal levels in agricultural soils (WRC 1997) (**Table 4.1**).

Table 4.1: Maximum permissible metal and inorganic content in South African soils (mg kg^{-1}) (WRC 1997).

Metal	mg kg^{-1}	Metal	mg kg^{-1}
As	2	Hg	0.5
Cd	2	Ni	50
Cr	80	Pb	6.6
Cu	6.6	Zn	46.5

The family Hyacinthaceae includes geophytes with a bulb as the underground storage organ. These bulbs are widely used in South African traditional medicine to alleviate a variety of symptoms (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). The chemistry, bioactivity and ethnobotanical aspects of Hyacinthaceae have been summarized by POHL, CROUCH and MULHOLLAND (2000). Many important Hyacinthaceae species have been recommended for cultivation (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006).

Despite the importance of monitoring heavy metal accumulation in medicinal plants coupled with increasing emphasis on cultivation, it is interesting to note that no work has been done on heavy metal accumulation in South African medicinal plants grown as agricultural crops. Thus, the aim of these experiments was to assess the effect of Cd on *Albuca setosa*, *Eucomis autumnalis*, *Eucomis humilis* and *Merwilla plumbea* (Hyacinthaceae), placing emphasis on the medicinally used bulbs.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

Stock plants raised in the shade house at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus (29° 37.55 S; 30° 24.13 E), were used for this experiment. Plants were transferred into pots containing sterilized, acid washed quartz sand and fertilized with 50% Hoagland's nutrient solution (HS) (HOAGLAND

and SNYDER 1933) (**Appendix A**). At the beginning of spring, a one-month adaptation period was given to all plants and only healthy plants were chosen for experiments. *Albuca setosa* and *Eucomis sp.* were grown in greenhouses whilst *M. plumbea* plants were grown in controlled environment chambers (16:8 h light and dark conditions with a photosynthetic photon flux density of 80.4 ± 3.5 µmol m^{-2} s^{-1} at 25 °C). The pots were arranged in a randomized block design.

All reagents used were analytical grade. To avoid nutrient deficiency, HS was added to the plant root environment weekly until the experiment was terminated. Where indicated, Cd (in the form of $CdCl_2^.H_2O$) was added to the HS. Hoagland's nutrient solution (without Cd) served as control. Additional watering of plants (200 ml per pot) took place every alternate day.

4.2.1.1 *Albuca setosa* Jacq.

Mature plants of equal size (bulb diameter: 5.5 ± 1.2 cm; plant age: 2-3 years old) were planted in 30 cm pots containing sterile quartz sand with 16 plants per treatment. Hoagland's nutrient solution supplemented with Cd at 5 mg l^{-1} was added weekly (250 ml per pot) and plants were harvested at 4, 8 and 12 weeks.

4.2.1.2 *Eucomis autumnalis* (Mill.) Chitt. and *Eucomis humilis* Baker

Eucomis plants (bulb diameter: 5.0 ± 1.0 cm; plant age: 2-3 years old) were grown in 12 cm pots with five plants per treatment. Hoagland's nutrient solution supplemented with Cd at 2 mg l^{-1} was added weekly (100 ml per pot). The experiment was terminated after 6 weeks.

4.2.1.3 *Merwilla plumbea* (Lindl.) Speta

Merwilla plumbea plants (bulb diameter: 1.2 ± 0.3 cm; plant age: 1-2 years old) were grown in individual pots (13.5 x 10 cm) with 10 replicates per treatment. Hoagland's nutrient solution supplemented with Cd at 2, 5 or 10 mg l^{-1} was added weekly (100 ml per pot). The experiment was terminated after 6 weeks.

4.2.2 Sample preparation and data collection

At harvest, plants were lightly washed to remove any particles of sand that may adhere to the surface. Growth parameters including leaf length, number of leaves, leaf fresh weight, bulb size, fresh/dry weight of bulb, root length, number of roots and fresh weight of roots were recorded. Thereafter, the plant parts were cut into small pieces and dried at 50 °C for approximately 72 h. Once dry, the individual plant parts were ground into fine powders (< 0.5 mm) using an IKA A11 (IKA Works, Inc.) analytical mill. The powders were placed into air-tight containers and stored in the dark at room temperature until analysis.

4.2.3 Chlorophyll analysis

The chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll content of plant leaf material was determined according to INSKEEP and BLOOM (1984; 1985). Fresh plant material (0.1 g) was homogenized in 10 ml N,N-dimethylformamide (DMF). The plant material was placed in the dark for 24 h on a shaker maintained at 10 °C. The absorbance of the extracts was measured using a Varian Cary 50 Spectrophotometer at 647 nm (maximum for chl b) and 664.5 nm (maximum for chl a) using a 1 ml quartz curvette. Absolute chlorophyll concentrations (Chl a, Chl b and total chlorophyll) were quantified using the simultaneous equations of INSKEEP and BLOOM (1985):

$$\text{Chl a} = 12.70_{A664.5} - 2.79_{A647}$$

$$\text{Chl b} = 20.70_{A647} - 4.62_{A664.5}$$

$$\text{Total chlorophyll} = 17.91_{A647} + 8.08_{A664.5}$$

Each experiment was replicated five times, and results were expressed as mg chlorophyll per g fresh weight.

4.2.4 Elemental analysis

Elemental analysis was done by ICP-OES as described in Section 3.2.3.

4.2.5 Data analysis

Growth data from different treatments were analyzed using one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package and either Tukey's or paired t-tests were used to separate means at a 5% level of significance.

4.3 RESULTS AND DISCUSSION

4.3.1 *Albuca setosa*

The symptoms of Cd toxicity in plants are easily identifiable with the most general symptoms being stunting and chlorosis (DAS, SAMANTARAY and ROUT 1997). The effects of Cd on growth parameters of leaves, bulbs and roots of *A. setosa* are summarized in **Figure 4.1**. After 4 weeks, leaf length and fresh weight of leaves were significantly reduced by Cd at 5 mg l^{-1}. The fresh weight of bulbs showed significant ($p < 0.05$) reduction at 4 and 12 weeks; although at 8 weeks the weight of the Cd-treated bulbs were comparable to that of the control. Although root length and number of roots were not significantly affected by Cd after 4 weeks, fresh weight of Cd-treated roots was significantly lower compared to control (15.3 g and 25.8 g respectively). The reduction in fresh weight of roots was caused by suppression of lateral root growth (**Figure 4.2**). At 12 weeks, root length of Cd-treated plants was significantly lower than the control (34.8 and 46.1 cm respectively).

POLEC-PAWLAK, RUZIK, ABRAMSKI, CIURZYNSKA and GAWRONSKA (2005) found that Cd reduced the growth of root hairs, lateral root formation and biomass in *Arabidopsis thaliana* (L.) Heynh. WANG, ZOU, DUAN, JIANG and LIU (2007) reported similar findings whereby the roots of maize (*Zea mays* L.) appeared thinner and more sparsely branched due to Cd toxicity. However, in accordance with findings of ARDUINI, MASONI, MARIOTTI and ERCOLI (2004), these changes in root
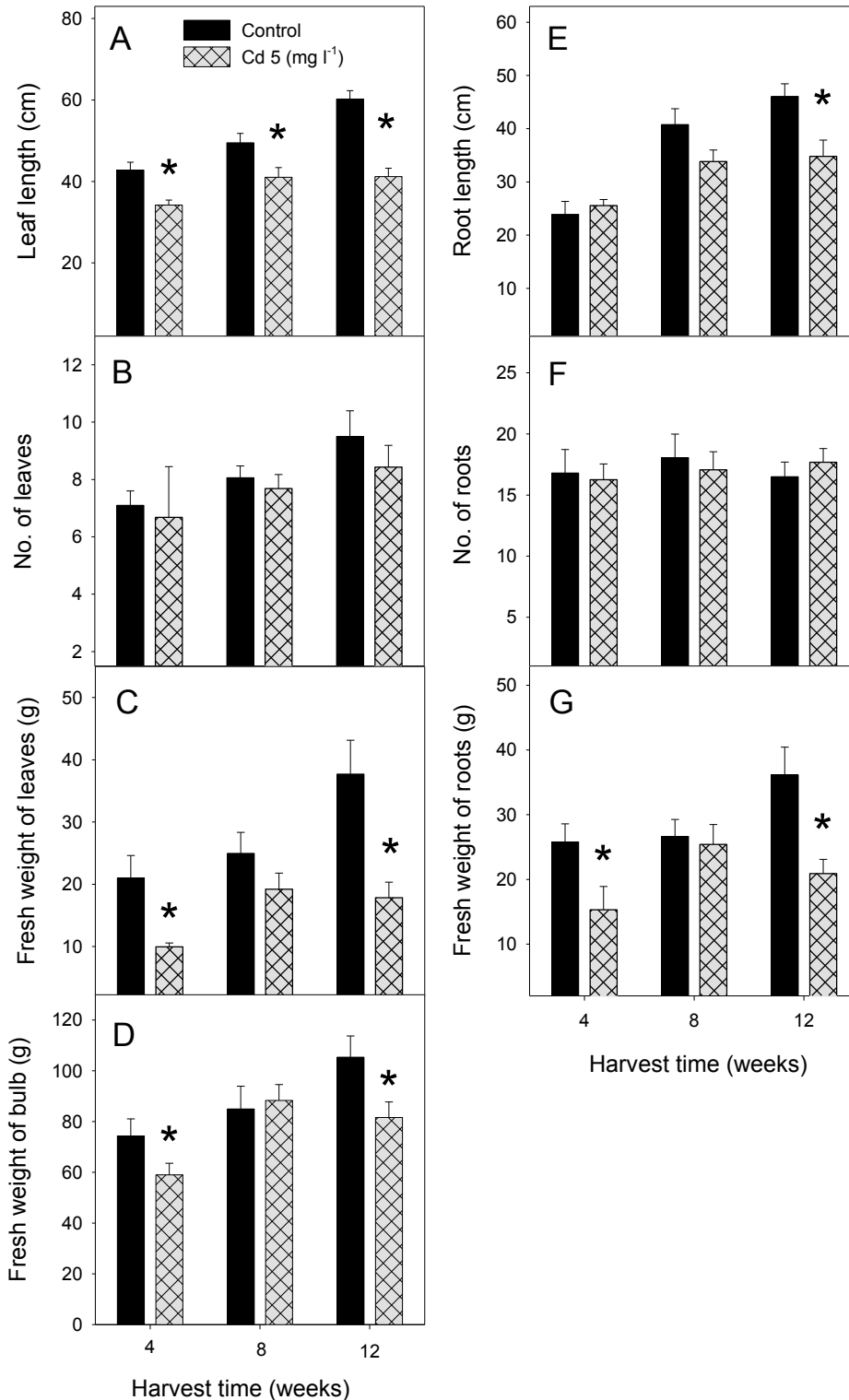
Figure 4.1: Effect of Cd (5 mg l⁻¹) application over 12 weeks on growth parameters of *Albuca setosa*. (A) leaf length, (B) number of leaves, (C) fresh weight of leaves, (D) fresh weight of bulbs, (E) root length, (F) number of roots, (G) fresh weight of roots. An asterisk (*) denotes a significant difference from the control ($p < 0.05$). Error bars indicate S.E.
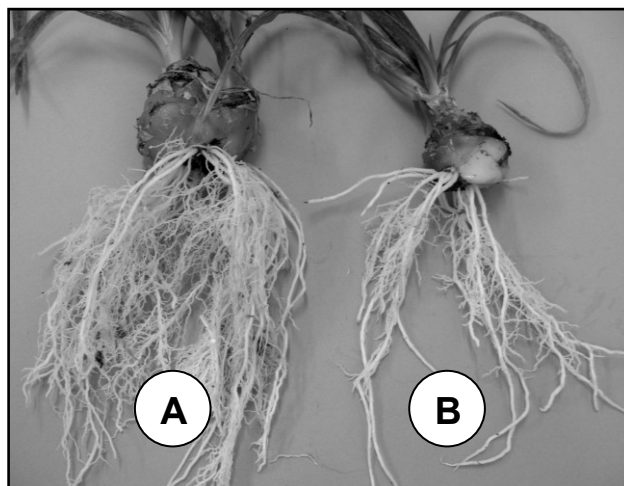
Figure 4.2: Effect of Cd (5 mg l^{-1}) application on root growth and development of *Albuca setosa* after 4 weeks. (A) Control (B) Cd-treated.

morphology did not affect the Cd supply to the above ground parts of the plant, which increased with increasing age.

Treatment of *A. setosa* plants with Cd led to accumulation of high metal concentrations. Cadmium content in the roots reached 110 mg kg^{-1} after 8 weeks yet decreased after 12 weeks to 98 mg kg^{-1} (**Figure 4.3**). The decrease in Cd concentration between 8 and 12 weeks may be due to the renewal of the most active parts of the below ground mass which has been reported for Cd tolerant species (DAS, SAMANTARAY and ROUT 1997). The roots stored 53% of the total Cd after 4 weeks, yet only 39 and 31% after 8 and 12 weeks respectively. Similar results were reported in *Miscanthus sinensis* L. var Giganteus, where after 1 month of Cd treatment, the capability of the roots to retain Cd was reduced and the metal passed passively into the rhizome thereafter (ARDUINI, MASONI, MARIOTTI and ERCOLI 2004).

Cadmium content in the medicinally used *A. setosa* bulbs increased with time. After 12 weeks the bulbs contained 36.9 mg kg^{-1}. The total mass of Cd in the bulbs was 0.8 mg, after 4 weeks, corresponding to 30% of the Cd in the whole plant. At 8 and 12 weeks the total mass of Cd in the bulbs increased to 2.7 and 3.0 mg respectively corresponding to 37 and 46% of the total Cd in the plant.
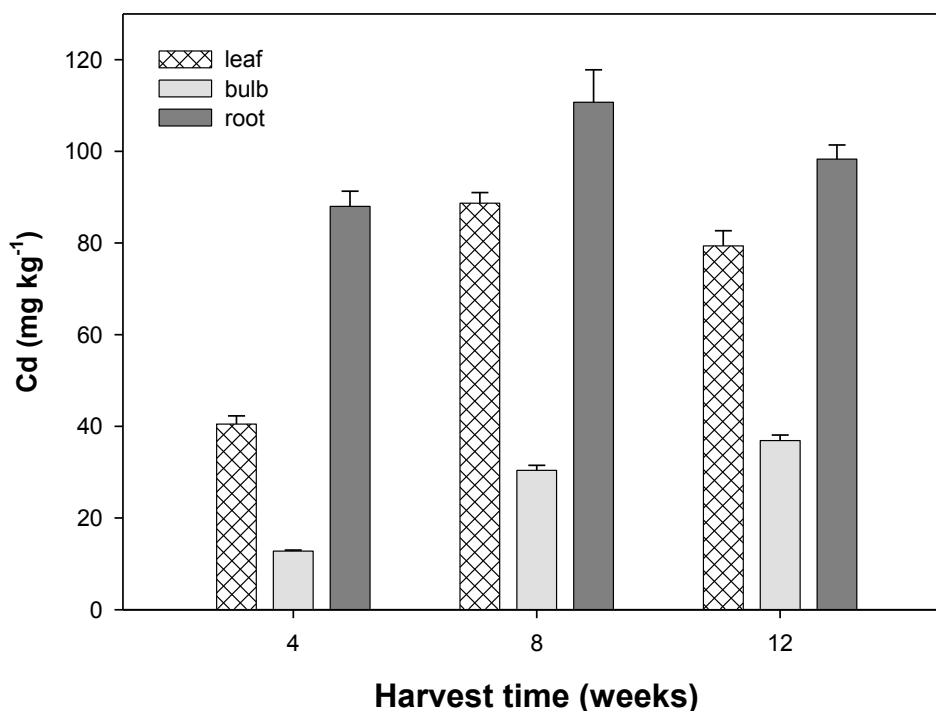
Figure 4.3: Cadmium accumulation (mg kg^{-1}) in leaves, bulbs and roots of *Albuca setosa* after 4, 8 and 12 weeks of growth with Cd applied at 5 mg l^{-1}. Error bars indicate S.E. Cadmium was undetected in the control plants.

According to MCGRATH and ZHAO (2003), a Cd hyperaccumulator should be able to accumulate 100 mg Cd kg^{-1} in dry leaves. In this study, *A. setosa* accumulated the highest concentration of Cd content in the leaves after 8 weeks (89 mg Cd kg^{-1}).

ALEXANDER, ALLOWAY and DOURADO (2006) evaluated the effect of Cd (4.2 mg kg^{-1}) on 6 commonly grown vegetables (5 cultivars per vegetable). Results were as follows: carrot (1.2 to 2.5 mg kg^{-1}), spinach (4.2 to 6.9 mg kg^{-1}), pea (0.2 to 0.4 mg kg^{-1}), french bean (0.01 to 0.1 mg kg^{-1}), onion (3.3 to 4.0 mg kg^{-1}) and lettuce (7.9 to 9.1 mg kg^{-1}). *Albuca setosa* accumulated considerably more Cd than the above-mentioned vegetables, when supplied with Cd at 5 mg l^{-1}. Although medicinal plants are not consumed as regularly or in the same quantity as vegetables, the accumulation of Cd to high concentrations still raises safety issues.

4.3.2 *Eucomis* species

No visible symptoms or growth abnormalities were seen for either *Eucomis* species after the 6 week Cd (2 mg l^{-1}) treatment (results not shown). By comparing different plant organs, it was observed that with a Cd supply level of 2 mg l^{-1}, *E. autumnalis* stored more Cd in the leaves (8.3 mg kg^{-1}), bulbs (4.9 mg kg^{-1}) and roots (26.7 mg kg^{-1}) than *E. humilis* (0.99 mg kg^{-1}, 1.3 mg kg^{-1} and 13 mg kg^{-1} respectively) (**Figure 4.4**). Total Cd concentration in *E. autumnalis* was more than double that of *E. humilis* (40.2 and 15.3 mg kg^{-1} respectively).
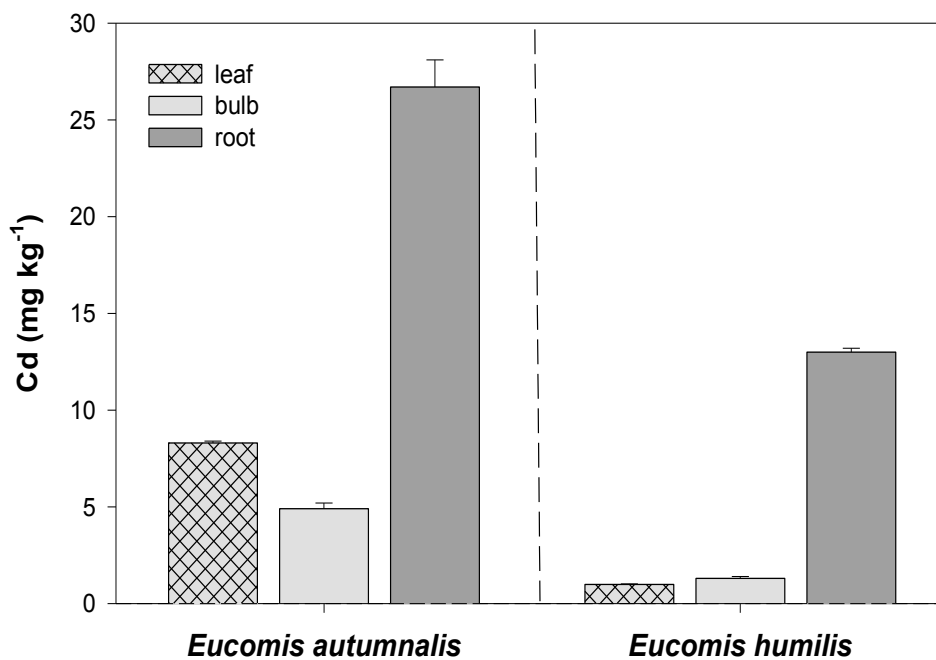
Figure 4.4: Cadmium accumulation (mg kg^{-1}) in leaves, bulbs and roots of *Eucomis autumnalis* and *Eucomis humilis* after 6 weeks growth in Cd (2 mg l^{-1}). Error bars indicate S.E. Cadmium was undetected in the control plants.

STOLT, SNELLER, BRYNGELSSON, LUNDBORG and SCHAT (2003) reported similar results for *Triticum* species where no significant differences in the growth response to Cd was observed between plant species (*T. aestivum* vs. *T. turgidum*), or within the species (Thasos vs. Tjalve and Topdur vs. Grandur) however a difference in Cd accumulation was recorded.

Bulbs of *E. autumnalis* contained 16 times more Cd than the WHO guideline of 0.3 mg kg^{-1} when supplied with Cd at 2 mg l^{-1}. The bulbs are widely used in decoctions administered as enemas or emetics to treat a variety of symptoms and are taken during pregnancy to facilitate labor. KURIWAKI, NISHIJO, HONDA, TAWARA, NAKAGAWA, HORI and NISHIJO (2005) studied the effects of Cd exposure on pregnant rats. In addition to the Cd detected in the foetal liver, it was suggested that Cd may inhibit Ca, Cu, Na and K uptake and transportation across the placenta. Similarly, Fe and Zn transportation from the placenta to the foetus was negatively affected. The study concluded that Cd exposure decreases the elemental concentration in the foetal liver and kidney, which may in turn influence foetal development and metabolism. Thus, caution is urged, especially for pregnant users, as a large number of these bulbs are used in traditional medicine.

4.3.3 *Merwilla plumbea*

The effects of Cd treatments on growth parameters of *M. plumbea* are presented in **Figure 4.5**. Cadmium concentrations of up to 10 mg l^{-1} had no significant effect on leaf length, number of leaves, bulb size, bulb dry weight, root length or number of roots. Compared with the control, fresh weight of leaves was significantly reduced by Cd at 2 mg l^{-1} (10.8 and 7.0 mg kg^{-1} respectively). Similarly, Cd application of 2 mg l^{-1} resulted in lower fresh weight of bulbs and roots. In spite of high stomatal resistance, Cd-treated plants maintain lower water content than untreated plants (ONCEL, KELES and USTUN 2000). This may be linked to damage of the root system (ONCEL, KELES and USTUN 2000).

