LIBRARY'S COPY

UPTAKE OF CADMIUM AND ITS EFFECT ON THE PHYSIOLOGY OF THE LIVERWORT *Dumortiera hirsuta* (SW) NEES AND THE MOSS *Atrichum androgynum* (CM) JAEG.

By

PUSELETSO JACINTA MAUTSOE

Submitted in fulfilment of the academic requirements for the degree of

MASTER OF SCIENCE

in the

DEPARTMENT OF BOTANY
FACULTY OF SCIENCE
UNIVERSITY OF NATAL
PIETERMARITZBURG

JUNE 1997

I certify that, unless specifically indicated to the contrary in the text, this dessertation is the result of my own work. The investigation was carried out in the Department of Botany, University of Natal, Pietermaritzburg from January 1995 to June 1997 under the supervision of Dr. R. P. Beckett.

P. J. Mautsoe

P. J. Mautsoe

ABSTRACT

In this thesis, the uptake kinetics of Cd by the liverwort *D. hirsuta* and the moss *A. androgynum* were investigated. In preliminary experiments, the toxicity of Cd to the bryophytes was investigated by characterising the effects of Cd on photosynthesis and K loss. Experiments were carried out to explain the existence of variation between different collections observed in uptake kinetics in the liverwort *D. hirsuta*. Photosynthesis in *D. hirsuta* was more sensitive to Cd than photosynthesis in *A. androgynum*. The sensitivity was directly related to intracellular Cd concentrations. *D. hirsuta* accumulated considerably higher concentration of intracellular Cd than *A. androgynum*. Cd caused intracellular K loss in *D. hirsuta* but not in *A. androgynum*.

Extracellular Cd uptake was rapid and independent of metabolism. Intracellular Cd uptake as a function of Cd concentrations followed Michaelis-Menten kinetics. Intracellular Cd uptake in *D. hirsuta* was affected by age of the plant, K pretreatment and the site where plants were collected. The moss *A. androgynum* displayed Cd uptake acclimation when uptake was measured at low temperatures. The results indicated that uptake kinetics could be affected by seasonal variation.

Tolerance of Cd in the moss *A. androgynum* could be induced by

pretreating the plants with low concentrations. The moss possibly excludes Cd from the cytoplasm and thus reduces the concentration of Cd in the cytoplasm to below toxic level.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and thanks to the following people who made this study possible.

I gratefully thank my supervisor Dr. R. P. Beckett, Department of Botany, University of Natal for his guidance and support through out the study. His patience, thoughtfulness, dedication and criticism were an inspiration.

Professor J. van Staden and Dr. M. Smith for their constructive criticism and practical advice.

Miss Angela Beaumont, Mr. J. V. Bandu and Electronic Microscope Unit staff for preparing the Plates in this study.

I am indebted to the academic and technical staff of Department of Botany, University of Natal, for their advice and assistance.

I sincerely thank the Government of Lesotho and the Department of Botany who provided finance for this study.

A special word of thanks to my mother, Mrs Limakatso Mautsoe for her continuos support, sacrifice and encouragement.

Finally, I thank my family, friends and collegues for their interest and inspiration in this study. May God bless.