The results clearly show that *M. plumbea* is more sensitive to low levels of Cd than higher levels. This disruption in homeostasis is a common phenomenon caused by Cd toxicity. Contrary to our findings, studies have suggested that low concentrations of heavy metals have a stimulatory effect on root growth and an inhibitory effect at higher concentrations (ONCEL, KELES and USTUN 2000; NYITRAI, BOKA, GASPAR, SARVARI, LENTI and KERESZTES 2003).
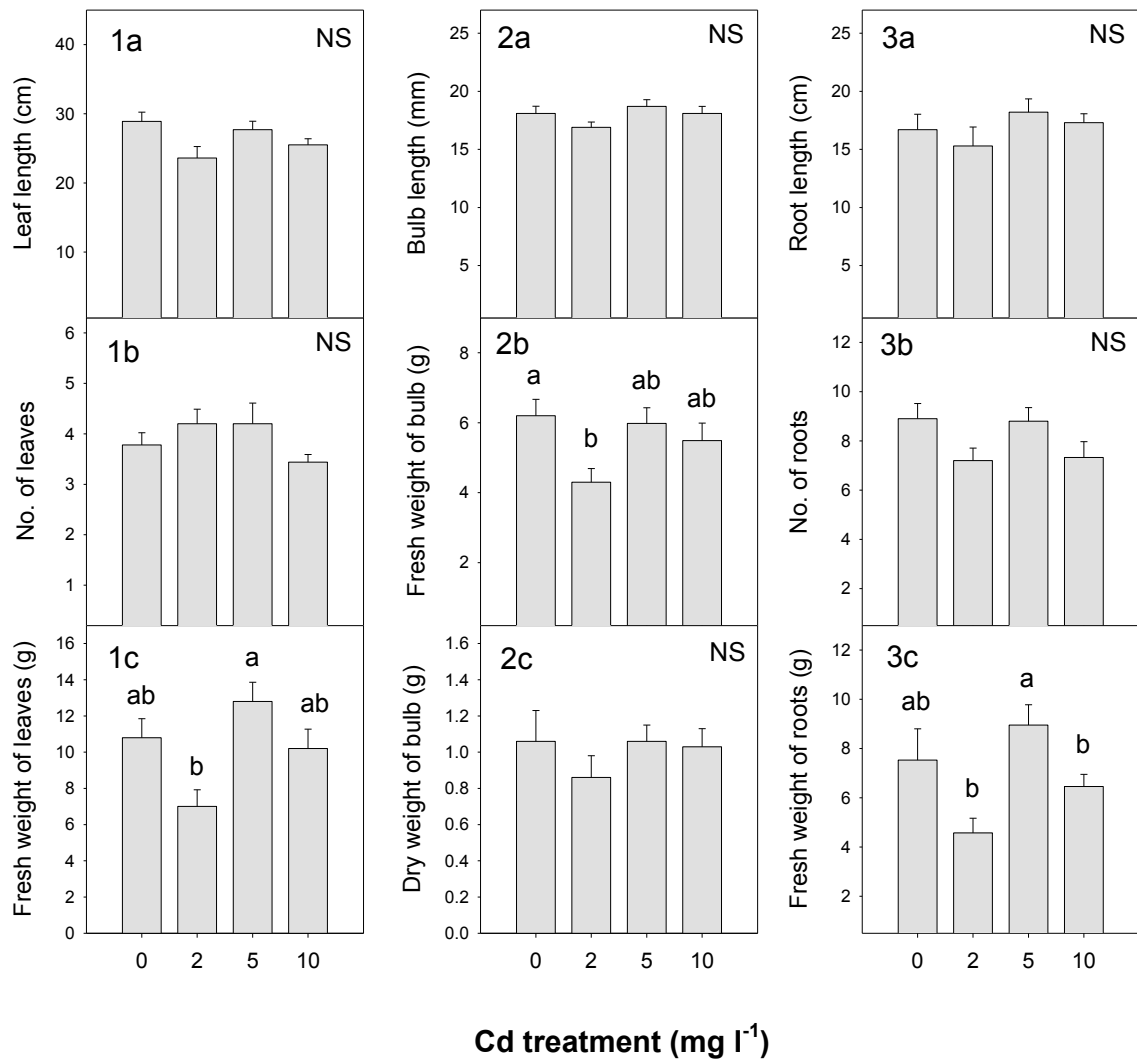
Figure 4.5: **Effect of Cd on growth of** *Merwilla plumbea* **(1a) leaf length, (1b) number of leaves, (1c) fresh weight of leaves, (2a) bulb length, (2b) fresh weight of bulb, (2c) dry weight of bulb, (3a) root length, (3b) number of roots, (3c) fresh weight of roots. Mean values with dissimilar letter(s) are significantly different (***p* < 0.05**). NS = non significant. Error bars indicate S.E.**

Leaf chlorophyll content decreased with increasing Cd concentrations (**Table 4.2**). These findings are in agreement with observations reported by RAI, KHATOON, BISHT and MEHROTRA (2005). On the contrary, the Cd hyperaccumulator, *Sedum alfredii* Hance, increased its total chlorophyll, chlorophyll a and chlorophyll b content by 32, 30 and 46% when exposed to Cd at 112 mg l^{-1} (ZHOU and QIU 2005).

Table 4.2: Effect of Cd application on leaf chlorophyll content (mg chlorophyll per g fresh weight) of *Merwilla plumbea*. Mean values (± S.E.) in a column for each treatment with dissimilar letter(s) are significantly different ($p < 0.05$).

Cd treatment (mg l^{-1})	Chlorophyll a	Chlorophyll b	Total Chlorophyll
0	8.7 ± 0.85 a	2.4 ± 0.26 a	11.2 ± 1.1 a
2	7.4 ± 0.69 ab	2.2 ± 0.19 ab	9.6 ± 0.88 ab
5	5.3 ± 0.30 b	1.6 ± 0.10 b	7.0 ± 0.39 b
10	6.4 ± 0.76 ab	1.8 ± 0.20 ab	8.3 ± 0.96 ab

The distribution of Cd in leaves, bulbs and roots differed with increasing Cd concentrations. *Merwilla plumbea*, supplied with Cd at 2, 5 and 10 mg l^{-1}, accumulated 44.4, 32.5 and 143.5 mg kg^{-1} of the element in their roots, respectively (**Figure 4.6**). A study by SUN, ZHOU and DIAO (2008) revealed that the hyperaccumulator, *Solanum nigrum* L. accumulated 83.2 mg kg^{-1} and 80 mg kg^{-1} in the leaves and roots respectively when exposed to Cd at 10 mg kg^{-1}. In comparison, when exposed to Cd at 10 mg l^{-1}, *M. plumbea* accumulated only 8.3 mg kg^{-1} in the leaves, yet 86% (143.5 mg kg^{-1}), was detected in the roots.
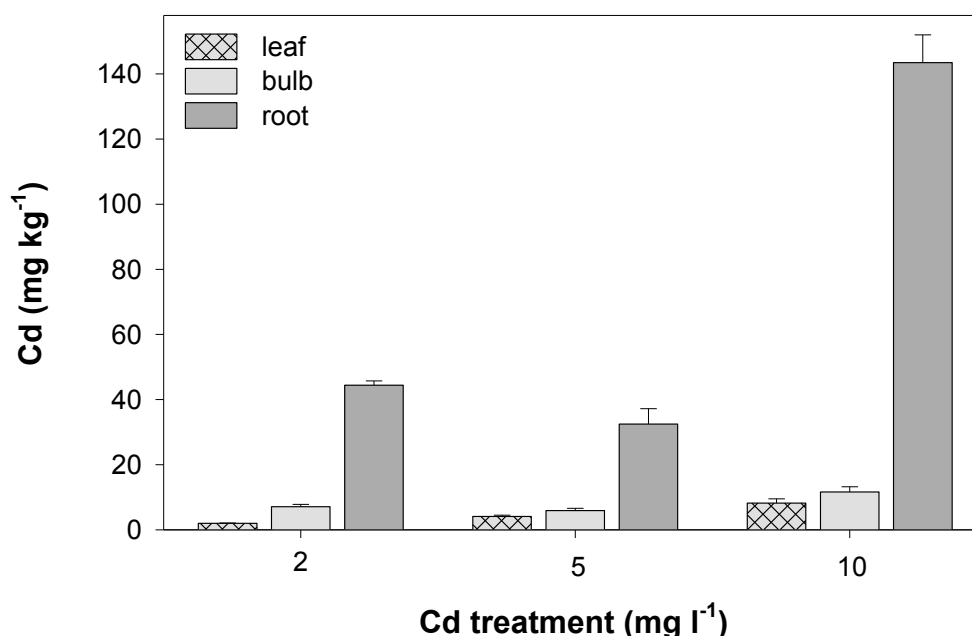
Figure 4.6: Cadmium accumulation (mg kg^{-1}) in leaves, bulbs and roots of *Merwilla plumbea* after 6 weeks growth in Cd (2, 5 and 10 mg l^{-1}). Error bars indicate S.E. Cadmium was undetected in the control plants.

The bulbs of *M. plumbea* accumulated Cd 7.1, 5.9 and 11.6 mg kg^{-1} when supplied with Cd at 2, 5 and 10 mg l^{-1} respectively, corresponding to 12, 9 and 6% of Cd in the whole plant (**Figure 4.6**). This ability of *M. plumbea* to accumulate Cd in the much sought after and widely used bulbs is disturbing.

Merwilla plumbea is medicinally used for both adults and children. It has been suggested that Cd exposure in children may have a larger impact on renal function, particularly tubular reabsorption, than exposure in an adult (TRZCINKA-OCHOCKA, JAKUBOWSKI, RAZNIEWSKA, HALATEK and GAZEWSKI 2004). Thus, when administering medicinal plants, especially to children, care must be taken when using Cd acummulatory species.

The medicinally used Hyacinthaceae bulbs examined accumulated Cd above the recommended WHO limit of 0.3 mg kg^{-1}. The ability of *A. setosa, E. autumnalis, E. humilis* and *M. plumbea* to accumulate Cd, illustrates a potential Cd exposure route to the consumers of South African traditional medicine. As Cd exposure can produce a variety of adverse effects on human health (**Section 2.5.2**), monitoring Cd levels in soils is strongly advocated for the cultivation of South African medicinal plants.

4.4 SUMMARY

- Application of various Cd concentrations to *A. setosa, E. autumnalis, E. humilis* and *M. plumbea* indicate that Cd is readily accumulated and distributed to various plant parts of these species.
- Cd at 5 mg l^{-1} reduced growth of *A. setosa*, the most obvious symptom being the negative effect on lateral root growth.
- Cd content in *A. setosa* bulbs increased with time and reached 37 mg kg^{-1} after 12 weeks.
- Cd at 2 mg l^{-1} had no effect on growth parameters of either *E. autumnalis* or *E. humilis*. However, a substantial difference in Cd accumulation was detected.
- When supplied with Cd at 2 mg l^{-1}, *M. plumbea* accumulated more Cd in the bulbs (7.1 mg kg^{-1}) than *E. autumnalis* and *E. humilis* (4.9 and 1.3 mg kg^{-1} respectively).

- Cd accumulated primarily in the roots with distribution in plant parts of Hyacinthaceae species as follows:

  A. setosa, E. autumnalis: roots>leaves>bulbs

  E. humilis, M. plumbea: roots>bulbs>leaves

5   Antagonistic/synergistic effect of Cd on microelements

5.1 INTRODUCTION

5.1.1 Interaction of microelements in higher plants

Interactions of microelements may be both antagonistic and synergistic, and their imbalance may cause chemical stress in plants (KABATA-PENDIAS 2001). Antagonism occurs when the combined physiological effect of two or more elements is less than the sum of their independent effects, and synergism occurs when the combined effects of these elements is greater (KABATA-PENDIAS 2001).

A common dietary uptake pathway of metals by humans and animals is through crops irrigated with contaminated wastewater or grown on polluted soils. Continuous build up of heavy metals in the soil may reduce crop yield, thus affecting the nutritional status and incomes of farming communities (HEIKENS 2006). Increasing evidence indicates that heavy metals are not only directly hazardous to humans but that greater problems emerge due to antagonism with microelements (**Table 5.1**). Thus the interaction between heavy metals and essential microelements on uptake and distribution in crops is of public concern (LIU, LI, XU, LIANG, LU, YANG and ZHU 2003). Elemental antagonism in plants affects metal accumulation in different plant organs (ZHOU and QIU 2005).

Table 5.1: Examples of studies on elemental translocation following heavy metal stress.

Plant species	Main findings related to element translocation	Reference
Brassica oleracea L. var. Botrytis cv. Maghi	• Heavy metal excess (Co>Cu>Cr) restricted concentrations of Fe in leaves • The translocation of P, S, Mn, Zn and Cu from roots to tips was affected most significantly by Co and least by Cr	CHATTERJEE and CHATTERJEE (2000)

Plant species	Main findings related to element translocation	Reference
Brassica oleracea L. var. Golden Aker	• With an increase in Pb supply in the media, the concentration of Pb and Zn in the plant increased whereas P, S, Fe, Mn and Cu decreased in various plant parts	SINHA, DUBE, SRIVASTAVA and CHATTERJEE (2006)
Brassica rapa L; *Lactuca sativa* L.	• After Se and Zn application, Pb and Cd content decreased markedly while Mn and Mg increased • Antagonism of Se and Zn against Pb and Cd in plants was suggested	HE, LV and WANG (2004)
Citrullus vulgaris cv. Ludhiana	• On exposure to Cr, the concentration of P, Mn and Zn increased in all plant parts whereas Cu and S decreased	DUBE, TEWARI, CHATTERJEE and CHATTERJEE (2003)
Hordeum vulgare L.	• Cd addition to nutrient solution led to dramatic reduction of Fe concentration in shoots, and Cu, Mn and Zn concentrations in the shoots and roots	WU and ZHANG (2002)
Hordeum vulgare L.	• Significant genotypic difference was found in microelement concentrations • Correlation analysis showed that only Mn accumulation was synergetic with Cd accumulation, despite a slightly positive relationship between Cd and Zn, Cu, or Fe in accumulation in barley grains	CHEN, DONG, WANG, WU, ZHANG, LI, CHEN, CHEN and WEI (2007)
Hypericum perforatum L.	• Accumulation of Ni was accompanied by increases of Mo, showing a synergistic effect between Ni and Mo • Significant decrease in Fe uptake from the culture medium (antagonism Ni/Fe) was observed	MURCH, HAQ, RUPASINGHE and SAXENA (2003)
Lolium perenne L.	• Cr-induced toxicity resulted in a modification of mineral content in roots and leaves, especially for Ca, Mg and Fe	VERNAY, GAUTHIER-MOUSSARD and HITMI (2007)
Lupinus albus L. cv. Multolupa	• Cd addition reduced P, K, Fe, Mn and Zn concentrations in the shoot and Mn in the root	ZORNOZA, VAZQUEZ, ESTEBAN, FERNANDEZ-PASCUAL and

Plant species	Main findings related to element translocation	Reference
		CARPENA (2002)
Oryza sativa L.	• Certain cultivars were more tolerant to soil Cd stress, while others were very sensitive • Positive correlations between Cd and Fe, Cd and Zn, Cd and Cu existed, but no significant correlation between Cd and Mg • Relationship between Cd and Mn varied with the different plant organs • Interactions of Cd and Fe, Zn, Cu were synergetic in uptake and translocation from root to shoot	LIU, LI, XU, LIANG, LU, YANG and ZHU (2003)
Oryza sativa L.	• Root tissue rather than iron plaque on the root surface is a barrier to Cd uptake and translocation within rice plants, and the uptake and translocation of Cd appear to be related to Fe nutritional levels in the plants	LIU, ZHANG and ZHANG (2007)
Phyllanthus amarus Schumach. & Thonn.*; Solanum nigrum* L.	• After addition of Cr, concentrations of Mn and Zn increased. Cu concentrations were less affected	RAI, KHATOON, RAWAT and MEHROTRA (2007)
Pteris vittata L.	• Arsenic uptake increased the uptake of K, P, Fe, Mn and Zn. However, Ca and Mg concentrations decreased	CAO, MA and TU (2004)
Raphanus sativus L. cv. Jaunpuri	• Lead accumulation reduced the concentration of Fe and S in shoots and increased the concentration of P and S in roots	GOPAL and RIZVI (2008)
Sedum alfredii Hance	• Ca in the root increased in the presence of Cd, while the Ca in the leaves and stems decreased with increasing Cd in the nutrient solution, indicating that the translocation of Ca to stems and leaves could be depressed by the concentration of Cd in the nutrient solution	ZHOU and QIU (2005)
Spartina alterniflora Loisel	• Organic As caused the highest Na root concentrations and simultaneously the lowest plant K levels (antagonism K-Na) • A significant increase in leaf Ca concentrations was found after application of organic As • Inorganic As significantly increased the concentrations of	CARBONELL, AARABI, DELAUNE, GAMBRELL and PATRICK (1998)

Plant species	Main findings related to element translocation	Reference
	B (root), Cu (root) and Cu (shoot)	
Spinacea oleracea L. cv. Banarasi	• Recovery from Cr toxicity was most noticeable when Fe was supplied through root and foliar spray, simultaneously	SINHA, DUBE and CHATTERJEE (2005)
Trigonella corniculata L.	• Due to the application of Ni, Fe content in the plant increased whereas the Cu and Zn decreased	PARIDA, CHHIBBA and NAYYAR (2003)
Triticum aestivum L.; *Zea mays* L.	• Cd and Zn order of transfer in different plant tissues was root>stem>grain • The transfer ratio of Cd was lower than that of Zn • Increasing Cd and Zn contents in soils could increase the accumulations of Cd and Zn in crops	NAN, LI, ZHANG and CHENG (2002)
Triticum aestivum L.	• The effect of Cd on nutrient concentrations in plants varied among elements, plant parts and genotypes • Cd treatment caused more P, K and Mn accumulation in roots, probably due to the inhibition of their translocation to the shoots	ZHANG, FUKAMI and SEKIMOTO (2002)
Zea mays L.	• Mn and Zn content in the plant was reduced by Cd in the media and progressively decreased with increasing Cd concentrations • Fe uptake in Cd-treated plants was greater than control • Cu increased with prolonged treatment time, and plants concentrated Cu in the roots more than in above-ground parts after treatments with different Cd concentrations	WANG, ZOU, DUAN, JIANG and LIU (2007)

The similarity in chemical properties between Cd and Zn, and their association in the environment can lead to interactions between these two elements (MCKENNA, CHANEY and WILLIAMS 1993). They are usually found together in ores and compete with each other for various ligands. Thus, interaction between these two elements in biological systems is likely to occur. It is also suggested that the toxic effects of Cd may be preventable or treatable by Zn (WAJDA, KUTERNOZINSKA and PILIPOWICZ 1989; DAS, SAMANTARAY and ROUT 1997). In **Chapter 3**, it was interesting to note that *Agathosma betulina* (sample 2), *Dioscorea dregeana* (sample

1) and *Eucomis autumnalis* (sample 4) containing the highest Cd content (**Figures 3.1** and **3.2**), also contained the highest Zn content (**Tables 3.4** and **3.5**).

In **Chapter 4** it was demonstrated that *Merwilla plumbea,* South Africa's top selling medicinal bulb (MANDER 1998), readily took up and distributed Cd to various parts of the plant. Currently, the South African guideline for the maximum permissible Zn concentration in the soil is 46.5 mg kg^{-1}, reduced from 185 mg kg^{-1} in 1991 (HERSELMAN, STEYN and FEY 2005). There are many reports of Cd/Zn antagonistic and synergistic effects (CHAKRAVARTY and SRIVASTAVA 1997; CHAOUI, GHORBAl and EL FERJANI 1997; ARAVIND and PRASAD 2005). Thus, it is imperative to investigate these effects in plants, especially those that will enter the food chain.

Interactions of Cd and other microelements in soil have not been fully explained (LIU, LI, XU, LIANG, LU, YANG and ZHU 2003). Further studies investigating the relationship between Cd and other elements are essential to supplement the current knowledge on Cd accumulation in crops (WU and ZHANG 2002).

5.1.2 Indigenous leafy vegetables

The availability of indigenous vegetables has reduced drastically due to excessive cultivation of field crops which in turn has led to a loss of wild vegetables and habitat change (ODHAV, BEEKRUM, AKULA and BAIJNATH 2007). *Tulbaghia violacea* (Alliaceae) is one of the few plants of which the bulbs are used medicinally and the leaves are used as a vegetable (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). This gives it not only medicinal, but also horticultural potential. There is little data regarding leafy vegetables, especially in urban areas (MENCH 1998). Investigating indigenous vegetables can help to alleviate malnutrition and contribute towards community development which could lead to establishing local entrepreneurs (REINTEN and COETZEE 2002).

Leafy vegetables, in general, have a greater possibility of accumulating heavy metals in their edible parts than have grain or fruit crops (MAPANDA, MANGWAYANA, NYAMANGARA and GILLER 2007). In addition, leafy vegetables are also more

vulnerable to heavy metal contamination from motor vehicle emissions (NABULO, ORYEM-ORIGA and DIAMOND 2006). ODHAV, BEEKRUM, AKULA and BAIJNATH (2007) stated that further research is required with regards to toxic compounds in traditionally consumed foods in South Africa.

Thus, the aim of the following experiments was to determine the effect of Cd and Zn combinations on uptake, distribution and microelemental content in the heavily used *M. plumbea* and to investigate the effect of Cd uptake and microelemental distribution in *T. violacea.*

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

Stock plants of *M. plumbea* and *T. violacea,* raised in the shade house at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus (29° 37.55 S; 30° 24.13 E), were used for this experiment. Plants were transferred into pots containing sterilized, acid washed quartz sand and fertilized with 50% Hoagland's nutrient solution (HS) (HOAGLAND and SNYDER 1933) (**Appendix A**). At the beginning of spring, a one-month adaptation period was given to plants and only healthy plants were chosen for experiments. *Tulbaghia violacea* plants were grown in the greenhouse whilst *M. plumbea* plants were grown in controlled environment chambers (16:8 h light and dark conditions with a photosynthetic photon flux density of 80.4 ± 3.5 µmol m^{-2} s^{-1} at 25 °C). The pots were arranged in a randomized block design. Cadmium in the form of CdCl$_2$·H$_2$O was added to the HS. Hoagland's nutrient solution (without metals) served as control. Additional watering of plants (200 ml per pot) took place every alternate day.

5.2.1.1 *Merwilla plumbea* (Lindl.) Speta

Merwilla plumbea plants (bulb diameter: 1.0 ± 0.4 cm; plant age: 1-2 years old) were grown in individual pots (13.5 x 10 cm) in plant growth chambers with 16 plants per treatment. Five treatments were evaluated: (1) HS (control); (2) HS + 2 mg Cd l^{-1} (single); (3) HS + 2 mg Cd l^{-1} + 50 mg Zn l^{-1} (combination); (4) HS + 2 mg Cd l^{-1} + 100

mg Zn I^{-1} (combination) and (5) HS + 2 mg Cd I^{-1} + 150 mg Zn I^{-1} (combination). The experiment was terminated after 6 weeks. The Cd/Zn nutrient solution was added weekly (100 ml per pot) and plants were harvested after 6 weeks.

5.2.1.2 *Tulbaghia violacea* Harv.

Tulbaghia violacea plants of various size classes: small (± 8 - 10 g), medium (± 16 - 20 g) and large (± 80 – 95 g) were planted in 30 cm pots containing sterile quartz sand with 16 plants per treatment. Cadmium nutrient solution (250 ml per pot) was added at concentrations of 2 and 5 mg I^{-1}. The experiment was terminated after 6 weeks.

5.2.2 Sample preparation and data collection

After 6 weeks, the plants were harvested, measured and processed as outlined in Section 4.2.2.

5.2.3 Chlorophyll analysis

The chlorophyll content in the leaf material was quantified as described in Section 4.2.3.

5.2.4 Elemental analysis

Elemental analysis was done by ICP-OES as outlined in Section 3.2.3.

5.2.5 Data analysis

The effects of different treatments were analyzed using one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package and Tukey's test was used to separate means at a 5% level of significance.

5.3 RESULTS AND DISCUSSION

5.3.1 *Merwilla plumbea*

The addition of Cd had no significant effect on the leaf length, number of leaves or leaf fresh weight of this bulbous plant (**Figure 5.1**). However, an increase of Zn in the media lowered leaf growth parameters demonstrating a negative or toxic effect. Bulb and root growth was not significantly affected by Cd or Cd/Zn combination treatments. Likewise, chlorophyll content fluctuated but no significant effect was caused by Cd or Cd/Zn combinations (**Table 5.2**).

Table 5.2: Effect of Cd/Zn treatments on leaf chlorophyll content (mg chlorophyll per fresh weight) of *Merwilla plumbea*. Mean values (± S.E) in a column for each treatment with dissimilar letter(s) are significantly different ($p < 0.05$).

Treatment (mg l^{-1})	Chlorophyll a	Chlorophyll b	Total Chlorophyll
HS	7.2 ± 0.77 a	2.4 ± 0.28 a	9.6 ± 1.06 a
HS + Cd 2 mg l^{-1}	7.8 ± 0.22 a	2.5 ± 0.07 a	10.3 ± 0.29 a
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	7.4 ± 0.38 a	2.4 ± 0.12 a	9.8 ± 0.51 a
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	6.0 ± 0.60 a	2.0 ± 0.20 a	8.0 ± 0.81 a
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	6.6 ± 0.64 a	2.1 ± 0.20 a	8.6 ± 0.84 a

When Cd at 2 mg l^{-1} was added to HS, leaves, bulbs and roots accumulated Cd to 3.6, 9.8 and 91 mg kg^{-1} respectively. Due to limited stock plants, smaller plants were used in this experiment, compared to the *M. plumbea* plants used in the experiments outlined in **Chapter 4.** It is interesting to note that the smaller plants accumulated more Cd in the leaves, bulbs and roots (3.6, 9.8 and 91 mg kg^{-1} equating to 16, 18 and 65% of the total mass respectively) than the larger plants which accumulated only 2.0, 7.1 and 44 mg kg^{-1} (6, 12 and 82% of the total mass respectively), when supplied with the same Cd concentration (2 mg l^{-1}). This emphasizes the need to investigate the effect of plant size in heavy metal accumulation (discussed in **Section 5.3.2**).
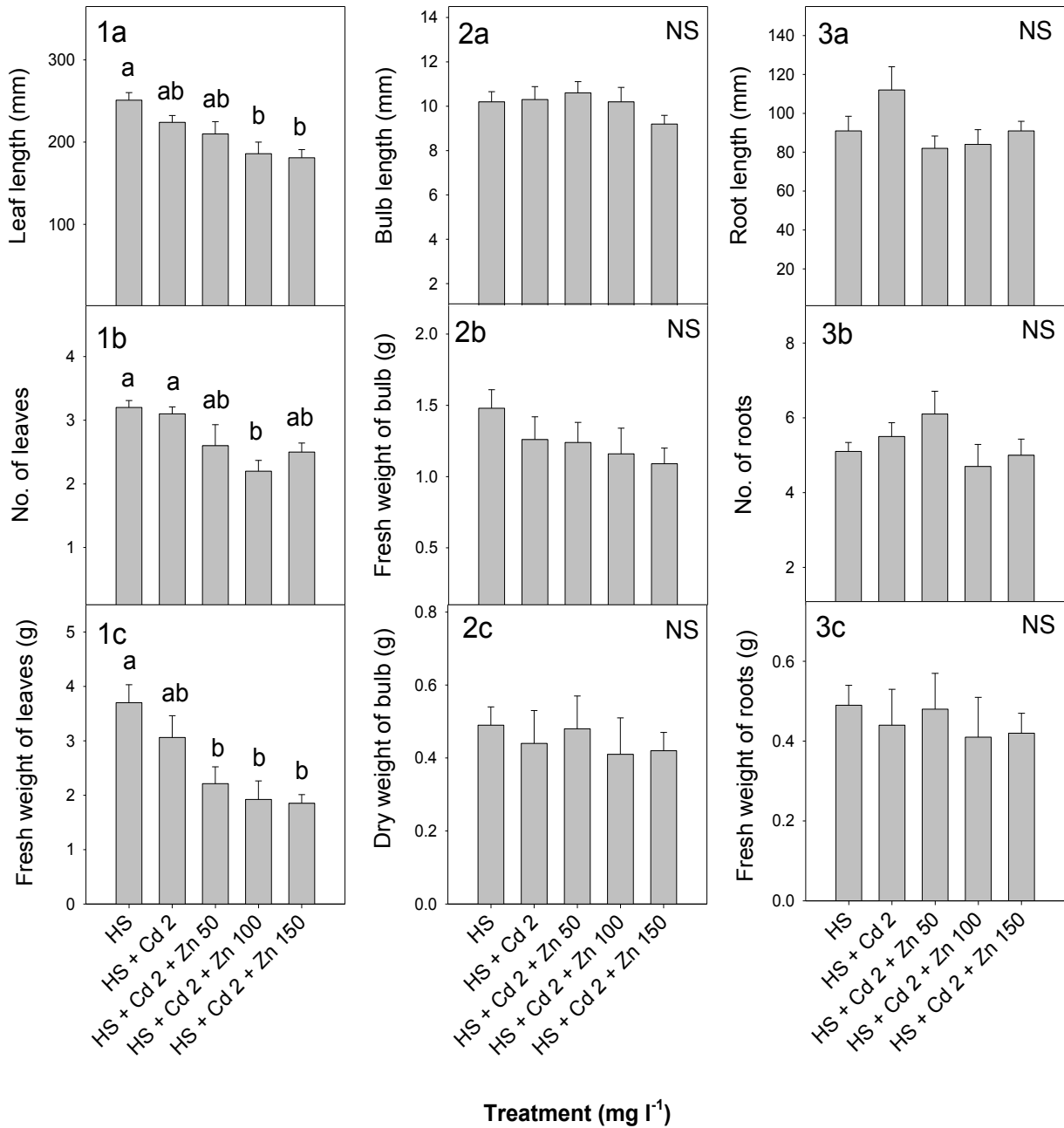
Figure 5.1: Effect of HS (control), HS + Cd 2 mg l^{-1} (single), HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1} (combination), HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1} (combination) and HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1} (combination) on growth parameters of *Merwilla plumbea.* (1a) leaf length, (1b) number of leaves, (1c) fresh weight of leaves, (2a) bulb length, (2b) fresh weight of bulb, (2c) dry weight of bulb, (3a) root length, (3b) number of roots, (3c) fresh weight of roots. Mean values with dissimilar letter(s) are significantly different (*p* < 0.05). NS = non significant. Error bars indicate S.E.
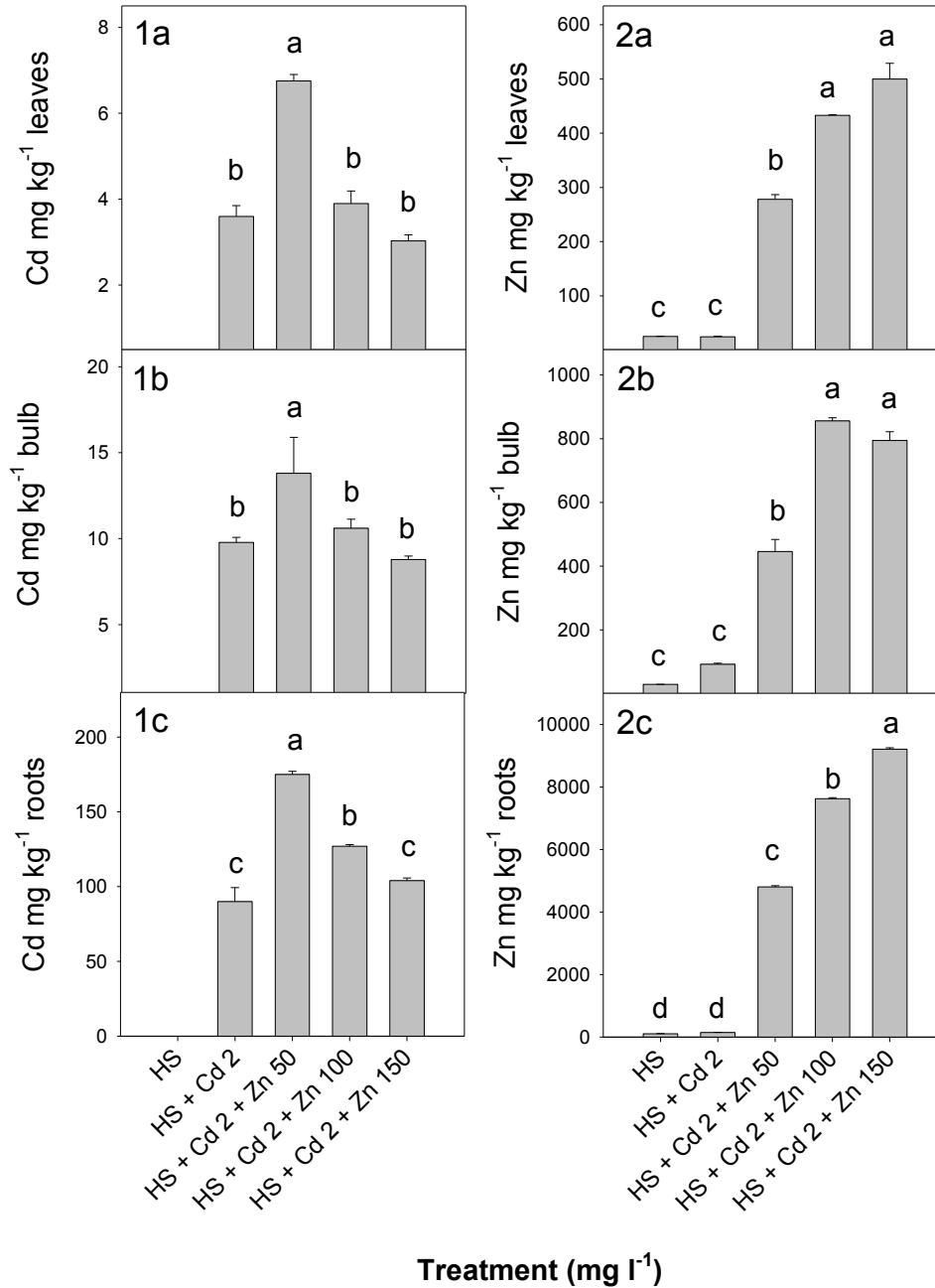
Figure 5.2: Cadmium (1 a,b,c) and Zn accumulation (2 a,b,c) in *Merwilla plumbea* after 6 weeks growth in HS (control), HS + Cd 2 mg l^{-1} (single), HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1} (combination), HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1} (combination) and HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1} (combination). Mean values with dissimilar letter(s) are significantly different ($p < 0.05$). Error bars indicate S.E. Cadmium was undetected in the control plants.

Cadmium/Zn interactions seem to be inconsistent as there are reports of both antagonism and synergism between these two elements in the uptake-transport process (KABATA-PENDIAS and PENDIAS 1984). When grown in 2 mg Cd l^{-1} + 50 mg Zn l^{-1} *M. plumbea* accumulated Cd to 6.8, 13.8 and 175 mg kg^{-1} in the leaves, bulbs and roots respectively (**Figure 5.2**). Thus, the addition of Zn caused an increase in Cd accumulation. However, with a further increase of Zn in the media, Cd accumulation decreased thereby suggesting an antagonistic effect of Zn on Cd uptake. Similarly, MCKENNA, CHANEY and WILLIAMS (1993) found that an increase in Zn application reduced Cd accumulation in lettuce and spinach. Zinc readily accumulated in the leaves, bulbs and roots of *M. plumbea* and increased steadily with an increase of Zn in the media (**Figure 5.2**).

Boron concentrations in the leaves and roots were not significantly affected by the addition of Cd (**Table 5.3**). However, with an increase of Zn in the media, leaf B content increased while the content in the bulbs and roots decreased. Boron is relatively immobile in plants, but because it is translocated in the xylem, it is largely stored in older leaves (KABATA-PENDIAS and PENDIAS 1984).

Copper and Mo levels were not significantly affected by Cd or Cd/Zn combinations. The present results show that Fe levels were not significantly affected by Cd. This is in contrast to findings by ZHOU and QIU (2005) whereby Fe concentrations in *Sedum alfredii* Hance (Crassulaceae) increased significantly in the presence of Cd. However, with the addition of Zn to the Cd-containing media, total Fe concentration (leaves + bulbs + roots) increased.

Manganese concentrations in leaves and roots significantly increased in the presence of Cd at 2 mg l^{-1}. Similar findings have been reported by PENG, LUO, YOU, LIAN, LI and SHEN (2008). WU, DONG, CAI, CHEN and ZHANG (2007) suggested that by sustaining higher Mn concentrations in the chloroplast, cell membrane and cell organs, Cd tolerance may be improved. The addition of Zn at 50 mg l^{-1} caused a further Mn increase in all *M. plumbea* plant parts.

The present results highlight the necessity to investigate fundamental physiology criteria of plants which are highly recommended for cultivation. An increase in Zn in

Table 5.3: Effect of various Cd/Zn treatments on microelement distribution in *Merwilla plumbea.*
Mean values (± S.E.) with dissimilar letter(s) are significantly different ($p < 0.05$).

Treatment	Leaf	Bulb	Root
B (mg kg^{-1})			
HS	57.0 ± 2.5 b	28.2 ± 0.88 a	72.1 ± 4.6 a
HS + Cd 2 mg l^{-1}	60.1 ± 2.1 b	24.8 ± 0.90 b	72.0 ± 8.7 a
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	71.2 ± 1.7 a	14.9 ± 0.37 d	39.3 ± 1.8 c
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	67.5 ± 1.1 a	20.0 ± 0.75 c	53.1 ± 0.08 ab
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	65.5 ± 2.3 ab	20.2 ± 0.54 c	46.5 ± 0.66 bc
Cu (mg kg^{-1})			
HS	8.6 ± 0.75 a	10.6 ± 0.72 a	121 ± 6.6 a
HS + Cd 2 mg l^{-1}	7.9 ± 0.95 a	10.7 ± 0.57 a	113 ± 17.6 a
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	9.8 ± 1.3 a	7.7 ± 1.20 a	99 ± 2.01 a
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	8.5 ± 0.10 a	9.1 ± 0.29 a	112 ± 0.45 a
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	9.4 ± 0.02 a	9.5 ± 0.75 a	98 ± 1.40 a
Fe (mg kg^{-1})			
HS	94 ± 3.4 c	108 ± 1.5 ab	1696 ± 134 b
HS + Cd 2 mg l^{-1}	109 ± 4.4 c	103 ± 1.6 ab	1495 ± 0.0 b
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	170 ± 5.6 a	77 ± 3.5 b	2493 ± 97.3 a
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	151 ± 3.7 b	81 ± 0.8 ab	2426 ± 88.1 a
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	184 ± 1.9 a	124 ± 20.8 a	2384 ± 78.6 a
Mn (mg kg^{-1})			
HS	15.2 ± 0.06 d	8.6 ± 0.23 b	69.5 ± 5.9 c
HS + Cd 2 mg l^{-1}	20.28 ± 0.21 c	8.9 ± 0.65 ab	111 ± 1.7 b
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	30.6 ± 0.73 a	9.5 ± 0.06 ab	118 ± 7.1 b
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	26.1 ± 0.02 b	10.8 ± 0.73 a	144 ± 3.4 a
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	25.9 ± 0.23 b	9.2 ± 0.12 ab	110 ± 2.5 b
Mo (mg kg^{-1})			
HS	0.55 ± 0.01 a	0.38 ± 0.03 a	0.54 ± 0.01 a
HS + Cd 2 mg l^{-1}	0.49 ± 0.01 a	0.41 ± 0.03 a	0.59 ± 0.10 a
HS + Cd 2 mg l^{-1} + Zn 50 mg l^{-1}	0.57 ± 0.05 a	0.27 ± 0.09 a	0.40 ± 0.01 a
HS + Cd 2 mg l^{-1} + Zn 100 mg l^{-1}	0.51 ± 0.00 a	0.23 ± 0.01 a	0.45 ± 0.01 a
HS + Cd 2 mg l^{-1} + Zn 150 mg l^{-1}	0.54 ± 0.01 a	0.23 ± 0.03 a	0.47 ± 0.01 a

the media caused a decrease in Cd accumulation in *M. plumbea*. Thus, we can conclude that increasing Zn levels in soils may be a possible solution reducing toxic Cd in *M. plumbea.*

5.3.2 *Tulbaghia violacea*

In small sized plants, compared to control, fresh weight of leaves, bulbs and roots, leaf and root length, and number of roots, were not significantly affected by the Cd treatments (**Table 5.4**). However, the addition of Cd at 5 mg l^{-1} significantly decreased the number of leaves. Leaf length, fresh weight of leaves and number of roots of the medium sized plants decreased when supplied with Cd at 2 mg l^{-1}. Fresh weight of bulbs decreased when supplied with Cd at 2 and 5 mg l^{-1}. However, Cd at 5 mg l^{-1} had no significant effect on root growth. Growth parameters of the large size plants were unaffected by increasing concentrations of Cd.

Cadmium accumulation in bulbs of small and medium sized plants increased with increasing Cd concentration (**Figure 5.3**). However, the Cd concentration in the bulbs of the large sized plants remained the same (8.7 mg kg^{-1}) when supplied with Cd at 2 or 5 mg l^{-1}. Accepting that bulb diameter is positively correlated with plant age and fitness (WILLIAMS, BALKWILL and WITKOWSKI 2007), younger plants (small sized plants) accumulated more Cd in the leaves than the older plants (medium and large sized plants). Leaf Cd content was the same for medium and large sized plants (1.4 mg kg^{-1}) when supplied with Cd at 2 mg l^{-1} compared with 2.1 mg kg^{-1} in the leaves of the small plants. With an increase of Cd to 5 mg l^{-1}, the small plants accumulated 5.5 mg kg^{-1} in the leaves, once again higher than medium and large sized plants which accumulated 3.1 and 2.9 mg kg^{-1} respectively.

Urban agriculture has gained increasing recognition and acceptance as a survival strategy for poor urban dwellers in a developing world (NABULO, ORYEM-ORIGA and DIAMOND 2006). MAPANDA, MANGWAYANA, NYAMANGARA and GILLER (2007) evaluated Cd concentrations in *Brassica juncea* (L.) Czern. and *Brassica napus* L. leaves from gardens irrigated with wastewater from the Mukuvisi River and partially treated sewage effluent at Pension farm in Harare, Zimbabwe. Cadmium leaf concentrations ranged from 0.7 to 2.4 mg kg^{-1}, and emphasis was placed on potential

INSERT TABLE 5.4
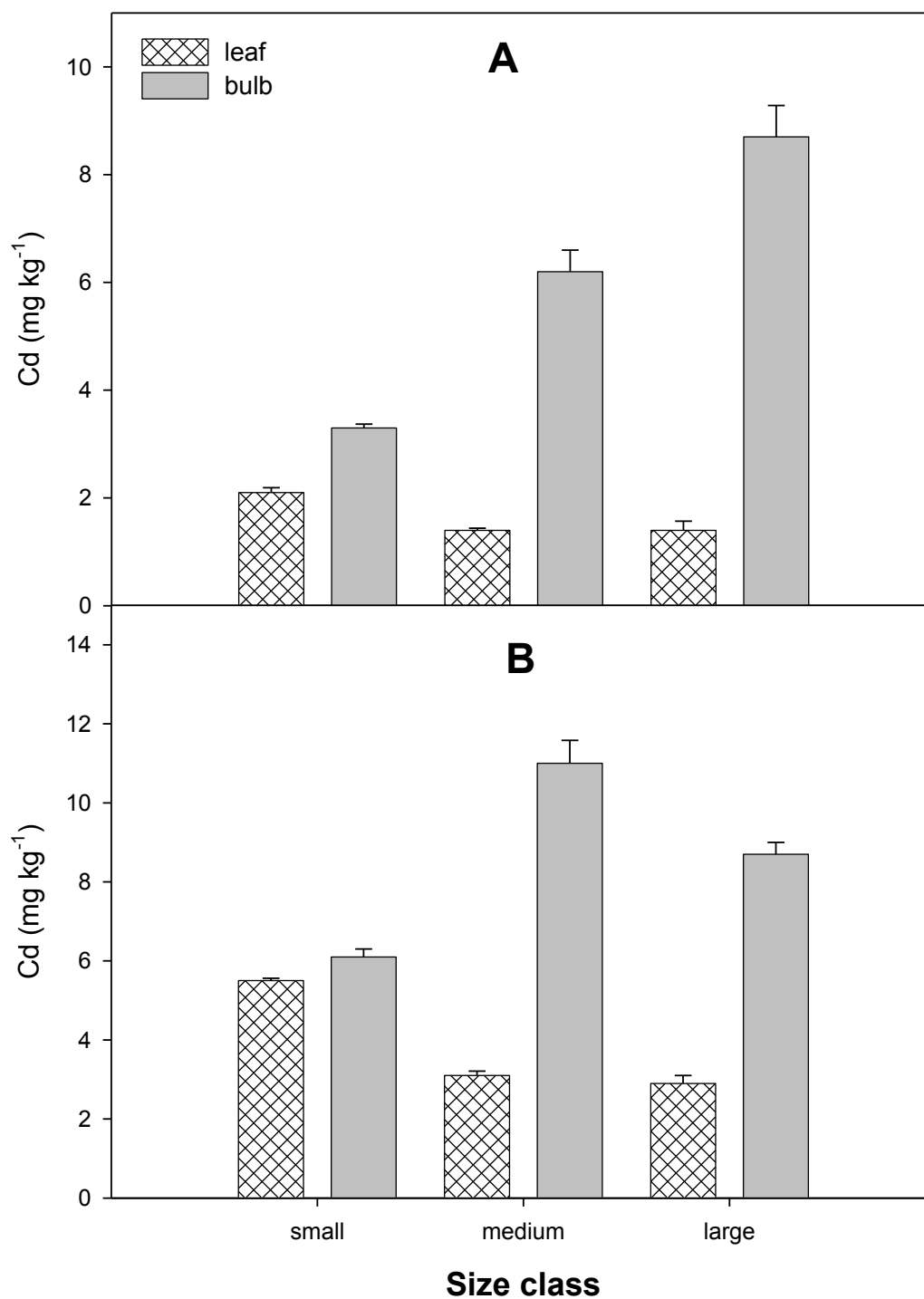
Figure 5.3: Cadmium accumulation (mg kg^{-1}) in leaves and bulbs of *Tulbaghia violacea* after 6 weeks growth in (A) Cd at 2 mg l^{-1} and (B) Cd at 5 mg l^{-1}. Error bars indicate S.E. Cadmium was undetected in the control plants.

public health hazards. Although growing locally available crops can create socio-economic benefits, consumer safety must be safeguarded.