CONTENTS

PAGE

ABSTRACT i

ACKNOWLEDGEMENTS iii

CONTENTS iv

LIST OF FIGURES x

LIST OF TABLES xii

LIST OF PLATES xv

CONTENTS

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION 1

PARTICULATE UPTAKE 4

IONIC UPTAKE 4

Extracellular uptake 5

Intracellular uptake 7

THE EFFECTS OF METALS ON BRYOPHYTE PHYSIOLOGY 10

HEAVY METAL TOLERANCE IN PLANTS 11

THE MECHANISMS OF METAL TOLERANCE 14

INTRODUCTION TO THE PRESENT STUDY 17

CHAPTER TWO

MATERIALS AND METHODS

PLANT MATERIAL 18

METAL UPTAKE AND DETERMINATION OF CELLULAR LOCATION . . . 18

CATION ANALYSIS 19

THE EFFECT OF K PRETREATMENT ON Cd UPTAKE BY *D. hirsuta* 21

THE EFFECT OF PLANT COLLECTION SITE ON Cd UPTAKE by *D. hirsuta* 21

DETERMINATION OF PHOTOSYNTHETIC AND RESPIRATION RATES
. . . . 22

TOXICITY OF Cd TO *D. hirsuta* AND *A. androgynum*
. . . . 23

TEMPERATURE ACCLIMATION IN *A. androgynum* 24

INDUCTION OF TOLERANCE 24

CHAPTER THREE

EFFECT OF Cd ON PHOTOSYNTHESIS AND POTASSIUM LOSS IN THE LIVERWORT *D. hirsuta* AND THE MOSS *A. androgynum*

INTRODUCTION 26

RESULTS 26

DISCUSSION 28

CONCLUSION 33

CHAPTER FOUR

FACTORS AFFECTING Cd UPTAKE IN THE LIVERWORT *Dumortiera hirsuta*

INTRODUCTION 34

RESULTS AND DISCUSSION 36

Cd uptake as a function of time 36

Cd uptake as a function of concentration 40

The effect of light on uptake 42

The effect of temperature on Cd uptake 42

The dependence of Cd uptake on plant age 45

The effect of competing ions on Cd uptake 47

The effect of K pretreatment on Cd uptake 49

The effect of plant collection site on Cd uptake . . 51

CONCLUSION 56

CHAPTER FIVE

FACTORS AFFECTING THE Cd UPTAKE IN THE MOSS *Atrichum androgynum*

INTRODUCTION 57

RESULTS AND DISCUSSION 57

Cd uptake as a function of time 61

Cd uptake as a function of concentration 61

Cd uptake as a function of temperature 64

The effect of inhibitors on Cd uptake 64

The effect of storage temperature on Cd uptake . . . 67

CONCLUSION 72

CHAPTER SIX

THE INDUCTION OF HEAVY METAL TOLERANCE IN THE MOSS *Atrichum androgynum*

INTRODUCTION 73

RESULTS AND DISCUSSION 73

CONCLUSION 81

CHAPTER SEVEN

GENERAL CONCLUSIONS

SENSITIVITY OF *D. hirsuta* AND *A. androgynum* TO Cd 82

Cd UPTAKE IN *D. hirsuta* AND *A. androgynum* 83

THE INDUCTION OF Cd TOLERANCE IN *A. androgynum* 89

REFERENCES 92

LIST OF FIGURES

PAGE

CHAPTER 1

Figure 1.1. Generalized dose response curve 3

CHAPTER 2

Figure 2.1. Diagramatic representation of the sequential elution technique 20

CHAPTER 4

Figure 4.1. Cd uptake by *D. hirsuta* as a function of time 38

Figure 4.2. Cd uptake by *D. hirsuta* as a function of concentration 41

Figure 4.3. Cd uptake by *D. hirsuta* as a function of temperature 44

PAGE

CHAPTER 5

Figure 5.1. Cd uptake by *A. androgynum* as a function of time 60

Figure 5.2. Cd uptake by *A. androgynum* as a function of concentration 62

Figure 5.3. Cd uptake by *A. androgynum* as a function of temperature 63

Figure 5.4. Effect of storage temperature on Cd uptake by *A. androgynum* 69

CHAPTER 6

Figure 6.1. Effect of pretreatment with low concentrations of Cd on photosynthesis and K loss in *A. androgynum* 76

LIST OF TABLES

PAGE

CHAPTER 3

Table 3.1. The effect of Cd on
photosynthesis and K loss in *D. hirsuta* 27

Table 3.2. The effect of Cd on
photosynthesis and K loss in *A. androgynum*
after 0 and 24 h following incubation in Cd 29