Boron content in both leaves and bulbs of small sized *T. violacea* plants significantly decreased when supplied with Cd at 5 mg l^{-1} (**Table 5.5**). However, in the bulbs of the medium and large sized plants, B content was not significantly affected by Cd at either 2 or 5 mg l^{-1}. Copper content in the bulbs of the small and large sized plants significantly decreased when supplied with Cd at 2 mg l^{-1}, whilst Cu content in bulbs of medium sized plants was significantly lowered by Cd at 5 mg l^{-1}.

Iron content in bulbs of small sized plants was not affected when supplied with Cd at 2 mg l^{-1}, however Fe levels decreased significantly when supplied with Cd at 5 mg l^{-1} (48.7 and 23.7 mg l^{-1} respectively). Leaf Fe content in the small sized plants was not significantly affected by Cd treatments. At 2 mg Cd l^{-1}, Fe levels in the bulbs of medium sized plants was lowered significantly compared to non-Cd-treated bulbs (38.3 and 144 mg kg^{-1} respectively), with a similar trend seen in the bulbs of large sized plants (59.5 and 124 mg kg^{-1} respectively). As reported earlier in the case of *M. plumbea* (**Section 5.3.1**), Cd at 2 mg l^{-1} did not significantly affect Fe levels in *T. violacea* despite reports by ZHOU and QIU (2005) stating that Cd increased Fe content. Conversely, the leaf Fe levels in the large sized *T. violacea* plants significantly increased when supplied with Cd at 2 mg l^{-1}, when compared to the control (119 and 60.2 mg kg^{-1} respectively). This may be due to the age and coping ability of the plant. Medium sized plants showed a similar trend with regards to B and Fe translocation. When supplied with Cd at 2 mg l^{-1}, leaf B and Fe content decreased. However when supplied with Cd at 5 mg l^{-1}, leaf B and Fe content increased (**Table 5.5**).

Manganese and Mo content in bulbs of small sized plants was not significantly affected by the presence of Cd. However, in medium sized bulbs, the content of these microelements was significantly decreased by Cd at 5 mg l^{-1}. In the case of bulbs of large sized plants, Mn content was significantly decreased when supplied with Cd at 2 mg l^{-1} with Mn leaf content decreasing with an increase in Cd (**Table 5.5**). Similar results have been reported whereby Cd toxicity caused a reduction in

Table 5.5: Effect of various Cd treatments on microelement distribution (mg kg⁻¹) in *Tulbaghia violacea*. Mean values (± S.E.) with dissimilar letter(s) are significantly different (*p* < 0.05).

Size class	Treatment (Cd mg l⁻¹)	Leaf	Bulb	Leaf	Bulb
		B (mg kg⁻¹)		Cu (mg kg⁻¹)	
small	0	40.5 ± 2.23 a	12.2 ± 0.68 a	2.75 ± 0.03 b	7.73 ± 0.26 a
	2	37.1 ±1.51 a	11.1 ± 0.35 ab	5 .70 ± 0.06 a	3.06 ± 0.36 b
	5	30.0 ± 1.72 b	9.93 ± 0.29 b	0.85 ± 0.01 c	3.15 ± 0.28 b
medium	0	43.4 ± 1.21 b	15.3 ± 0.43 a	4.43 ± 0.28 a	10.5 ± 0.20 a
	2	38.7 ± 1.39 b	13.8 ± 0.48 a	5.41 ± 0.04 a	11.5 ± 0.59 a
	5	52.9 ± 2.48 a	12.8 ± 0.75 a	3.20 ± 0.37 b	3.6 ± 0.23 b
large	0	36.3 ± 0.44 b	15.8 ± 0.87 a	3.82 ± 0.30 b	14.2 ± 0.21 a
	2	48.4 ± 0.45 a	14.6 ± 0.32 a	3.22 ± 0.11 b	6.23 ± 0.68 b
	5	29.1 ± 0.71 c	15.3 ± 0.81 a	4.83 ± 0.22 a	4.15 ± 0.11 c
		Fe (mg kg⁻¹)		Mn (mg kg⁻¹)	
small	0	81.8 ± 3.71 a	53.4 ± 3.91 a	30.0 ± 0.49 ab	10.8 ± 0.21 a
	2	84.1 ± 2.59 a	48.7 ± 0.92 a	32.1 ± 1.12 a	11.9 ± 0.56 a
	5	77.1 ± 5.42 a	23.7 ± 1.54 b	28.7 ± 0.59 b	10.8 ± 0.33 a
medium	0	81.3 ± 3.26 a	144 ± 3.95 a	20.93 ± 0.15 a	21.8 ± 0.63 a
	2	67.2 ± 1.71 b	38.3 ± 1.26 b	22.3 ± 0.37 b	20.1 ± 0.44 a
	5	72.4 ± 3.26 ab	34.3 ± 0.83 b	21.7 ± 0.04 ab	15.7 ± 0.45 b
large	0	60.2 ± 1.66 b	124 ± 2.1 a	21.7 ± 0.18 a	18.9 ± 1.06 a
	2	119 ± 2.26 a	59.5 ± 4.1 b	19.8 ± 0.26 b	12.9 ± 0.64 b
	5	55.7 ± 2.58 b	62.0 ± 3.0 b	17.9 ± 0.13 c	15.8 ± 0.51 ab
		Mo (mg kg⁻¹)		Zn (mg kg⁻¹)	
small	0	1.31 ± 0.01 b	2.15 ± 0.06 a	16.8 ± 0.25 b	14.6 ± 0.14 a
	2	1.51 ± 0.02 a	2.19 ± 0.02 a	48.5 ± 3.41 a	15.2 ± 0.52 a
	5	1.26 ± 0.02 b	2.24 ± 0.18 a	12.8 ± 1.22 b	12.6 ± 0.46 b
medium	0	1.35 ± 0.00 a	3.34 ± 0.13 a	24.3 ± 1.01 a	30.9 ± 1.91 a
	2	1.31 ± 0.02 a	3.03 ± 0.08 a	21.6 ± 0.80 a	29.5 ± 0.55 a
	5	1.38 ± 0.02 a	2.51 ± 0.02 b	23.0 ± 0.07 a	26.5 ± 0.81 a
large	0	0.69 ± 0.01 b	2.08 ± 0.23 ab	25.2 ± 0.71 a	35.3 ± 0.47 a
	2	0.79 ± 0.05 ab	2.79 ± 0.11 a	25.2 ± 3.31 a	31.1 ± 0.41 b
	5	0.86 ± 0.02 a	1.69 ± 0.12 b	27.6 ± 2.22 a	28.6 ± 0.59 c

leaf Mn content (CHIZZOLA 2001; GARDEA-TORRESDEY, PERALTA-VIDEA, MONTES, DE LA ROSA and CORRAL-DIAZ 2004).

The only Cd/Zn interaction observed was in the small sized plants whereby plants supplied with Cd at 2 mg l^{-1} accumulated Zn to 48.5 mg kg^{-1} in the leaves compared to 16.8 mg kg^{-1} in the control. However, when supplied with Cd at 5 mg l^{-1}, only 12.8 mg Zn kg^{-1} accumulated in the leaves suggesting Cd/Zn antagonism at 5 mg Cd l^{-1}.

The use of traditional and indigenous leafy vegetables by local people is still relatively under-developed in South Africa (ODHAV, BEEKRUM, AKULA and BAIJNATH 2007). The results presented in this study include the first report on the nutritional status of *T. violacea* leaves. The findings indicate that the leaves may be an important supplementation vegetable and the micronutrient content can be compared with similar South African traditional leafy vegetables such as *Amaranthus dubius* Mart. ex. Thell., *Centella asiatica* (L.) Urb. and *Solanum nodiflorum* Jacq. (ODHAV, BEEKRUM, AKULA and BAIJNATH 2007). In this study, plant size had an important effect with regards to elemental distribution. However, the most noticeable result is the Cd accumulation in the leaves.

This study indicated that *T. violacea* has the ability to accumulate Cd. In addition, age of the plant plays an important factor with regards to Cd accumulation and elemental distribution.

5.4 SUMMARY

Merwilla plumbea

- Although Cd readily accumulated in the plant when supplied with Cd at 2 mg l^{-1}, the addition of Zn at 50 mg l^{-1} to the Cd-containing media led to a higher Cd accumulation. However, further increase of Zn in the media (100 and 150 mg l^{-1}) showed an antagonistic effect of Zn on Cd uptake.
- B concentrations in the leaves and roots were not significantly affected by the addition of Cd to the media. However, with the addition of supplementary Zn to the media, leaf B content increased while the B content in the bulbs and roots decreased.

- Cu and Mo levels were not significantly affected by Cd or Cd/Zn combinations.

- Fe levels in the plant were not significantly affected by Cd in the media. However, with an increase of Zn to 150 mg l^{-1} in the Cd-containing media, Fe content in the leaves, bulbs and roots increased.

- Compared to the control, Cd and Cd/Zn applications caused an increase in Mn content in leaves, bulbs and roots.

Tulbaghia violacea

- Cd at 5 mg l^{-1} significantly decreased the number of leaves in small sized plants, however leaf length, fresh weight of leaves, and bulb and root parameters were not significantly affected by Cd treatments.

- Leaf length, fresh weight and number of leaves of the medium sized plants decreased when supplied with Cd at 2 mg l^{-1}.

- Small sized plants accumulated more Cd in the leaves than medium or large sized plants.

- Cd accumulation in the bulbs increased with increasing Cd in the media.

- B content in both leaves and bulbs of small sized plants significantly decreased when supplied with Cd at 5 mg l^{-1}. However, in the bulbs of medium and large sized plants, B content was not significantly affected by Cd at 2 or 5 mg l^{-1}.

- Cu content in bulbs of small and large sized plants significantly decreased with 2 mg Cd l^{-1} in the media. Cu content in bulbs of medium sized plants was significantly lowered by 5 mg Cd l^{-1}.

- Application of Cd at 5 mg l^{-1} lowered leaf Cu, Fe, Mo and Zn content in small and medium sized plants.

6 Effect of nutrient supply on accumulation of microelements in *Dioscorea* species

6.1 INTRODUCTION

6.1.1 Cultivation of South African *Dioscorea* species

The majority of species of the genus *Dioscorea* are perennial, herbaceous climbers that form rhizomes and tubers as storage organs (BURKILL 1960; PURSEGLOVE 1972; VAN STADEN and FOWLDS 1992) and are distributed in tropical regions of Africa, America and Asia (TERUI and OKAGAMI 1993). Several species of *Dioscorea* are amongst the principle sources of diosgenin, which can be converted to medicinally important steroids (VAN STADEN and FOWLDS 1992). These steroids are used as contraceptives and anti-inflammatory agents (BRUNETON 1995). None of the South African species of *Dioscorea* are used commercially, but there are several American, Chinese and Indian species of importance in the extraction of steroidal saponins which are hydrolysed to diosgenin (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).

Two highly utilized medicinal species in South Africa are *Dioscorea dregeana* (Kunth) Dur. & Schinz and *Dioscorea sylvatica* (Kunth) Eckl. The tubers, used for their sedative properties to treat ailments such as hysteria, convulsions and epilepsy (CROUCH, SYMMONDS, SPRING and DIEDERICHS 2006), are illegally uprooted from indigenous forests (DOLD and COCKS 2002) and are traded at informal markets across the country (CUNNINGHAM 1993). Such circumstances have suggested the necessity for commercializing many *Dioscorea* species in Africa (OKOLE and ODHAV 2004).

Considering the high demand for *Dioscorea* species, there is very little information available on propagation practices of South African species. *Dioscorea* species are generally regarded as demanding on soil fertility (O'SULLIVAN and JENNER 2006), thus understanding the fate of microelements in the plants as affected by nutrient

levels in the growth media is important in order to develop management practices. Furthermore, mineral composition homeostasis under stress is an important aspect in the understanding of heavy metal tolerance. This study investigated the effect of nutrient levels on microelemental uptake and distribution in *Dioscorea dregeana* and *Dioscorea sylvatica*.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design

Three-week-old seedlings grown in Petri dishes were transplanted in 20 cm pots filled with sterile, acid-washed quartz sand and moistened with Hoagland's nutrient solution (HS) (HOAGLAND and SNYDER 1933) (**Appendix A**) of various strengths depending on the treatments. Each pot consisted of four seedlings with six replications per treatment. The pots were arranged randomly in the plant growth chambers under 16:8 h light and dark conditions with a photosynthetic photon flux density of 80.4 ± 3.5 µmol m^{-2} s^{-1} at 25 °C.

6.2.1.1 *Dioscorea dregeana* (Kunth) Dur. & Shinz.

Three-week-old seedlings were treated immediately after repotting. Two different nutrient trials were conducted. In the first trial, seedlings were subjected to low (10%), half (50%) and full (100%) strength HS. For each treatment, potting sand was moistened with 75 ml of the respective solution twice weekly. The experiment was terminated after 6 weeks. In the second trial, the effects of deficiency of three macronutrients, N, P and K were studied by eliminating each one of these from 50% HS. The seedlings were treated by adding 75 ml of half-strength HS without N or P or K (-N,-P,-K) twice weekly. The 50% HS containing NPK was used as a control. The experiment was terminated after 6 weeks.

6.2.1.2 *Dioscorea sylvatica* (Kunth) Eckl.

A nine-week adaptation period allowed the seedlings to establish before the start of the treatments. Healthy nine-week-old plants were subjected to Zn stress by

adjusting the HS to contain Zn at 100, 200 or 300 mg l^{-1}, in the form of $ZnSO_4 \cdot 7H_2O$. Nutrient solution (50% HS) with no additional Zn served as a control. Treatments were added once weekly (75 ml per pot) for 4 weeks.

6.2.2 Sample preparation and data collection

The plants were harvested, measured and processed as outlined in Section 4.2.2.

6.2.3 Chlorophyll analysis

The chlorophyll content in the leaf material was quantified as described in Section 4.2.3.

6.2.4 Elemental analysis

Elemental analysis was done by ICP-OES as described in Section 3.2.3.

6.2.5 Data analysis

Growth data with different treatments were analyzed using one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package and either Tukey's or paired t-tests were used to separate means at a 5% level of significance.

6.3 RESULTS AND DISCUSSION

6.3.1 *Dioscorea dregeana*

6.3.1.1 Nutrient levels

Various degrees of nutrient levels (10, 50 and 100%) in the media had no significant ($p > 0.05$) effect on shoot and root length (**Table 6.1**). On the other hand, application of both 50% and 100% HS significantly increased seedling fresh weight, leaf area, aerial shoot length and dry weight of the shoot relative to 10% HS. The number of

INSERT TABLE 6.1

leaves per seedling was significantly higher for seedlings grown in 50% HS compared to seedlings grown in 10% and 100% HS. In addition, the highest percentage of aerial shoots was recorded in 50% HS. It has been reported that low Fe supply lowers tuber yield of potatoes (*Solanum tuberosum* L.) (CHATTERJEE, GOPAL and DUBE 2006). However, subjecting *D. dregeana* seedlings to various nutrient levels showed no significant change with respect to tuber size.

The percentage of nutrient solution had an effect on elemental distribution (**Table 6.2**). Seedlings grown in 10% HS contained a higher total B, Fe and Mo content compared to seedlings grown in 50% and 100% HS. Whereas seedlings grown in 100% HS contained the lowest total B, Fe and Zn (**Table 6.2**).

Root Fe influx is regulated by the Fe status of the plant (COHEN, FOX, GARVIN and KOCHIAN 1998). In agreement with our findings for *D. dregeana*, FOX, SHAFF, GRUSAK, NORVELL, CHEN, CHANEY and KOCHIAN (1996) revealed that Fe-deficient *Pisum sativum* L. seedlings exhibited significantly higher rates of root Fe uptake than Fe-sufficient seedlings.

The capability of root tissues to hold Cu against transport to shoots under conditions of Cu excess has been observed (KABATA-PENDIAS and PENDIAS 1984). Plants grown in 50% HS contained a significantly higher Cu content in the tubers/roots (107 mg kg^{-1}) compared to plants grown in 10% and 100% (23.2 and 29.1 mg kg^{-1} respectively). It has been suggested that stability and homeostasis of mineral composition under nutrient deficiency stress plays an important role in Cu-tolerance of plants (KE, XIONG, CHEN and CHEN 2007). XIONG, LI and XU (2002) revealed that low nutrient strength in the growth medium stimulated Cu accumulation by *Brassica pekinensis* Lour., while high nutrient strength reduced Cu concentration. This was true for *D. dregeana* when comparing results for Cu content after applications of 50% and 100% HS.

Table 6.2: Effect of varying nutrient levels (HS – Hoagland's nutrient solution) on microelement distribution (mg kg^{-1}) in *Dioscorea dregeana*. Mean values (± S.E.) with dissimilar letter(s) are significantly different ($p < 0.05$).

Treatment (HS %)	Leaves	Tubers + roots
B (mg kg^{-1})		
10	43.9 ± 3.17 b	86.1 ± 4.67 a
50	56.2 ± 1.38 a	26.2 ± 0.90 b
100	39.1 ± 0.36 b	34.4 ± 2.78 b
Cu (mg kg^{-1})		
10	6.6 ± 0.23 b	23.2 ± 1.63 b
50	9.3 ± 0.41 a	107 ± 6.53 a
100	6.9 ± 0.05 b	29.1 ± 1.02 b
Fe (mg kg^{-1})		
10	560 ± 12.1 a	790 ± 1.65 a
50	347 ± 25.1b	664 ± 27.1 b
100	292 ± 8.0 b	460 ± 7.6 c
Mn (mg kg^{-1})		
10	19.2 ± 1.03 c	25.2 ± 1.84 ab
50	58.2 ± 0.12 a	29.5 ± 0.42 a
100	29.8 ± 0.15 b	21.7 ± 0.93 b
Mo (mg kg^{-1})		
10	1.49 ± 0.24 a	2.25 ± 0.26 a
50	0.84 ± 0.05 b	1.29 ± 0.08 b
100	0.87 ± 0.02 ab	1.88 ± 0.16 ab
Zn (mg kg^{-1})		
10	42.6 ± 2.55 a	74.8 ± 5.32 a
50	35.7 ± 0.64 b	120 ± 4.16 b
100	24.7 ± 0.40 c	62.9 ± 2.88 a

The accumulation of Cu and Mn corresponded to the different mobility of these microelements. A large proportion of Cu taken up by the plants was retained in the roots while Mn was accumulated primarily in the leaves. Similar findings in *Hordeum vulgare* L. have been reported by DEMIREVSKA-KEPOVA, SIMOVA-STOILOVA, STOYANOVA, HOLZER and FELLER (2004). Total Cu and Zn was highest when grown in the 50% HS (116.7 and 155.8 mg kg^{-1} respectively). Copper-Zn antagonistic interactions have been observed whereby the uptake of one element competitively

inhibited the other (KABATA-PENDIAS and PENDIAS 1984). However, such a Cu-Zn antagonism was not seen in *D. dregeana.*

It is clear that nutrient percentage plays an important role in nutrient distribution and accumulation which in turn affects plant growth and development. NARULA, KUMAR and SRIVASTAVA (2005) revealed that the presence of Cu in the growth media stimulated diosgenin production in *Dioscorea bulbifera* L. Thus, the current study indicates the need for further investigation regarding the effect of nutrient supply on phytochemical yield (discussed in **Chapter 8**).

6.3.1.2 Deficiency of N, P and K

The effects of macronutrient deficiencies on the expression of genes involved in primary metabolism in the shoot (evidence for increased carbohydrate concentrations and altered biomass, and the consequences of these changes on the growth and morphology of the plant root system) was reviewed by HERMANS, HAMMOND, WHITE and VERBRUGGEN (2006).

In the present work, the relative concentration of elements in plant tissues compared to a control was adopted. In *D. dregeana*, deficiency of each macronutrient (N,P,K) showed a negative effect on seedling growth. The size of the tuber and root dry weight did not increase or decrease significantly with the elimination of N, P or K (**Table 6.1**). The rest of the growth parameters of the seedlings in the presence of N,P and K (control) gave significantly better growth performance than the application of basal nutrient without N, P or K. Reduction in plant growth due to macronutrient deficiencies has been reported (YEH, LIN and WRIGHT 2000).

Insufficient N significantly lowered total B content in the leaves and tubers/roots (**Table 6.3**). Boron content was (non-significantly) lower in P-deficient plants compared to control, especially in leaves (40.6 and 56.2 mg kg^{-1} respectively). Boron-phosphorous interactions in soils are related to the interference of phosphate ions with B mobility (KABATA-PENDIAS and PENDIAS 1984). Potassium deficiency had no significant effect on B content in leaves or tubers/roots of *D. dregeana*.

Table 6.3: Effect of macronutrient deficiency (N, P or K) on microelement distribution (mg kg^{-1}) in *Dioscorea dregeana*. An asterisks (*) denotes a significant difference from the control (*p* < 0.05; ± S.E).

Treatment	Leaves	Tubers + roots
	B (mg kg^{-1})	
Control	56.2 ± 1.38	26.2 ± 0.90
- N	28.5 ± 1.38 *	17.2 ± 0.94 *
- P	40.6 ± 5.47	24.3 ± 0.25
- K	43.1 ± 3.95	25.9 ± 1.57
	Cu (mg kg^{-1})	
Control	9.3 ± 0.41	107 ± 6.53
- N	11.6 ± 1.60	95.9 ± 3.18
- P	15.2 ± 0.76	172 ± 9.49 *
- K	21.6 ± 3.04	89.2 ± 0.72a
	Fe (mg kg^{-1})	
Control	347 ± 25.1	664 ± 27.1
- N	118 ± 4.04 *	318 ± 25.7 *
- P	433 ± 18.5	815 ± 51.9
- K	398 ± 26.2	761 ± 47.1
	Mn (mg kg^{-1})	
Control	58.2 ± 0.12	29.5 ± 0.42
- N	34.4 ± 7.04 *	26.9 ± 0.41
- P	79.8 ± 1.53 *	39.3 ± 1.17 *
- K	75.1 ± 0.40 *	36.3 ± 0.09 *
	Mo (mg kg^{-1})	
Control	0.84 ± 0.05	1.29 ± 0.08
- N	0.49 ± 0.11 *	1.11 ± 0.02
- P	0.95 ± 0.04	1.66 ± 0.13
- K	0.97 ± 0.08	2.22 ± 0.08 *
	Zn (mg kg^{-1})	
Control	35.7 ± 0.64	120 ± 4.16
- N	32.1 ± 6.45	69.3 ± 1.56 *
- P	66.8 ± 3.05 *	124 ± 6.51
- K	38.4 ± 2.71	103 ± 6.41

Deficiency of N, P and K increased leaf Cu content. In agreement with our findings, lack of P and K resulted in higher Cu concentrations in maize stalks (LI, ZHOU, CANG, ZHANG, FAN and QIN 2007). Compared with control, the omission of P significantly increased the Cu content in the underground plant material (107 and 172 mg kg^{-1} respectively).

Omission of N significantly lowered the total Fe content in *D. dregeana* (**Table 6.3**). Compared to the control, P deficiency caused a (non-significant) Fe increase in leaves and tubers/roots. HIRSCH, MARIN, FLORIANI, CHIARENZA, RICHAUD, NUSSAUME and THIBAUD (2006) reported that P deficiency promoted an alteration of Fe storage from the vacuole to the chloroplasts in *Arabidopsis thaliana* L. Omission of K had no significant effect on total Fe content. However, sufficient K supply has been reported to reduce translocation of Fe from roots to shoots, especially in upper leaves, in rice (*Oryza sativa* L.), thus ameliorating toxicity (LI, YANG and LUO 2001).

Compared to the control, leaf Mn content significantly decreased due to N deficiency (**Table 6.3**). However, the Mn content in tubers/roots remained constant. Lack of P and K significantly increased total Mn content in *D. dregeana*. Addition of P has shown a favorable effect in managing the severity and progression of Mn toxicity symptoms in potatoes (SARKAR, PANDEY, SUD and CHANEMOUGASOUNDHARAM 2004).

The lack of N caused a decrease in leaf Mo content. However, the tubers/roots remained comparable to the control (**Table 6.3**). Phosphorous deficiency had no effect on Mo accumulation and distribution. However, low K significantly increased Mo accumulation in tubers/roots.

Omission of N significantly lowered Zn content in the tubers/roots. However, leaf Zn content remained comparable to the control. Lack of P significantly increased leaf Zn content. On the contrary, GIANQUINTO, ABURAYYAN, DITOLA, PICCONTINO and PEZZAROSSA (2000) found that the addition of P to plants grown at low Zn supply reduced leaf Zn concentration in *Phaseolus vulgaris* cv. Borlotto nano. The Zn levels in K-reduced plants were comparable to those in the control plants.

The use of fertilizers is often essential in order to obtain a larger yield. However, the relatively low cost of certain fertilizers and the misinterpretation of the relationship between fertilizer application and crop yield have encouraged some farmers to over-fertilize crops (CUI, CHEN, LI, XU, SHI and ZHANG 2006). The application of fertilizers has been shown to have an effect on phytochemical yield. Nitrogen supply to *Hypericum perforatum* L. (St. Johns wart) plants had a profound impact on the levels of phytochemicals in the leaves. Decreasing the levels of N resulted in an increase in secondary metabolite yield amounting to 2.4 – 3.3 fold (BRISKIN, LEROY and GAWIENOWSKI 2000). Likewise, a lower N concentration significantly increased hypoxide levels in *Hypoxis hemerocallidea* Fisch.Mey & Ave-Lall. (Hypoxidaceae) (MCALISTER and VAN STADEN 1995), a popular medicinal plant recommended to treat HIV/AIDS. Similarly, artemisinin content of the dried leaves of *Artemisia annua* L. was significantly affected by N applications (OZGUVEN, SENER, ORHAN, SEKEROGLU, KIRPIK, KARTAL, PESIN and KAYA 2008).

It is clear from the above mentioned examples that elemental ratios in soils directly influence phytochemical yield. Consequently, directly impacting on the quality of medicinal plants. Proper nutrient application can not only improve the tuber size, thus increasing yield, but may also improve the quality of the diosgenin containing *D. dregeana*.

6.3.2 *Dioscorea sylvatica*

Zinc is an essential trace element needed for plant growth and development. It is an important co-factor required for the structure and function of numerous proteins as well as DNA synthesis (GROTZ and GUERINOT 2006; VAN DER PERK 2006). However, excess Zn can cause toxicity. Zinc phytotoxicy has been reviewed by ROUT and DAS (2003).

Zinc frequently exceeds concentrations of 300 mg kg^{-1} in contaminated soils (VAN DER PERK 2006). It is clear from the results that *D. sylvatica* was negatively affected by elevated Zn in the nutrient solution (**Figure 6.1**). Bulb length, bulb dry weight and root fresh weight showed a significant decrease when supplied with Zn at 100 mg l^{-1} compared to the control. Bulb length declined by over 50% when supplied with Zn at

100, 200 and 300 mg l^{-1}. There was a statistically significant decrease in all growth parameters between Zn supply of 100 and 200 mg l^{-1}, however, no significant decrease in growth parameters was seen between 200 and 300 mg Zn l^{-1}.

The chlorophyll content in the older leaves (collected from lowest part of vine) were more affected by an increase in Zn than the young leaves (collected from top part of vine) (**Table 6.4**). When supplied with Zn at 300 mg l^{-1}, chlorophyll content in the young leaves was only slightly (yet significantly) lowered compared to the control. However, when supplied with the same Zn content in the media (300 mg l^{-1}), chlorophyll content in older leaves significantly decreased compared to the control. When given luxury supplies of Zn, several plant species translocate appreciable quantities of this metal from older leaves to generative organs; but under Zn-deficiency conditions, the same species mobilize little, if any, Zn from old leaves. Zinc is likely to be concentrated in mature leaves (KABATA-PENDIAS 2001). It is this toxic Zn concentration in older leaves, which could cause a decrease in chlorophyll. Decrease in chlorophyll content due to Zn toxicity has been reported (VAILLANT, MONNET, HITMI, SALLANON and COUDRET 2005).

According to MARSCHNER (1995), the average Zn concentration in plant shoot dry matter sufficient for adequate growth is 20 mg kg^{-1}. This was true for plants grown in 50% HS (no Zn increase), whereby leaves of *D. sylvatica* contained 21.1 mg kg^{-1} (**Table 6.5**) and achieved maximum growth (**Figure 6.1**). Total Zn content in the plant increased with increasing Zn in the media, and reached 1 151, 1 920 and 7 642 mg kg^{-1} in the leaves, bulbs and roots respectively, when supplied with Zn at 300 mg l^{-1}. Although this is a substantial level, it is clear from the reduction in growth parameters that the plant was under severe stress. LI, YANG, YANG and HE (2006) evaluated Zn accumulation and subcellular distribution in leaves of a hyperaccumulating ecotype and a non-hyperaccumulating ecotype of *Sedum alfredii* Hance. For the hyperaccumulating ecotype of *S. alfredii,* the cell wall and the vacuole played a very important role in Zn tolerance and hyperaccumulation.
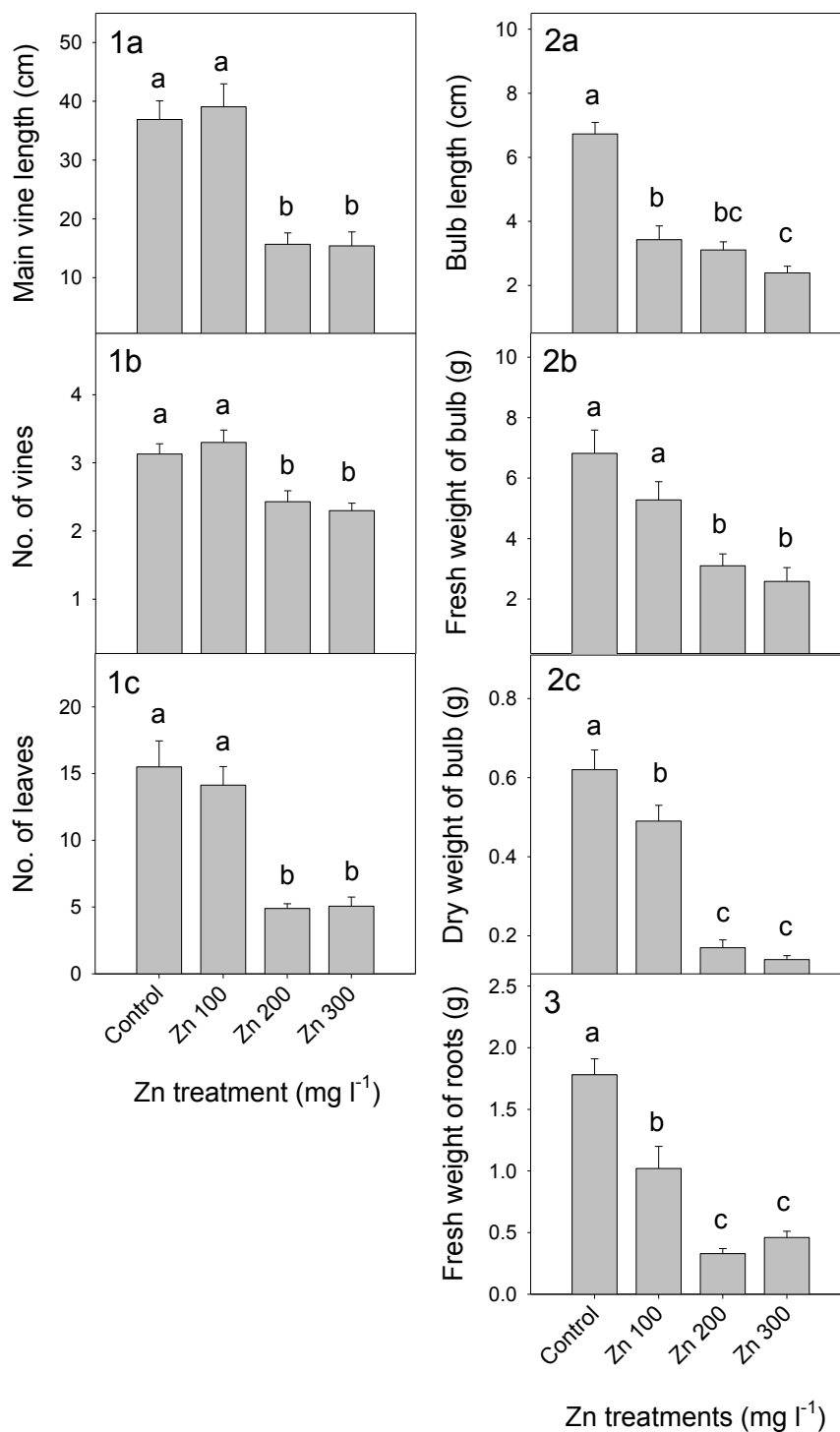
Figure 6.1: **Effect of Zn (100, 200 and 300 mg l^{-1}) after 4 weeks on growth parameters of** ***Dioscorea sylvatica.*** **(1a) main vine length, (1b) number of vines, (1c) number of leaves, (2a) bulb length, (2b) fresh weight of bulb, (2c) dry weight of bulb, (3) fresh weight of roots. Mean values with dissimilar letter(s) are significantly different (*p* < 0.05). Error bars indicate S.E.**

Table 6.4: Effect of excess Zn on leaf chlorophyll content (mg chlorophyll per fresh weight) in (A) young and (B) old leaves of *Dioscorea sylvatica*. Mean values (± S.E.) with dissimilar letter(s) are significantly different ($p < 0.05$).

A			
Treatment (Zn mg l^{-1})	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Control	10.5 ± 0.37 a	3.45 ± 0.11 a	13.9 ± 0.49 a
100	7.37 ± 0.28 b	0.75 ± 0.18 b	8.12 ± 0.35 c
200	9.64 ± 1.78 a	3.80 ± 0.17 a	13.4 ± 0.35 ab
300	8.62 ± 0.33 ab	3.24 ± 0.14 a	11.9 ± 0.46 b

B			
Treatment (Zn mg l^{-1})	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Control	16.1 ± 0.73 a	5.58 ± 0.36 a	21.6 ± 1.09 a
100	7.28 ± 0.89 b	2.46 ± 0.34 b	9.75 ± 1.22 b
200	5.28 ± 0.56 bc	2.00 ± 0.22 b	7.27 ± 0.78 bc
300	4.21 ± 0.22 c	0.40 ± 0.09 c	4.61 ± 0.38 c

As seen for *D. sylvatica*, roots often contain much more Zn than do aerial parts, particularly if the plants are grown in Zn-rich media. With luxury levels of soil Zn, this element may be translocated from the roots and accumulated in the shoots of the plant (KABATA-PENDIAS 2001).

Leaf B and Cu contents were not significantly affected by Zn concentrations of up to 200 mg l^{-1} (**Table 6.5**). However, when supplied with Zn at 300 mg l^{-1}, Cu in the leaves and bulbs decreased while root Cu content significantly increased compared to the control, thus indicating a redistribution of Cu due to Zn toxicity. CAYTON, REYES and NEUE (1985) indicated that an increase in Zn enhanced translocation of Cu and Mn in *Oryza sativa* L.

Iron content in leaves and roots was significantly lowered due to an increase in Zn. Iron-Zn antagonism is widely recognized – an excess of Zn leads to a reduction in Fe concentrations in plants (KABATA-PENDIAS and PENDIAS 1984). KAYA and HIGGS (2001) evaluated the effectiveness of P and Fe supplements in nutrient concentrations of *Lycopersicon esculentum* cvs. Blizzard, Liberto, Calytosp grown at

Table 6.5: Effect of excess Zn on microelement distribution (mg kg^{-1}) in *Dioscorea sylvatica*. Mean values (± S.E.) with dissimilar letter(s) are significantly different ($p < 0.05$).

Treatment (mg l^{-1})	Leaves	Tubers	Roots
B (mg kg^{-1})			
Control	46.0 ± 1.71 a	20.1 ± 0.00 ab	38.2 ± 1.42 a
Zn 100	50.7 ± 1.80 a	19.1 ± 1.09 ab	37.8 ± 2.01 a
Zn 200	45.1 ± 1.33 a	17.4 ± 1.24 b	26.4 ± 0.51 b
Zn 300	36.4 ± 0.79 a	22.1 ± 1.06 a	35.2 ± 0.66 a
Cu (mg kg^{-1})			
Control	8.97 ± 0.66 a	28.4 ± 4.99 a	150 ± 4.11 c
Zn 100	8.73 ± 0.21a	18.7 ± 0.55 a	128 ± 3.61 c
Zn 200	8.18 ± 0.05 a	25.1 ± 1.01 a	216 ± 8.30 a
Zn 300	6.55 ± 0.10 b	20.9 ± 2.83 a	177 ± 2.31 b
Fe (mg kg^{-1})			
Control	165 ± 1.3 a	188 ± 6.1 a	1784 ± 14 a
Zn 100	106 ± 2.1 b	244 ± 11 a	1424 ± 32 b
Zn 200	94.6 ± 5.7 bc	196 ± 11 a	1178 ± 9 c
Zn 300	89.0 ± 1.6 c	233 ± 32 a	938 ± 13 d
Mn (mg kg^{-1})			
Control	28.9 ± 0.25 b	12.3 ± 0.29 a	32.3 ± 0.73 a
Zn 100	35.7 ± 0.41 a	11.7± 1.33 a	29.2 ± 0.33 bc
Zn 200	20.2 ± 0.13 d	8.5 ± 0.34 a	30.4 ± 0.16 ab
Zn 300	26.6 ± 0.06 c	10.1 ± 2.22 a	28.1 ± 0.23 c
Mo (mg kg^{-1})			
Control	0.67 ± 0.01 a	1.94 ± 0.11 a	4.2 ± 0.32 a
Zn 100	0.37 ± 0.02 b	0.95 ± 0.26 c	2.8 ± 0.27 b
Zn 200	0.24 ± 0.01 c	1.32 ± 0.05 ab	1.6 ± 0.03 c
Zn 300	0.27 ± 0.01 c	1.22 ± 0.03 b	1.7 ± 0.08 c
Zn (mg kg^{-1})			
Control	22.1 ± 1.22 c	43.0 ± 2.54 d	201 ± 8.91 d
Zn 100	1134 ± 12.3 b	882 ± 23.3 c	2666 ± 39.1 c
Zn 200	1201 ± 6.6 a	1679 ± 66.5 b	5051 ± 63.9 b
Zn 300	1151 ± 8.9 b	1920 ± 65.5 a	7642 ± 1.44 a

high Zn. The study revealed an increase in dry weight and chlorophyll concentrations and decrease Zn concentrations in leaves and roots.

Compared to the control, Zn supplied at 100 mg l^{-1} significantly increased the leaf Mn content. However a further Zn increase in the media to 300 mg l^{-1} lowered the leaf Mn content considerably (**Table 6.5**). Similarly, Mo-Zn antagonism was observed whereby increasing Zn lead to a decrease in total Mo content. However, Mn content in the tubers was not significantly affected by the increase in Zn. CAYTON, REYES and NEUE (1985) revealed that the increase of Zn in the culture media increased uptake of Mn in rice.

Dioscorea sylvatica was sensitive towards excess Zn in the media. Zinc phytotoxicity was evident from the decrease in growth parameters and reduction in chlorophyll content. The evaluation of the redistribution of micronutrients following Zn toxicity is an important step towards the development of the much sought after *D. sylvatica* as an agricultural crop.

6.4 SUMMARY

Dioscorea dregeana

- Plants grown in 10% Hoagland's nutrient solution (HS) contained higher total B, Fe and Mo levels compared to seedlings grown in 50% and 100% HS.
- Seedlings grown in 100% HS had the lowest total B, Fe and Zn contents.
- Insufficient N and P lowered total B content in the plant. However, K deficiency had no effect on B content in leaves or tubers and roots.
- Deficiency of N, P and K had no effect on leaf Cu content. However, the omission of P significantly increased the Cu content in the underground plant mass.
- Compared to the control, P deficiency resulted in a Fe increase in the leaves, tubers and roots.
- Lack of P and K significantly increased total Mn content.

Dioscorea sylvatica

- After application of various Zn concentrations (100, 200 and 300 mg Zn l^{-1}), there was a significant decrease in all growth parameters between Zn at 100 and 200 mg l^{-1}, however, no statistically significant decrease was seen with a further increase in Zn to 200 and 300 mg l^{-1}.

- Compared to the control, bulb length declined by more than 50% when grown in Zn at 100, 200 and 300 mg l^{-1}.

- Chlorophyll content in older leaves was more affected by an increase in Zn than in younger leaves.

- Total Zn content in the plants increased with increasing Zn in the media – most of the Zn was detected in the roots.

- Relationships between Zn uptake and microelemental distribution differed in the different plant parts.

- Certain microelements appear to be redistributed due to Zn toxicity.

7 Effect of Cu, Cd, Hg, Pb and Zn on germination and seedling growth of some species of Hyacinthaceae

7.1 INTRODUCTION

7.1.1 Seed propagation of South African medicinal plants

To date, seed propagation remains the most feasible and the cheapest option for the cultivation of wild medicinal plants. Recently, research has been conducted on germination rates and seedling establishment for some important South African medicinal plant species (KULKARNI, SPARG and VAN STADEN 2005; KAMBIZI, ADEBOLA and AFOLAYAN 2006; KULKARNI, SPARG and VAN STADEN 2006). However, there is no information on the response of South African medicinal plants to microelements and heavy metals. Seedlings are more sensitive to essential and non-essential elements than mature plants, as their defence mechanisms are not yet fully developed (LIU, ZHANG, SHAN and ZHU 2005). Thus, optimizing nutrient levels could improve seedling vigor and survivability.

This study was conducted to determine the effects (sensitivity and tolerance) of metal elements on germination and seedling growth of *Bowiea volubilis*, *Eucomis autumnalis* and *Merwilla plumbea*, which are frequently proposed for cultivation and extensively used in traditional medicine in South Africa.

7.2 MATERIALS AND METHODS

7.2.1 Seed collection and storage

The seeds were collected when mature in 2005 from the University of KwaZulu-Natal Botanical Garden, and were stored in brown paper bags at room temperature (25 ± 2 °C) for a period of three months before being used for the experiment.

7.2.2 Experimental design

Seeds were surface decontaminated with 0.1% mercuric chloride for 2 mins and then rinsed thoroughly with distilled water prior to germination testing. *Bowiea volubilis* seeds were scarified with sand paper for optimum germination (KULKARNI, SPARG and VAN STADEN 2005).

Disposable Petri dishes (9 cm), each containing two Whatman No. 1 filter paper discs were used for germination trials. Four replications of 20 seeds per Petri dish were used and 3.5 ml of appropriate solution was added. The concentrations of Cu ($CuSO_4.5H_2O$) and Zn ($ZnSO_4.7H_2O$) tested were 1, 2, 5, 10, 20 and 50 mg l^{-1}. Cadmium ($CdCl_2.H_2O$), Hg ($HgCl_2$) and Pb ($CH_3COO)_2Pb.3H_2O$) concentrations tested were 0.5, 1 and 2 mg l^{-1}. Distilled water was used as the control treatment. Test solutions were replenished every other day to maintain moisture levels. Petri dishes containing seeds of *B. volubilis* and *M. plumbea* were placed in plant growth chambers set at 25 ± 0.5 °C with 16:8 h light and dark conditions. The photosynthetic photon flux density of lamps in the growth chamber was 80.4 ± 3.5 µmol m^{-2} s^{-1}. Germination (2 mm radicle emergence) was recorded every day. The experiment was terminated after 21 days when seedlings were developed. To achieve maximum germination of *E. autumnalis,* the seeds were germinated under constant dark conditions for 45 days at 20 ± 0.5 °C (KULKARNI, SPARG and VAN STADEN 2006) and germination was recorded every alternate day under a 'safe green light' (0.3 µmol m^{-2} s^{-1}).

7.2.3 Data collection

Germination percentage, shoot length (mm), root length (mm) and seedling fresh weight (mg) were recorded.

7.2.4 Data analysis

Germination and seedling growth data were analyzed using one-way analysis of variance (ANOVA) and Fisher's 95% confidence level ($p < 0.05$) was tested for pair-wise comparison. Percentage germination data were arcsine transformed before

analysis. MINITAB® (Minitab Inc., PA 16801-3008, USA) release 14 statistical software was used.

7.3 RESULTS AND DISCUSSION

Increasing concentrations of Cu had no significant effect on percentage germination of *B. volubilis*. A low concentration of Cu (1 mg l^{-1}) significantly increased shoot length and seedling fresh weight in comparison to the other concentrations (**Figure 7.1**). In the absence of Cu, the seedlings achieved the maximum root length. Increasing concentrations of Zn gradually decreased the percentage germination. Seedlings supplied with Zn at 50 mg l^{-1} showed significant inhibition of the shoot/root length. Significantly longer roots were recorded in the absence of Zn. When supplied with Zn at 2 mg l^{-1}, seedlings reached significantly greater fresh weights than with all other treatments (**Figure 7.1**).

Percentage germination of *E. autumnalis* seeds without Cu (control) was significantly better than seeds germinated in varying Cu concentrations (**Figure 7.2**). Copper supplied at 2 mg l^{-1} significantly decreased shoot length, whilst Cu at 1 mg l^{-1} significantly decreased root length. Seedling fresh weight was highest when supplied with Cu at 1 mg l^{-1}, which decreased with increasing concentrations. The effect of different Zn concentrations on germination and seedling growth were similar to the effects of Cu (**Figure 7.2**). These findings clearly suggest that the levels of Cu and Zn should be less than 1 mg l^{-1} for optimum growth of *E. autumnalis* seedlings.

Seeds of *M. plumbea* germinated under different concentrations of Cu showed no significant effect on the percentage germination (**Figure 7.3**). Conversely, shoot and root lengths significantly decreased in Cu at 2 and 1 mg l^{-1} respectively (**Figure 7.3**). None of the Zn concentrations tested significantly affected the percentage germination. The results of shoot/root length and seedling fresh weight showed some fluctuations. However, all the values of these parameters significantly decreased when supplied with Zn at 10 mg l^{-1} (**Figure 7.3**). The fact that Cu and Zn did not have a negative influence on percentage germination may be due to sporadic germination

Figure 7.1: Effect of microelements on percentage germination and seedling growth (21-day-old) of *Bowiea volubilis* under 16:8 h light and dark conditions at 25 ± 0.5 °C. Control (0) seeds were germinated with distilled water. Mean values with dissimilar letter(s) are significantly different ($p < 0.05$). NS = non-significant. Error bars indicate S.E.
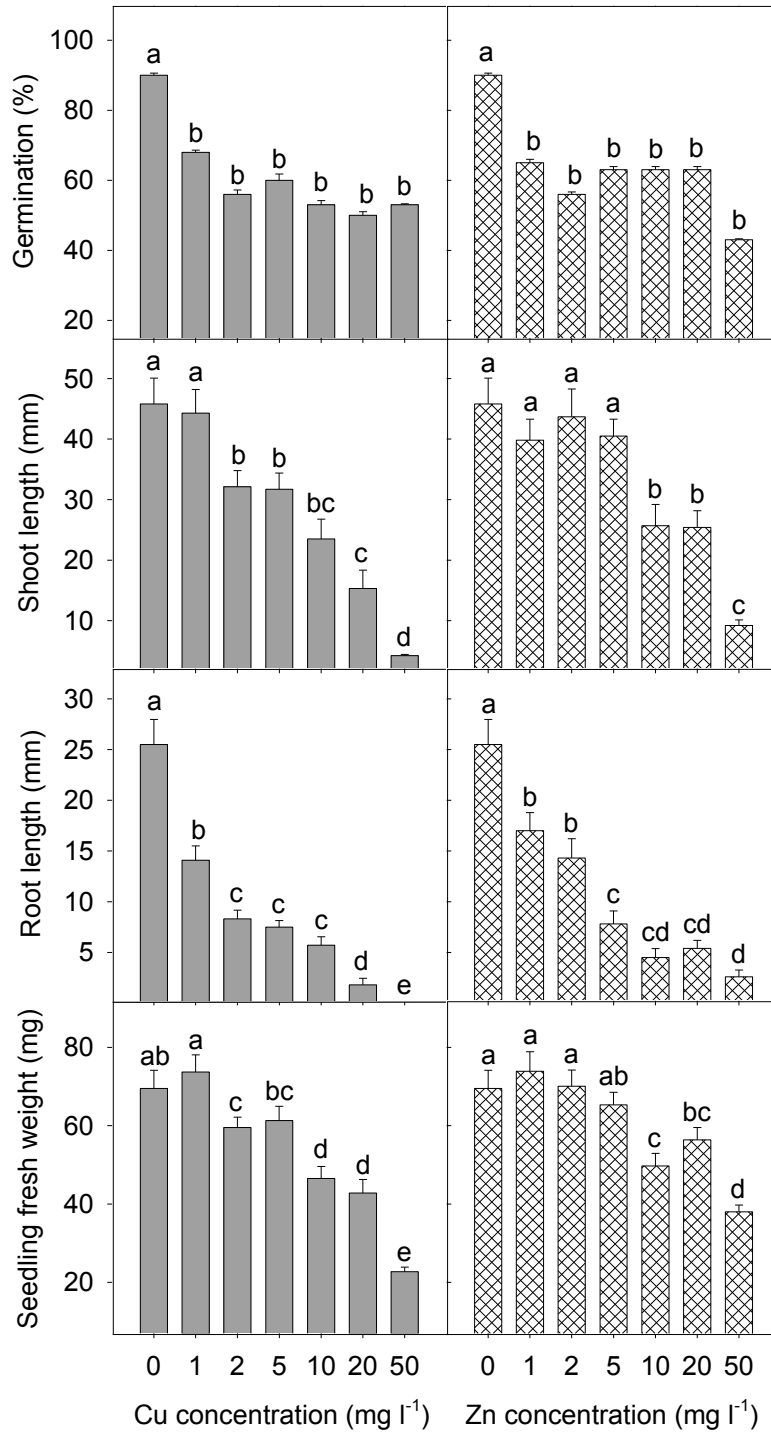
Figure 7.2: Effect of microelements on percentage germination and seedling growth (45-day-old) of *Eucomis autumnalis* under constant dark conditions at 20 ± 0.5 °C. Control (0) seeds were germinated with distilled water. Mean values with dissimilar letter(s) are significantly different (*p* < 0.05). Error bars indicate S.E.

of *M. plumbea* seeds. This was not the case for seedling growth. This result shows that even though percentage germination in *M. plumbea* was not affected by the higher concentrations, the lower concentrations of Cu and Zn affected seedling growth. This study indicates that the levels of Cu and Zn should be slightly lower than 1 mg l^{-1} for *M. plumbea*.

With the contamination of agricultural soils due to prolonged use of Cu- and Zn-containing herbicides, pesticides and fertilizers (for example, copper carbonate, copper oxychloride, zinc oxide, zinc sulfate), Cu and Zn tolerance in plants is of great importance. In this study, high concentrations of Cu and Zn did not exhibit detrimental effects on percentage germination of *B. volubilis* and *M. plumbea* seeds, but had a negative effect on seedling growth. Similar results have been reported for *Zea mays* L. (MAHMOOD, HUSSAIN, SAEED and ATHAR 2005).

This suggests that certain species utilize their own reserves, and therefore, there is less chance that metal ions will interfere until the process of germination is complete (STEFANI, ARDUINI and ONNIS 1991). However, this was not the case for *E. autumnalis*, where low concentrations of Cu and Zn had an inhibitory effect on germination (**Figure 7.2**). This study showed that although all three species belong to the same family, the response of germination to Cu and Zn was markedly different.

It is clear from the present results that seedling development was affected by increasing concentrations of both Cu and Zn. In all three wild-species tested, root growth was affected more than shoot growth. The findings are in agreement with similar work verifying that a high Cu supply usually inhibits root growth before shoot growth (CHEN, LIN and KAO 2000). This does not necessarily imply that roots are more sensitive to high copper concentrations, but rather that roots are the preferential sites for copper accumulation. In metal-polluted environments, roots are the primary contact zone with soil contaminants. The strong tendency of root tissues to accumulate Cu ions rather than transport them to the shoots has been observed under conditions of both Cu deficiency and excess (KABATA-PENDIAS 2001; FUENTES, DISANTE, VALDECANTOS, CORTINA and VALLEJO 2007). Most plant species and genotypes have great tolerance to excessive amounts of Zn, however,
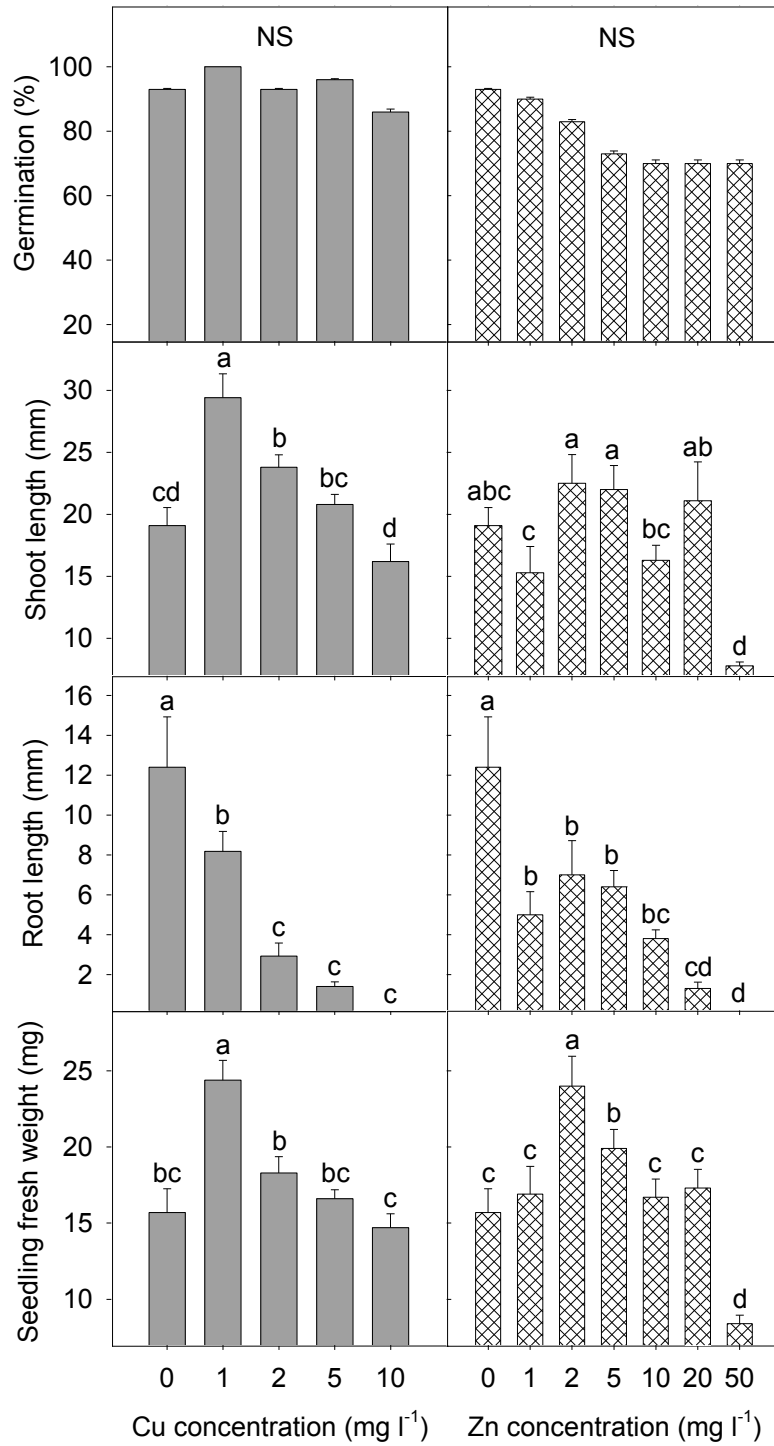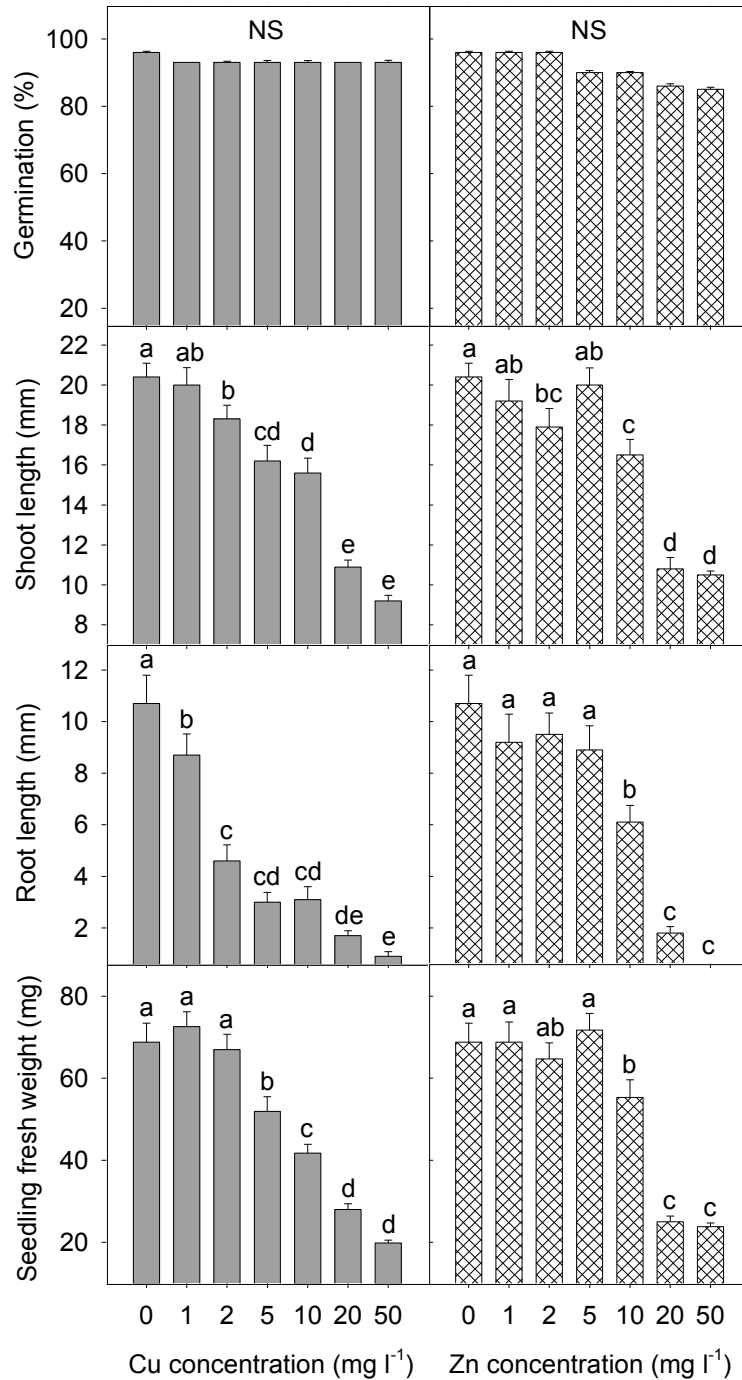
Figure 7.3: Effect of microelements on percentage germination and seedling growth (21-day-old) of *Merwilla plumbea* under 16:8 h light and dark conditions at 25 ± 0.5 °C. Control (0) seeds were germinated with distilled water. Mean values with dissimilar letter(s) are significantly different ($p < 0.05$). NS = non-significant. Error bars indicate S.E.

depression in growth is a common symptom of toxicity (KABATA-PENDIAS and PENDIAS 1984). A study by EL-GHAMERY, EL-KHOLY and ABOU EL-YOUSSER (2003) showed that Zn had an inhibitory effect on cell division in root tips causing a reduction in growth. Root growth of *B. volubilis* and *M. plumbea* was strongly inhibited when supplied with Zn at 50 mg I^{-1} and rooting of *E. autumnalis* was severely affected by Zn. These results indicate that all three species would suffer growth abnormalities when grown in agricultural soils containing or irrigated with high levels of Cu and Zn.

Mercury significantly reduced the percentage germination of *B. volubilis* seeds at all three concentrations examined (**Table 7.1**). In the case of *E. autumnalis*, Cd and Hg significantly lowered the percentage germination at 0.5 and 1 mg I^{-1} respectively (**Table 7.1**). The different concentrations of heavy metals (Cd, Hg and Pb) tested showed no significant effect on the percentage germination of *M. plumbea* seeds. Seedlings of *B. volubilis* had a significantly lower root length at 2 mg Cd I^{-1}. Lead promoted the root growth when supplied at 0.5 mg I^{-1}, but showed a large variation in growth for 1 and 2 mg I^{-1}. Increasing Hg concentrations decreased the root length of *B. volubilis* seedlings (**Table 7.1**). Seedling growth of *E. autumnalis* was not affected significantly by any of the concentrations of heavy metals tested. Cadmium (1 mg I^{-1}) significantly decreased the shoot/root length and seedling weight of *M. plumbea* (**Table 7.1**). Whereas Pb and Hg showed a significantly negative effect on shoot length when supplied at 0.5 mg I^{-1}.

Cadmium is not an essential element to plants but contamination therewith could lead to anatomical and physiological changes (CHAOUI and EL FERJANI 2005; LIU, ZHANG, SHAN and ZHU 2005). As discussed in **Section 2.2.2**, Cd pollution of South African rivers and dams has been reported (FATOKI and AWOFOLU 2003; OKONKWO and MOTHIBA 2005). Consequently, irrigating these medicinal plants with high levels of Cd-polluted water may have an adverse growth effect.

Table 7.1: Effect of heavy metals on germination and seedling growth of medicinal plant species of the Hyacinthaceae. Mean values ± S.E. with dissimilar letter(s) are significantly different ($p < 0.05$).

Plant species	Treatment (mg l^{-1})		Shoot length (mm)	Root length (mm)	Seedling weight (mg)	Germination (%)
B. volubilis	Cd	0	19.1 ± 1.4 a	12.4 ± 2.5 a	15.7 ± 1.5 a	93 ± 0.4 a
		0.5	17.2 ± 1.5 a	7.6 ± 1.0 b	17.1 ± 1.2 a	90 ± 0.4 a
		1	19.5 ± 1.6 a	7.4 ± 1.6 b	15.4 ± 1.2 a	90 ± 0.6 a
		2	17.2 ± 1.4 a	5.1 ± 0.7 b	17.0 ± 1.0 a	90 ± 0.6 a
	Pb	0	19.1 ± 1.4 a	12.4 ± 2.5 a	15.7 ± 1.5 b	93 ± 0.4 a
		0.5	23.3 ± 2.5 a	13.8 ± 2.7 a	21.5 ± 2.0 a	98 ± 0.4 a
		1	21.0 ± 1.7 a	7.1 ± 1.5 b	18.8 ± 1.7 ab	93 ± 0.7 a
		2	22.9 ± 1.6 a	11.8 ± 2.4 ab	22.1 ± 1.9 a	93 ± 0.4 a
	Hg	0	19.1 ± 1.4 a	12.4 ± 2.5 a	15.7 ± 1.5 a	93 ± 0.4 a
		0.5	15.0 ± 0.8 a	12.2 ± 0.3 ab	13.8 ± 0.3 a	80 ± 1.5 b
		1	20.1 ± 1.9 a	9.6 ± 1.9 ab	15.1 ± 1.8 a	73 ± 1.2 b
		2	17.2 ± 1.6 a	7.6 ± 1.2 b	14.8 ± 1.2 a	80 ± 0.7 b
E. autumnalis	Cd	0	45.8 ± 4.2 a	25.5 ± 2.4 a	69.5 ± 4.6 a	90 ± 0.5 a
		0.5	35.4 ± 4.2 a	21.0 ± 1.3 a	60.3 ± 3.2 a	63 ± 0.9 b
		1	41.1 ± 3.4 a	25.2 ± 1.9 a	69.5 ± 3.5 a	56 ± 0.7 b
		2	35.1 ± 3.5 a	24.0 ± 1.5 a	64.3 ± 4.4 a	63 ± 1.2 b
	Pb	0	45.8 ± 4.2 a	25.5 ± 2.4 a	69.5 ± 4.6 a	90 ± 0.5 a
		0.5	44.3 ± 3.6 a	22.7 ± 2.0 a	79.9 ± 5.9 a	85 ± 0.9 a
		1	44.2 ± 3.6 a	24.5 ± 1.4 a	73.1 ± 3.8 a	86 ± 0.9 a
		2	39.1 ± 3.7 a	21.0 ± 1.1 a	67.0 ± 4.2 a	86 ± 0.9 a
	Hg	0	45.8 ± 4.2 a	25.5 ± 2.4 a	69.5 ± 4.6 a	90 ± 0.5 a
		0.5	44.1 ± 3.1 a	21.7 ± 1.5 a	68.0 ± 3.6 a	86 ± 0.9 ab
		1	46.8 ± 3.9 a	19.8 ± 2.1 a	64.7 ± 3.9 a	83 ± 0.4 b
		2	46.2 ± 3.0 a	21.0 ± 1.8 a	66.9 ± 3.7 a	83 ± 0.4 b
M. plumbea	Cd	0	20.4 ± 0.6 a	10.7 ± 1.1 a	68.8 ± 4.6 a	96 ± 0.4 a
		0.5	12.5 ± 0.4 b	8.3 ± 1.0 ab	50.0 ± 4.0 b	94 ± 1.1 a
		1	12.7 ± 0.5 b	4.3 ± 0.5 b	42.3 ± 3.6 b	94 ± 0.7 a
		2	15.3 ± 0.5 b	5.8 ± 0.5 b	57.3 ± 3.1 ab	93 ± 0.7 a
	Pb	0	20.4 ± 0.6 a	10.7 ± 1.1 a	68.8 ± 4.6 a	96 ± 0.4 a
		0.5	16.0 ± 0.6 bc	7.6 ± 0.6 a	52.6 ± 2.1 b	100 ± 0 a
		1	13.4 ± 0.5 c	8.8 ± 0.6 a	58.0 ± 2.1 ab	95 ± 0.7 a
		2	18.0 ± 0.4 ab	10.6 ± 0.4 a	72.0 ± 2.0 a	100 ± 0 a
	Hg	0	20.4 ± 0.6 a	10.7 ± 1.1 a	68.8 ± 4.6 a	96 ± 0.4 a
		0.5	15.5 ± 0.1 b	10.0 ± 0.4 a	62.8 ± 2.5 a	95 ± 0.7 a
		1	15.6 ± 0.4 b	8.1 ± 0.7 a	57.7 ± 2.1 a	100 ± 0 a
		2	16.2 ± 0.7 b	8.2 ± 0.8 a	55.0 ± 3.8 a	95 ± 0.7 a

Lead has received much attention as a major chemical pollutant of the environment (NRIAGU, BLANKSON and OCRAN 1996; NRIAGU, JINABHAI, NAIDOO and COUTSOUDIS 1996; KABATA-PENDIAS 2001). There are many reports regarding stimulatory (ONCEL, KELES and USTUN 2000; NYITRAI, BOKA, GASPAR, SARVARI, LENTI and KERESZTES 2003) and inhibitory (JAJA and ODOEMENA 2004) effects of low concentrations of Pb on plant growth. This study similarly showed that Pb at 0.5 mg l^{-1} promoted and inhibited seedling growth of *B. volubilis* and *M. plumbea* respectively (**Table 7.1**).

Mercury is considered one of the most readily accumulated toxic metal elements. It accumulates in living organisms causing harmful damage (SU, ZHU and DU 2005). At low concentrations of Hg, seedling growth of rice and cucumber was inhibited (DU, ZHU, LIU and ZHAO 2005; CARGNELUTTI, TABALDI, SPANEVELLO, DE OLIVEIRA JUCOSKI, BATTISTI, REDIN, LINARES, DRESSLER, DE MORAES FLORES, NICOLOSO, MORSCH and SCHETINGER 2006). This study showed that the seedling growth (shoot + root length) of all Hyacinthaceae species examined was reduced when supplied with Hg at 0.5 mg l^{-1}.

This study indicates that the maximum permissible concentrations of Cd (2 mg kg^{-1}), Cu (6.6 mg kg^{-1}), Hg (0.5 mg kg^{-1}), Pb (6.6 mg kg^{-1}) and Zn (46.5 mg kg^{-1}) in South African agricultural soils set by the Water Research Commission (1997) (**Table 4.1**) may be too high for growing these wild medicinal plants. This study therefore recommends separate threshold limits of metal elements for important traditional medicinal plants.

7.4 SUMMARY

- The negative impact of microelements and heavy metals was pronounced at the post-germination stage.
- All three medicinal plant species studied showed a similar trend of sensitivity with increasing concentrations of essential elements (Cu and Zn). However, these species responded differently to Cd, Hg and Pb.

- In general, elevated Cu and Zn concentrations affected root growth more than shoot growth.

- All three species would suffer growth abnormalities in agricultural soils irrigated with or containing high levels of Cu and Zn.

- Due to heavy metal toxicity, seedling growth was below that of the control, in most cases.

- Results from this study therefore suggest heavy metal threshold limits for the cultivation of important traditional medicinal plants.

8   Effect of Cd on biological activity of select medicinal plants

8.1 INTRODUCTION

8.1.1 Secondary metabolites

Some of the crucially important molecules of life include carbohydrates (composed of sugar units), proteins (made up of amino acids), and nucleic acids (based on nucleotides). Despite the extremely varied characteristics of living organisms, the pathways for generally modifying and synthesizing these products (apart from minor variations) are effectively the same in all organisms. These processes are collectively known as primary metabolism, with the compounds involved known as primary metabolites (DEWICK 2002).

In contrast to these primary metabolic pathways, which generally produce compounds common to most organisms, secondary metabolism is concerned with compounds that have a much more limited distribution in nature and are found only in specific organisms or groups of organisms and are an expression of an individual species (DEWICK 2002). Secondary metabolites are those compounds produced by plants that are not directly essential for basic life functions (THEIS and LERDAU 2003). Ecologically speaking, plants have evolved secondary compounds as chemical defenses that can repel, stun, poison or kill threatening species (GURIB-FAKIM 2006). Furthermore, they are not necessarily produced under all conditions, and, in most cases, little is known about the function and benefit of these compounds to the individual organism (DEWICK 2002).

An environmental condition which may be stressful for one plant may be most favourable for another plant (MAHAJAN and TUTEJA 2005). Environmental factors such as water and temperature stress conditions are important criteria with regards to secondary metabolite production (ZOBAYED, AFREEN and KOZAI 2005; ZOBAYED, AFREEN and KOZAI 2007). Mineral elements are involved in the structure of some secondary metabolites, yet can also have adverse on their

regulation (POUTARAUD and GIRARDIN 2005). Thus, optimizing nutrient supply is a key factor in the quality of medicinal plants.

Recent work has demonstrated some of the effects of heavy metals on secondary metabolites (**Table 8.1**). Such environmental changes could have serious effects on the quality, safety and efficacy of natural plant products produced by medicinal plant species.

Table 8.1: Examples of studies on heavy metal stress affecting secondary metabolite production.

Plant species	Main findings relating to secondary metabolites	Reference
Hypericum perforatum L.	• After Ni addition to the media, the plant completely lost the ability to produce or accumulate hyperforin and demonstrated a 15–20-fold decrease in the concentration of pseudohypericin and hypericin	MURCH, HAQ, RUPASINGHE and SAXENA (2003)
Dioscorea bulbifera L.	• The presence of Cu stimulated diosgenin production	NARULA, KUMAR and SRIVASTAVA (2005)
Phyllanthus amarus Schum. and Thonn	• Phyllanthin and hypophyllanthin, was enhanced by Cd stress	RAI, KHATOON, BISHT and MEHROTRA (2005)
Bacopa monnieri L.	• The level of bacoside-A increased due to increased Fe in the media	SINHA and SAXENA (2006)

8.1.2 Screening of medicinal plants for biological activity

The integration of South African traditional medicine with primary healthcare has founded a scientific rationale for the validation of indigenous medicinal plants (LIGHT, SPARG, STAFFORD and VAN STADEN 2005). *In vitro* tests, commonly known as biological assays, are routinely used in ethnopharmacological research to demonstrate the pharmacological value of traditionally used plant parts. Many South African plants have been screened for biological activity. The number of plant extracts assayed from the sampling of South African medicinal plants emphasizes the potential areas for future work. Variation in biological activity has been discussed

in terms of plant age, seasonal variation and geographical variation in harvest site (TAYLOR and VAN STADEN 2001; SHALE, STIRK and VAN STADEN 2005; BUWA and VAN STADEN 2007). However, to date little work has been done on the effect of heavy metals on biological activity of South African medicinal plants.

The aim of these experiments was to assess the effect of Cd on the antibacterial, antifungal and anti-inflammatory activities in selected medicinal plants. *Eucomis autumnalis*, *Eucomis humilis*, *Merwilla plumbea* and *Tulbaghia violacea* were selected based on reported usage with previously confirmed biological activity.

8.2 ANTI-INFLAMMATORY SCREENING

The complex processes of inflammation involve biosynthesis of prostaglandins that are accountable for the sense of pain (GAIDAMASHVILI and VAN STADEN 2006). The inhibitors of prostaglandin biosynthesis are considered as potential anti-inflammatory agents. Cyclooxygenase, a key enzyme in the biosynthesis of prostaglandins and leukotrienes from arachidonic acid (JÄGER and VAN STADEN 2005), exists in two isoforms, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (JÄGER and VAN STADEN 2005). Testing plant extracts for activity against COX-1 and COX-2 is a commonly used screening method.

8.2.1 Materials and methods

8.2.1.1 Experimental design and sample preparation

Experimental design and sample preparation for *Eucomis autumnalis* (Mill.) Chitt. and *Eucomis humilis* Baker are described in Chapter 4 (**Sections 4.2.1, 4.2.1.2 and 4.2.2**). Only the medicinally used bulbs were tested for COX-1 and COX-2 activity.

8.2.1.2 Extraction of plant material

The extraction procedure consisted of sonicating 0.5 g of powdered plant material in 5 ml ethanol for 30 mins in an ultrasonic bath at room temperature. The plant material was then filtered under vacuum through Whatman No. 1 filter paper discs

using a Büchner funnel. The filtered extracts were dried down in a forced draft at room temperature and stored at 5 °C.

Bulb extract residues were resuspended in ethanol to a concentration of 10 mg ml^{-1}.

8.2.1.3 COX-1 and COX-2 bioassays

The cyclooxygenase-1 and -2 assays were performed as described by NOREEN, RINGBOM, PERERA, DANIELSON and BOHLIN (1998) with slight modifications (ZSCHOCKE and VAN STADEN 2000). The COX-1 and COX-2 enzymes were purchased from Sigma Aldrich. The enzyme (10 µl) was activated with 50 µl cofactor solution (0.9 mM L-epinephrine, 0.49 mM glutathione and 1 µM hematin in 0.1 M Tris buffer, at pH 8). The enzyme solution (60 µl) and the sample solution (2.5 µl dissolved plant extract applied to 17.5 µl distilled water) were incubated for five mins at room temperature. The reaction started with the addition of 20 µl [^{14}C] arachidonic acid (16 Ci/mol, 30 µM) to each of the samples. The samples were incubated for 10 mins at 37 °C before the reaction was terminated by adding 10 µl of 2N HCl. Four µl of a 0.2 mg ml^{-1} carrier solution of unlabelled prostaglandins (PGE$_2$:PGF$_2$ 1:1 v/v) was added.

The ^{14}C-labelled prostaglandins synthesized during the assay were separated from the unmetabolized [^{14}C] arachidonic acid by column chromatography. Silica gel in hexane:dioxane:acetic acid 350:50:1 v/v/v) (eluent 1) was packed to a height of 3 cm in Pasteur pipettes. One ml of eluent 1 was added to each of the assay mixtures and this mixture applied to separate columns. The arachidonic acid was eluted from the column with 4 ml of eluent 1 and discarded. The labeled prostaglandins were subsequently eluted with 3 ml ethyl acetate:methanol 85:15 v/v (eluent 2) into scintillation vials. Scintillation fluid (4 ml) was added to each vial and the radioactivity measured using a Beckman LS 6 000LL scintillation counter.

The percentage inhibition of the test solutions was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank. IC$_{50}$ was calculated based on 5 readings, using Grafit Version 5 (Erithacus Software Ltd., U.K)

at a starting concentration of 250 µg ml^{-1}. Indomethacin (Fluka BioChemika) was included as a control. The experiment was performed in duplicate.

8.2.2 Results and discussion

Both the COX-1 and the COX-2 assays follow the same protocol, which facilitates comparisons between activities of the extracts on the two enzymes (ZSCHOCKE and VAN STADEN 2000). The IC$_{50}$ indicates how much of a particular substance is needed to inhibit a given biological process by half. It is clear from the results that compared to control plants, *E. autumnalis,* supplied with Cd at 2 mg l^{-1}, had higher COX-1 activity (**Table 8.2**). However, compared with the control, Cd-treated plants had reduced COX-2 activity (**Table 8.2**). The Cd-treated *E. humilis* bulbs showed lower activity than the control for both COX-1 and COX-2 activity, however it was more pronounced in COX-1. In general, *E. humilis* bulbs, which accumulated less Cd than *E. autumnalis* (1.3 and 4.9 mg kg^{-1} respectively) (**Figure 4.4**) were less affected by the Cd treatment for both COX-1 and COX-2.

Table 8.2: Prostaglandin synthesis inhibition expressed as IC$_{50}$ (µg ml^{-1} ± S.D.) of *Eucomis autumnalis* and *Eucomis humilis* bulbous extracts following Cd stress.

Plant species	Treatment (Cd mg l^{-1})	IC$_{50}$ (µg ml^{-1})	
		COX-1	COX-2
E. autumnalis	0	76.5 ± 16	81.5 ± 15
	2	26.7 ± 6.3	223 ± 9.9
E. humilis	0	47.7 ± 2.8	90.6 ± 4.4
	2	58.8 ± 1.4	97.3 ± 8.1
Indomethacin (µM)		2.2 ± 0.18	135.4 ± 7.6

The results can be interpreted in two ways. Firstly, the presence of Cd in the plant extract may affect the activity of the COX-1 and/or COX-2 enzymes in the bioassay. To verify this, one would need to test the activity of the Cd. However, testing pure Cd would not necessarily be valid due to the presence of other extracted substances which may interact with the Cd and modify its form. Secondly, the presence of Cd as an environmental stress may increase or decrease secondary metabolite production. To confirm this, one would need to quantify the effect of Cd on the biosynthesis of the active constituent. However, despite its wide use in South African traditional

medicine, the active compound in *Eucomis* species is unknown. Regrettably, the chemistry of very few South African medicinal plants has been studied in detail (DREWES, HORN and KHAN 2006).

ELGORASHI, STAFFORD, MULHOLLAND and VAN STADEN (2004) revealed that the biological activity of medicinally used *Cyrtanthus suaveolens* Schönland was caused not from the plant extract but from a commercially available pesticide, Captan, found in the plant. Thus, reporting of biological activity of crude plant extracts without the isolation and identification of the active ingredient raises concern that the activity may be due to contamination.

8.3 ANTIBACTERIAL SCREENING

8.3.1 Materials and methods

8.3.1.1 Experimental design and sample preparation

Experimental design and sample preparation for *Merwilla plumbea* (Lindl.) Speta. are described in Chapter 4 (**Sections 4.2.1, 4.2.1.3** and **4.2.2**). Only the medicinally used bulbs were tested for antibacterial activity.

8.3.1.2 Extraction of plant material

Previous work on *M. plumbea* concluded that an ethanolic extract gave the highest inhibitory activity against the bacterial strains examined (SPARG, VAN STADEN and JÄGER 2002). Thus, ethanol extracts, as described in **Section 8.2.1.2**, were screened for antibacterial activity using a routine procedure.

Bulb extract residues were resuspended in ethanol to a concentration of 50 mg ml^{-1}.

8.3.1.3 Minimum inhibitory concentration (MIC) bioassay

Each extract was bioassayed against two Gram-positive bacteria, *Bacillus subtilis* (ATCC No. 6051) and *Staphylococcus aureus* (ATCC No. 12600) and two Gram-

negative bacteria *Escherichia coli* (ATCC No. 11775) and *Klebsiella pneumoniae* (ATCC No. 13883). The bacterial strains were maintained on Mueller-Hinton nutrient agar (Biolab) at 4 °C.

The bioassay used for antibacterial screening was the microdilution method for minimum inhibitory concentration (MIC) determination as described by ELOFF (1998). Prior to use in the assay, suspension cultures were inoculated in Mueller-Hinton (MH) broth (Oxoid) from bacterial stock cultures and incubated overnight at 37 °C in a waterbath with an orbital shaker. For each of the four bacteria, 100 µl of redissolved extract (50 mg ml^{-1}) were two-fold serially diluted with 100 µl of sterile distilled water in a sterile 96-well microtitre plate (Greiner Labortechnik). A similar two-fold serial dilution of neomycin (Sigma) (100 µg ml^{-1}) was used as a positive control against each bacterium. Extraction solvent, extracts and bacteria-free controls were included as negative controls. The bacterial-saturated suspension cultures were diluted 1:100 with sterile MH broth, with 100 µl being added to each of the wells containing the test and control solutions. The plates were covered and incubated overnight at 37 °C. Bacterial growth was visualised by adding 40 µl of 0.2 mg ml^{-1} p-iodonitrotetrazolium chloride (Sigma) to each of the wells. The plates were incubated at 37 °C for a further 30 mins.

The MIC was taken as the lowest concentrations of plant extract to elicit an inhibitory effect on the growth (last well not to exhibit a colour change) of the test bacterium. The experiment was performed in duplicate.

8.3.2 Results and discussion

The presence of Cd, either as a result of Cd in the extract or the effect on secondary metabolite production, affected the antibacterial activity of *M. plumbea* against certain bacterial strains. When the plants were supplied with Cd at 2 mg l^{-1}, the bulbous extract was more active against the Gram-positive bacterium, *Bacillus subtilis,* compared to the control (**Table 8.3**). When supplied with Cd at 5 mg l^{-1}, the activity was lower than when supplied at 2 mg Cd l^{-1} (**Table 8.3**). The activity correlates to the Cd accumulation in the bulbs which was lowest when supplied with 5 mg Cd l^{-1} (**Figure 4.6**). When supplied with Cd at 5 and 10 mg l^{-1}, increased antibacterial

activity was seen against *Staphylococcus aureus*. Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae)* were least affected by the Cd-treated plants, with no change seen in activity against *E. coli* when plants were supplied with Cd up to 10 mg l^{-1}. However, activity against *Klebsiella pneumoniae* was shown to be greater than the control when grown in Cd at 10 mg l^{-1} (**Table 8.3**), which correlates with the high Cd accumulation of the plant (**Figure 4.6**).

Table 8.3: Antibacterial activity expressed as minimum inhibitory concentrations (MIC) of *Merwilla plumbea* (bulb) extracts against four common bacterial types.

Treatment (Cd mg l^{-1})	Bacteria[1] used (MIC (mg ml^{-1}))			
	B.s	**S.a**	**E.c**	**K.p**
0	6.25	6.25	6.25	6.25
2	3.13	6.25	6.25	6.25
5	6.25	3.13	6.25	6.25
10	3.13	3.13	6.25	3.13
Neomycin	3.13×10^{-2}	3.13×10^{-2}	1.56×10^{-2}	1.56×10^{-2}

[1]Bacteria: *B.s = Bacillus subtilis, E.c = Escherichia coli, K.p = Klebsiella pneumoniae, S.a = Staphylococcus aureus*

This study shows that Cd had an effect on the biological activity of *M. plumbea*, either as a result of Cd in the plant extract or due to the stress induced secondary metabolite production. Thus, medicinal plants showing high activity should be thoroughly investigated as the activity may be caused by environmental pollutants.

8.4 ANTIFUNGAL SCREENING

8.4.1 Materials and methods

8.4.1.1 Experimental design and sample preparation

Experimental design and sample preparation for *Tulbaghia violacea* Harv. are described in Chapter 5 (**Sections 5.2.1, 5.2.1.2** and **5.2.2**). Only the medicinally used bulbs were tested for antifungal activity.

8.4.1.2 Extraction of plant material

Previous work on *T. violacea* concluded that an ethanolic extract gave the highest inhibitory activity against *Candida albicans* (MOTSEI, LINDSEY, VAN STADEN and JÄGER 2003). Thus, ethanol was the chosen solvent for this experiment. Extraction procedure as described in **Section 8.2.1.2** was carried out.

Bulb extract residues were resuspended in dimethylsulfoxide (DMSO) to a concentration of 50 mg ml^{-1}. Recent investigations by ELOFF, MASOKO and PICARD (2007) advised that acetone and DMSO appear to be the safest solvents to use in fungal bioassays.

8.4.1.3 Minimum inhibitory concentration (MIC) bioassay

Each extract was bioassayed against *Candida albicans* (ATCC 10231). The serial dilution method described in **Section 8.3.1.3** was used for the antifungal screening. MOTSEI, LINDSEY, VAN STADEN and JÄGER (2003) used a serial microdilution assay in determining the antifungal activity and determined growth with an ELISA reader. However, measuring growth by turbidity measurement has several complications (ELOFF 1998). Thus, using a modified method by MASOKO, PICARD and ELOFF (2007), a growth indicator (p-iodonitrotetrazolium chloride) was used (as described in **Section 8.3.1.3**). Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 48 and 72 h. The experiment was done in duplicate.

8.4.2 Results and discussion

Contrary to previous reports on plant age influencing biological activity (FICOL, BILIA, MORELLI and TOME 2000; TAYLOR and VAN STADEN 2001), accepting that bulb diameter is positively correlated with plant age (WILLIAMS, BALKWILL and WITKOWSKI 2007), it is clear from **Table 8.4** that plant age had no effect on the antifungal activity of *T. violacea*.

Table 8.4: Antifungal activity expressed as minimum inhibitory concentrations (MIC) of *Tulbaghia violacea* **(bulb) extracts against** *Candida albicans.*

Plant size class	Treatment (Cd mg l^{-1})	Activity MIC (mg ml^{-1}) 48 h	72 h
small	0	6.25	6.25
	2	6.25	6.25
	5	6.25	6.25
medium	0	6.25	6.25
	2	6.25	6.25
	5	6.25	6.25
large	0	6.25	6.25
	2	6.25	6.25
	5	6.25	6.25
Amphotericin B		0.39×10^{-2}	0.39×10^{-2}

Regardless of the increasing accumulation of Cd in the bulbs (**Figure 5.3**), the antifungal activity was not affected (**Table 8.4**). These results correlate to the growth data whereby increasing levels of Cd in the media had little affect on the growth parameters (**Table 5.4**). Thus, assuming that *T. violacea* is a Cd tolerant plant, it is not surprising that the biological activity remained unchanged. This tolerance has been reported in other species. When supplied with Cd at 10 mg l^{-1} to the growth media, despite Cd accumulation in the leaves, the essential oil content in peppermint (Mentha x piperita L., cv. Mitchum) and basil (*Ocimum basilicum* L., cv. Broad Leaf Italian) was not affected (ZHELJAZKOV, CRAKER and XING 2006).

The findings reported in this chapter lay emphasis on heavy metal contamination as an important factor in the optimisation of quality control of plants used in traditional medicine. In addition, researchers should be aware of the impact of environmental contaminants when reporting on biological activity of crude plant extracts - especially since a large portion of South African medicinal plants tested for biological activity are obtained from informal medicinal markets, which, as demonstrated in **Chapter 3**, may contain an elevated heavy metal content. Consequently, the results of the reported biological activity may be skewed due to the presence of heavy metals in the medicinal plants tested.

8.5 SUMMARY

- Biological activity of *E. autumnalis* was more affected than that of *E. humilis.*

- *Eucomis autumnalis* supplied with Cd at 2 mg l^{-1} had greater COX-1 activity compared with the control. However, Cd suppressed the activity of COX-2.

- The Cd-treated *E. humilis* bulbs showed lower activity than the control and was more pronounced in COX-1.

- When supplied with Cd at 10 mg l^{-1}, *M. plumbea* bulbs showed higher antibacterial activity against three bacterial species. However, compared to the control, no change in activity was seen against *E. coli.*

- Neither bulb size nor Cd accumulation in the bulbs had an effect on antifungal activity of *T. violacea.*

- The development of optimized agricultural practices is essential for quality control.

9   General conclusions

The South African medicinal plant trade meets the primary health care needs of a large percentage of the population. However, inappropriate methods of collection, storage and processing result in the accumulation of potentially harmful substances in the products. These factors contribute towards a shortcoming with regards to South African medicinal plant products competing in international markets (TADMOR, JEFTHAS, GOLIATH, SMITH, LANGENHOVEN, ACQUAYE, JULIANI, LETCHAMO, RENAUD, ZIMBA, RASKIN, BROWN and SIMON 2002).

This thesis was an investigation into the occurrence and uptake of heavy metals by selected South African medicinal plants. To date, very little work has been done in this field. The experiments cover three broad areas: a) the random screening of medicinal plants being sold to the public to assess the levels of heavy metal contamination; b) uptake and distribution of heavy metals by selected medicinal plants and how these metals affect their biological activity and c) the effect of heavy metal contamination on germination and seedling growth.

Results revealed heavy metal contamination in some medicinal plants traded at informal street markets. Certain species are able to accumulate heavy metals while other species are more sensitive to elevated metal concentrations with their growth being affected. Metal accumulation also has the potential to change the biological activity of the plants. Thus, heavy metal contamination of medicinal plants, due to unregulated trade or poor cultivation regimes, has several consequences that compromise the quality, safety and efficacy of traditional medicines. Firstly, due to potential heavy metal accumulation, consumer safety is compromised. And secondly, both horticultural yield and phytochemical composition may be negatively affected. The findings of this thesis provide valuable information to the small-scale farmer for the safe and beneficial cultivation of South African medicinal plant species and increases awareness regarding heavy metal contamination in traditional medicinal plants.

Monitoring programmes for contaminants and toxins provide valuable contributions toward improving food safety, warn of actual and potential food scares, and facilitate assessment of potential health hazards (DOGHEIM, ASHRAF, ALLA, KHORSHID and FAHMY 2004). Whilst laboratory/greenhouse based studies are important for providing an insight into some of the fundamental mechanisms of heavy metal uptake, field trials are essential to gauge adaptation mechanism in plants (MCGRATH, LOMBI, GRAY, CAILLE, DUNHAM and ZHAO 2006). Unfortunately, a factor that contributes to the lack of medicinal plant research in South Africa is the lack of laboratories with the equipment and expertise to carry out multifaceted analyses (SCHULZ 2001; MULHOLLAND 2005).

Regrettably, South Africa is being left behind because of its lack of direction and focus to overcome the various challenges facing the medicinal plant trade. In a recent publication, BERGER (2006) states that the Traditional Health Practitioners Act of 2004 lacks sufficient methodology and useful guidelines for the application of traditional medicine. "While the document is long on rules and procedures it is extremely short on substantive statements that set out standards of skill, knowledge and training, or that address the safety and efficacy specifications required of materials and methods used in the practice of traditional medicine".

Much work needs to be done to improve the various aspects of the current extensive traditional medicinal plant trade. This would not only provide a product of quality and safety to the consumer, but also ensure that the continued use of medicinal plants is sustainable. The findings of this thesis establish a firm groundwork for the need to regulate and monitor the South African traditional medicinal plant trade against potentially harmful toxins.

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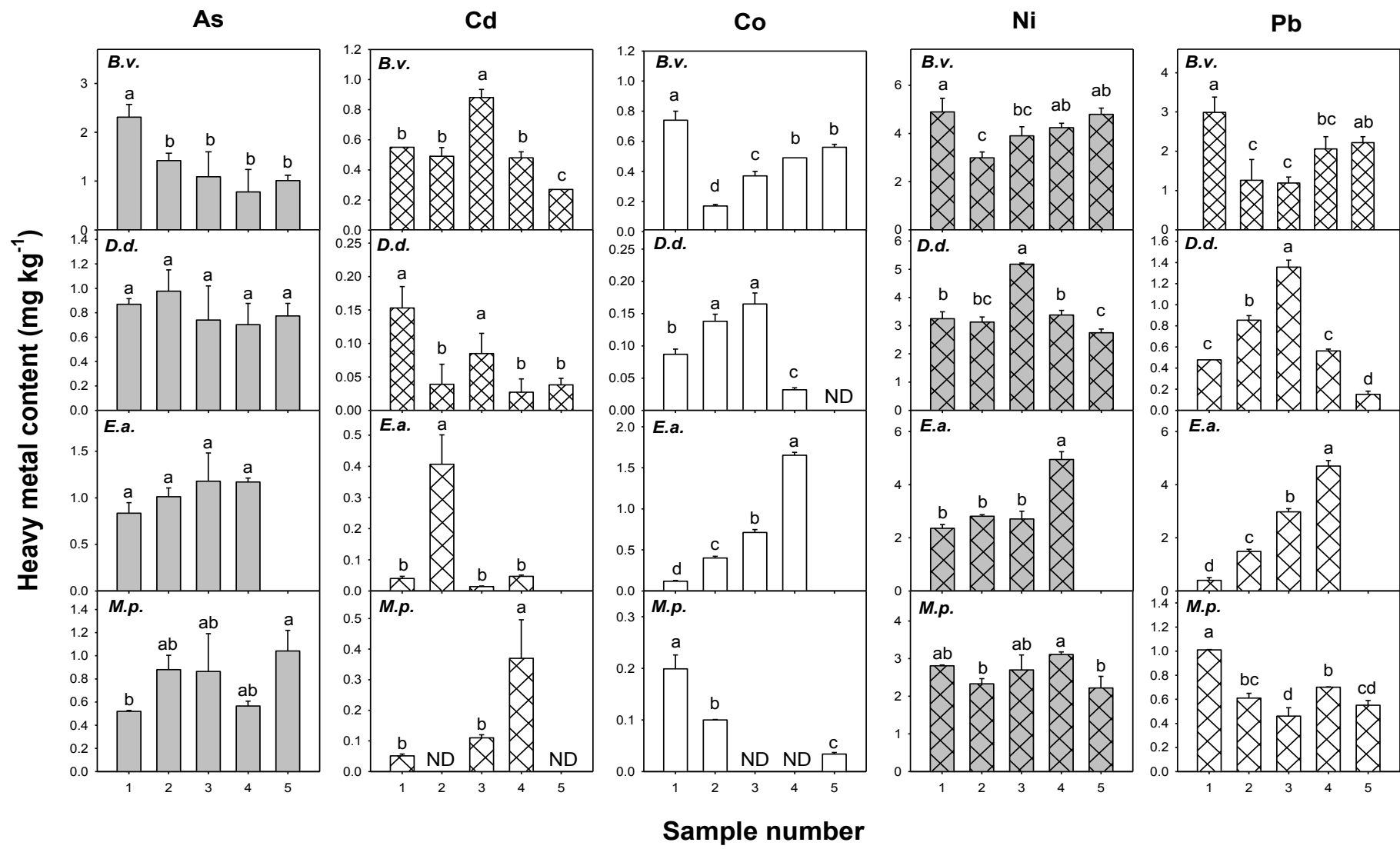
Figure 3.1: Variation in heavy metal content (mg kg^{-1}) in *Bowiea volubilis* bulbs (*B.v.*), *Dioscorea dregeana* tubers (*D.d.*), *Eucomis autumnalis* bulbs (*E.a.*) and *Merwilla plumbea* bulbs (*M.p.*). Mean ± S.D. (n=3). Mean values with dissimilar letter(s) are significantly different ($p < 0.05$). ND = not detected.
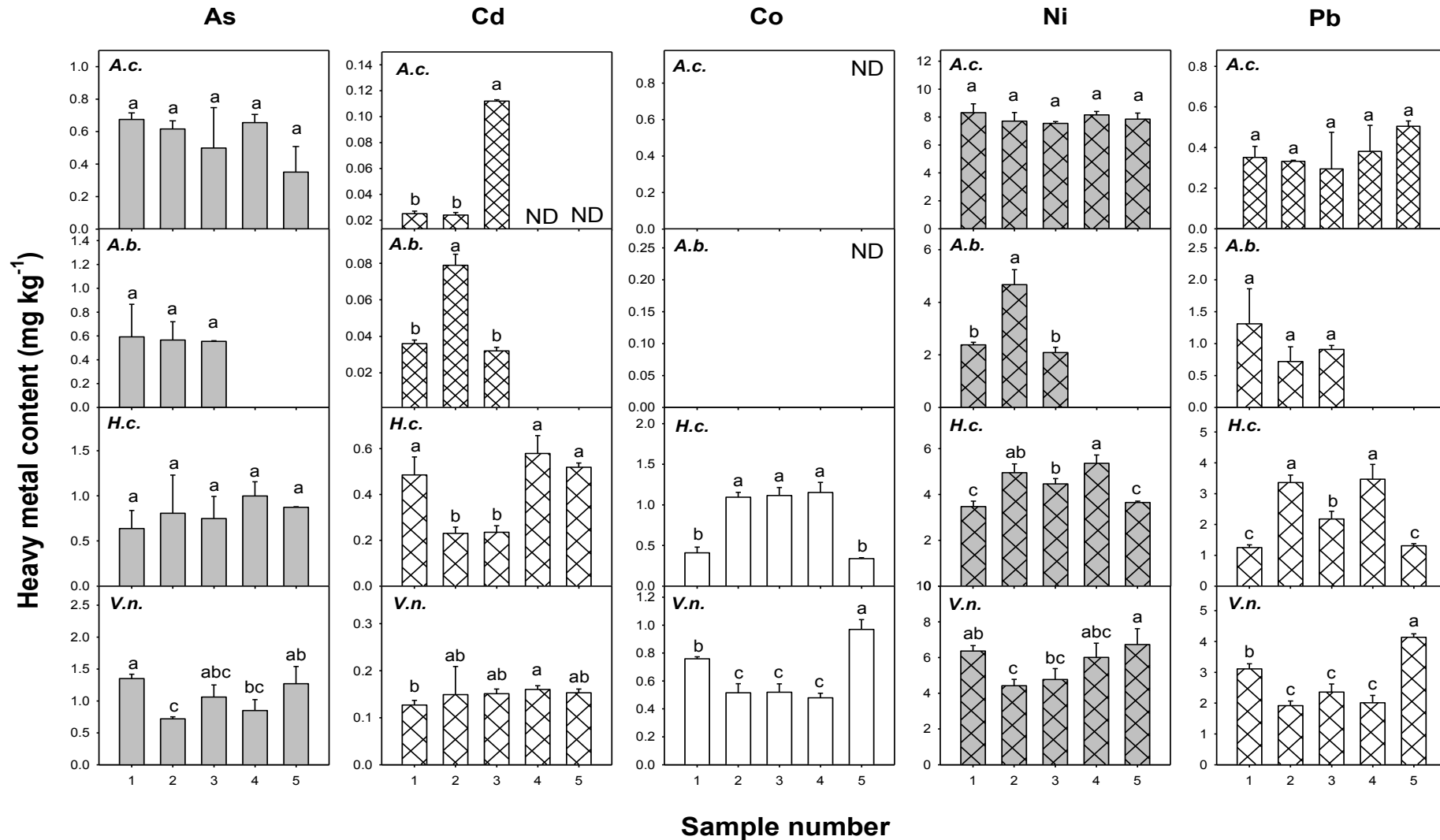
Figure 3.2: Variation in heavy metal content (mg kg⁻¹) in *Acacia caffra* roots (*A.c.*), *Agathosma betulina* leaves + stems (*A.b.*), *Helichrysum cymosum* leaves + stems (*H.c.*) and *Vernonia neocorymbosa* roots (*V.n.*). Mean ± S.D. (n=3). Mean values with dissimilar letter(s) are significantly different (*p* < 0.05). ND = not detected.

Table 3.4: Concentration of microelements (mg kg^{-1}) determined by ICP-OES in leaves + stems and roots of medicinal plants obtained from street markets (mean ± S.D.; n=3). Mean values with dissimilar letter(s) are significantly different ($p < 0.05$).

| Plant species	Sample no.	B	Cu	Fe	Mn	Mo	Zn
*Acacia caffra	1	14.5 ± 1.05 a	0.904 ± 0.393 a	111.9 ± 7.34 ab	32.2 ± 1.12 b	0.151 ± 0.011 a	4.51 ± 0.70 a
	2	15.2 ± 0.71 a	0.701 ± 0.125 a	114.9 ± 3.18 ab	32.5 ± 0.28 b	0.124 ± 0.024 ab	4.96 ± 0.35 a
	3	14.2 ± 1.16 a	0.675 ± 0.480 a	106.5 ± 10.4 b	35.1 ± 0.48 a	0.111 ± 0.008 ab	4.24 ± 0.49 a
	4	15.1 ± 0.29 a	1.208 ± 0.786 a	121.0 ± 5.94 ab	32.8 ± 1.06 b	0.112 ± 0.016 ab	7.28 ± 1.63 a
	5	15.1 ± 0.79 a	0.946 ± 0.910 a	132.9 ± 9.92 a	33.7 ± 0.29 ab	0.099 ± 0.002 b	5.41 ± 0.85 a
†*Agathosma betulina*	1	36.9 ± 0.69 c	2.50 ± 0.07 a	127.6 ± 2.86 a	2089 ± 121 ab	0.091 ± 0.003 b	7.00 ± 0.76 ab
	2	51.3 ± 0.41 a	3.25 ± 0.47 a	114.0 ± 3.04 b	2462 ± 52 a	0.192 ± 0.002 a	8.91 ± 1.03 a
	3	38.3 ± 0.35 b	2.43 ± 0.51 a	119.1 ± 4.22 b	1615 ± 5 b	0.076 ± 0.003 c	6.53 ± 0.31 b
†*Helichrysum cymosum*	1	23.9 ± 3.05 a	0.409 ± 0.070 b	444 ± 42.8 c	1257 ± 37 a	0.128 ± 0.021 b	60.0 ± 8.53 a
	2	17.2 ± 0.46 b	1.095 ± 0.059 a	1661 ± 34.6 a	432 ± 5 c	0.328 ± 0.052 a	47.7 ± 1.84 b
	3	22.4 ± 2.20 ab	1.115 ± 0.099 a	1017 ± 63.3 b	509 ± 9 c	0.203 ± 0.013 b	32.1 ± 0.30 c
	4	19.8 ± 2.11 ab	1.153 ± 0.124 a	1707 ± 193 a	1120 ± 150 b	0.201 ± 0.026 b	39.8 ± 2.04 bc
	5	20.4 ± 1.19 ab	0.339 ± 0.011 b	610 ± 17 c	480 ± 12 c	0.167 ± 0.008 b	34.7 ± 0.21 c
Vernonia neocorymbosa	1	10.5 ± 1.83 a	6.42 ± 0.72 ab	1746 ± 46 b	65.8 ± 1.04 a	0.189 ± 0.012 b	14.7 ± 1.96 a
	2	11.8 ± 0.47 a	5.36 ± 0.32 b	1052 ± 116 c	52.3 ± 2.64 b	0.182 ± 0.024 b	12.3 ± 0.59 ab
	3	11.5 ± 0.99 a	6.07 ± 0.68 ab	1209 ± 148 c	51.3 ± 4.32 b	0.272 ± 0.031 ab	12.7 ± 0.57 ab
	4	11.9 ± 0.39 a	5.56 ± 0.32 ab	1075 ± 32 c	45.9 ± 1.83 b	0.192 ± 0.033 b	11.7 ± 1.10 b
	5	12.5 ± 1.68 a	6.82 ± 0.41 a	2024 ± 28 a	66.4 ± 2.39 a	0.366 ± 0.066 a	13.5 ± 0.50 ab

†Leaf + stem / *Root

Table 3.5: Concentration of microelements (mg kg^{-1}) determined by ICP-OES in bulbs and tubers of medicinal plants obtained from street markets (mean ± S.D.; n=3). Mean values with dissimilar letter(s) are significantly different ($p < 0.05$).

Plant species	Sample no.	B	Cu	Fe	Mn	Mo	Zn
†*Bowiea volubilis*	1	13.2 ± 0.79 a	11.8 ± 0.50 a	1392 ± 89 a	59.3 ± 1.18 a	0.144 ± 0.000 b	26.7 ± 2.50 a
	2	9.2 ± 0.45 b	4.80 ± 0.18 cd	382 ± 13 e	16.3 ± 0.57 e	0.149 ± 0.011 ab	24.0 ± 1.08 ab
	3	6.7 ± 0.63 c	6.70 ± 0.25 b	622 ± 14 d	24.7 ± 1.09 d	0.173 ± 0.014 a	13.0 ± 0.77 c
	4	6.1 ± 0.60 c	4.21 ± 0.17 d	974 ± 24 c	31.3 ± 0.46 c	0.158 ± 0.010 ab	20.4 ± 0.89 b
	5	6.0 ± 0.77 c	5.51 ± 0.50 c	1153 ± 44 b	36.7 ± 0.67 b	0.172 ± 0.005 a	15.3 ± 0.42 c
Dioscorea dregeana	1	17.1 ± 0.10 a	9.86 ± 0.23 a	187 ± 2.05 c	115 ± 3.02 b	0.160 ± 0.007 b	53.6 ± 4.38 a
	2	13.9 ± 0.28 b	6.89 ± 0.25 b	304 ± 0.27 b	122 ± 0.69 a	0.135 ± 0.034 bc	22.8 ± 0.33 cd
	3	10.9 ± 0.72 c	5.67 ± 0.21 bc	345 ± 15.6 a	78 ± 2.43 c	0.364 ± 0.010 a	37.8 ± 2.20 b
	4	12.8 ± 0.78 b	5.62 ± 0.05 bc	159 ± 14.1 d	63 ± 2.76 d	0.093 ± 0.008 c	28.5 ± 0.06 c
	5	14.2 ± 0.52 b	4.54 ± 1.16 c	85 ± 2.54 e	38 ± 0.98 e	0.110 ± 0.008 c	22.5 ± 0.14 d
†*Eucomis autumnalis*	1	14.7 ± 0.50 a	7.01 ± 0.22 c	146 ± 3.67 d	23.4 ± 2.04 d	0.080 ± 0.002 c	21.3 ± 1.04 b
	2	12.0 ± 0.08 b	7.15 ± 0.28 c	657 ± 9.19 c	34.9 ± 1.32 c	0.086 ± 0.008 bc	38.4 ± 2.94 a
	3	12.8 ± 1.43 ab	10.3 ± 0.39 a	1673 ± 12.1 b	70.5 ± 2.26 b	0.098 ± 0.009 b	24.5 ± 1.32 b
	4	13.7 ± 0.63 ab	9.58 ± 0.12 b	2669 ± 86.9 a	104 ± 4.36 a	0.136 ± 0.001 a	24.7 ± 1.40 b
†*Merwilla plumbea*	1	14.0 ± 0.06 bc	1.522 ± 0.045 ab	517 ± 6.94 a	9.07 ± 0.61 d	0.188 ± 0.003 a	15.2 ± 0.20 d
	2	11.4 ± 0.81 e	1.750 ± 0.046 a	285 ± 5.97 b	11.0 ± 0.47 c	0.116 ± 0.006 b	22.5 ± 0.74 b
	3	12.3 ± 0.15 de	1.501 ± 0.134 ab	176 ± 3.00 d	7.27 ± 0.17 e	0.099 ± 0.017 b	18.7 ± 0.39 c
	4	13.2 ± 0.51 cd	1.333 ± 0.128 b	148 ± 1.30 e	14.0 ± 0.45 b	0.112 ± 0.008 b	15.6 ± 1.26 d
	5	15.1 ± 0.31 a	1.360 ± 0.083 b	216 ± 1.14 c	16.3 ± 0.81 a	0.108 ± 0.015 b	28.0 ± 0.34 a

†Bulb / *Tuber

Table 5.4: Effect of Cd application on growth parameters of *Tulbaghia violacea* of varying size classes after 6 weeks. Mean values (± S.E) with dissimilar letter(s) are significantly different ($p < 0.05$).

Size class	Treatment (Cd mg l^{-1})	Leaf length (cm)	No. of leaves	Fresh weight leaves (g)	Fresh weight bulbs (g)	Root length (cm)	No. of roots	Fresh weight roots (g)
small	0	28.3 ± 0.94 a	4.5 ± 0.23 a	8.66 ± 1.00 a	7.57 ± 0.85 a	14.5 ± 1.20 a	14.5 ± 1.11 a	20.6 ± 2.14 a
	2	26.3 ± 1.25 a	4.4 ± 0.28 a	7.89 ± 0.84 a	10.0 ± 1.01 a	14.9 ± 1.25 a	11.5 ± 0.94 a	20.2 ± 2.27 a
	5	27.4 ± 0.81 a	3.3 ± 0.18 b	6.16 ± 0.49 a	7.72 ± 0.48 a	15.9 ± 1.22 a	12.3 ± 0.97 a	16.2 ± 1.89 a
medium	0	38.9 ± 1.46 a	9.08 ± 0.41 a	33.7 ± 2.61 a	22.8 ± 1.98 a	18.1 ± 1.03 a	12.4 ± 0.78 ab	33.3 ± 3.67 a
	2	33.3 ± 1.38 b	7.50 ± 0.39 a	19.8 ± 1.52 b	17.02 ± 0.94 b	17.4 ± 0.93 a	9.00 ± 0.70 b	27.1 ± 1.78 a
	5	36.2 ± 1.75 ab	8.50 ± 0.62 a	24.7 ± 3.52 ab	16.4 ± 1.22 b	18.5 ± 1.26 a	13.6 ± 1.72 a	29.5 ± 4.76 a
large	0	43.1 ± 1.70 a	11.5 ± 0.43 a	57.9 ± 2.84 a	39.6 ± 3.35 a	19.7 ± 0.97 a	15.6 ± 1.14 a	73.8 ± 6.93 a
	2	46.8 ± 1.82 a	10.0 ± 0.51 a	56.1 ± 8.29 a	34.9 ± 2.76 a	22.4 ± 1.28 a	14.0 ± 1.67 a	79.1 ± 12.3 a
	5	48.0 ± 2.02 a	10.3 ± 0.48 a	62.3 ± 8.92 a	35.1 ± 2.73 a	23.0 ± 1.64 a	16.3 ± 1.58 a	82.2 ± 15.0 a

Table 6.1: Effect of varying nutrient levels (HS – Hoagland's nutrient solution) and macronutrient deficiency (N,P,K) on growth parameters of *Dioscorea dregeana*. Mean values (± S.E.) with dissimilar letter(s) are significantly different ($p < 0.05$).

Treatment	Shoot length (mm)	Root length (mm)	No. of leaves	Seedling fresh weight (g)	Total leaf area (cm^2)	Aerial shoot length (mm)	Aerial shoot formation (%)	Tuber diameter (cm)	Shoot dry weight (mg)	Root dry weight (mg)
Percentage of HS nutrient solution										
10%	60 ± 1.4 a	93 ± 4.5 a	1.2 ± 0.1 c	2.175 ± 0.072 b	51 ± 2.99 b	34 ± 9.48 b	16.7	1.08 ± 0.14 a	161 ± 10 b	202 ± 8 a
50%	61 ± 1.9 a	93 ± 4.2 a	2.3 ± 0.1 a	3.031 ± 0.295 a	114 ± 12.8 a	104 ± 13.3 a	79.0	0.93 ± 0.39 a	262 ± 26 a	160 ± 18 a
100%	60 ± 2.1 a	104 ± 4.5 a	1.8 ± 0.1 b	3.075 ± 0.285 a	130 ± 18.9 a	78 ± 13.5 a	37.5	0.92 ± 0.28 a	238 ± 24 a	159 ± 17 a
Deficiency of N, P and K										
Control	61 ± 1.9 b	93 ± 4.2 a	2.3 ± 0.1 a	3.031 ± 0.295 a	114 ± 12.8 a	104 ± 13.3 a	79.0	0.93 ± 0.39 a	262 ± 26 a	160 ± 18 a
-N	67 ± 1.8 a	111 ± 10.4 a	1.0 ± 0.1 b	1.545 ± 0.167 b	37 ± 3.03 b	8 ± 6.29 b	4.10	0.94 ± 0.07 a	127 ± 12 b	140 ± 20 a
-P	64 ± 2.1 ab	72 ± 6.0 b	1.2 ± 0.1 b	1.759 ± 0.122 b	47 ± 3.63 b	27 ± 10.0 b	29.0	1.03 ± 0.06 a	133 ± 10 b	127 ± 10 a
-K	62 ± 2.1 ab	71 ± 5.7 b	1.2 ± 0.1 b	1.720 ± 0.120 b	44 ± 5.23 b	14 ± 6.94 b	16.7	1.03 ± 0.06 a	119 ± 11 b	130 ± 14 a

Appendix A – Hoagland's Nutrient Solution

All chemicals of analytical grade, were made up with distilled water to give a final concentration as shown in Table A. These stocks were stored in the dark at 10 °C until required. Depending on the required concentration, either 1, 5 or 10 ml of each stock was added to 1.5 L water, to make up 10, 50 or 100% Hoagland's nutrient solution respectively.

Table A: Composition of stock solutions used to make up Hoagland's nutrient solution (HOAGLAND and SNYDER 1933).

Stock	Concentration
$Ca(NO_3)_2 \cdot 4H_2O$	0.75 M
KNO_3	0.75 M
$MgSO_4 \cdot 7H_2O$	0.30 M
KH_2PO_4	0.15 M
NaFeEDTA	2.3 mM
H_3BO_3	7.0 mM
$MnCl_2 \cdot 4H_2O$	1.37 mM
$ZnSO_4 \cdot 7H_2O$	0.12 mM
$CuSO_4 \cdot 5H_2O$	22 µM
H_2MoO_4	16 µM