Table 3.3. The effect of Cd on
photosynthesis and K loss in *A. androgynum*
after 0, 48 and 96 h following incubation in Cd 30

CHAPTER 4

Table 4.1.Effect of light on Cd
uptake by *D. hirsuta* 43

Table 4.2. Effect of plant age on
Cd uptake by *D. hirsuta* 46

Table 4.3. Effect of competing cations
on Cd uptake by *D. hirsuta* 48

PAGE

Table 4.4. Effect of K pretreatment on
Cd uptake by *D. hirsuta* 50

Table 4.5. Effect of plant collection
site on Cd uptake by *D. hirsuta* 53

CHAPTER 5

Table 5.1. Effect of inhibitors on
Cd uptake by *A. androgynum* 66

Table 5.2. Effect of storage temperature
on Cd uptake by *A. androgynum* 71

CHAPTER 6

Table 6.1. Effect of pretreatment with
low concentrations of Cd on subsequent
incubation in higher concentrations in
the moss in *A. androgynum* 79

PAGE

CHAPTER 7

Table 7.1. A comparison of the characteristics of C uptake by *D. hirsuta*, *A. androgynum*, *R. squarrosus* and *Peltigera* 85

LIST OF PLATES

PAGE

CHAPTER 4

Plate 4.1. The habitat, morphology
and cross section of *D. hirsuta* 37

Plate 4.2. A comparison of outer cell
wall of *D. hirsuta* collected from 3 sites 55

CHAPTER 5

Plate 5.1. The habitat, habit and
morphology of *A. androgynum* 58

CHAPTER 6

Plate 6.1. The effect of pretreatment
with low concentrations of Cd on the
physical appearance of *A. androgynum* 77

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

There are two ways in which heavy metals may be introduced into an environment. They can either come from natural sources like metal ore deposits, or they can come from man's activities like smelting industries, mines and car emissions. Thus heavy metals are ever present constituents of the atmosphere, natural water and soils. However, increased industrial activities have sometimes increased the concentrations of metals in the environment to such extent that they may become toxic to animal and plant life.

Heavy metals have always been regarded as elements with specific gravities greater than four or five. This included the Actinides and Lanthanides series of elements that are not normally regarded as "heavy". Nieboer & Richardson (1980) proposed a classification of elements based upon the co-ordination chemistry of metals and their interaction with biological systems. In this classification, two main types of metals are proposed. The class A metals bind to oxygen-containing ligands and class B metals bind to nitrogen- and sulphur-containing ligands. Metals which display intermediate binding preference are termed "borderline". This classification is used in this thesis.

For a given plant growing under a given set of conditions, it is possible to determine a "dose response curve" (Figure 1.1). Curve B-D is the response of a normal, wild type plant to an essential metal (eg. Cu, Zn) and shows deficient, adequate and toxic zones. Curve A-D is the reaction of a normal plant to a nonessential metal (eg. Cd) and exhibits only adequate and toxic zones. The adequate zone may be specified by the low and high concentrations that cause growth reductions of 5 to 10% from maximum. This is analogous to plant tissue analysis in which yield is plotted against metal concentration in tissue. The adequate zones lies between the concentrations for critical deficiency and critical toxicity (Bouma in Rauser, 1995). The work presented here will be concerned only with the toxicity zone of the dose response curve.

Metals are frequently available in solution as positively charged cations. However, much of the heavy metal burden of bryophytes in industrialized regions is present as particulate matter adsorbed to fly ash or carbonaceous automobile exhausts. The degree of dissolution of trapped particles, especially in acidic environments, is generally unknown and hence the availability of elements to plants is little understood. The elements associated with bryophyte cells can be attributed to four possible locations (Brown & Bates, 1990). These are: (1) trapped particulate matter; (2) intercellular soluble; (3) extracellular, bound to the cell wall on charged exchange sites or (4) intracellular.

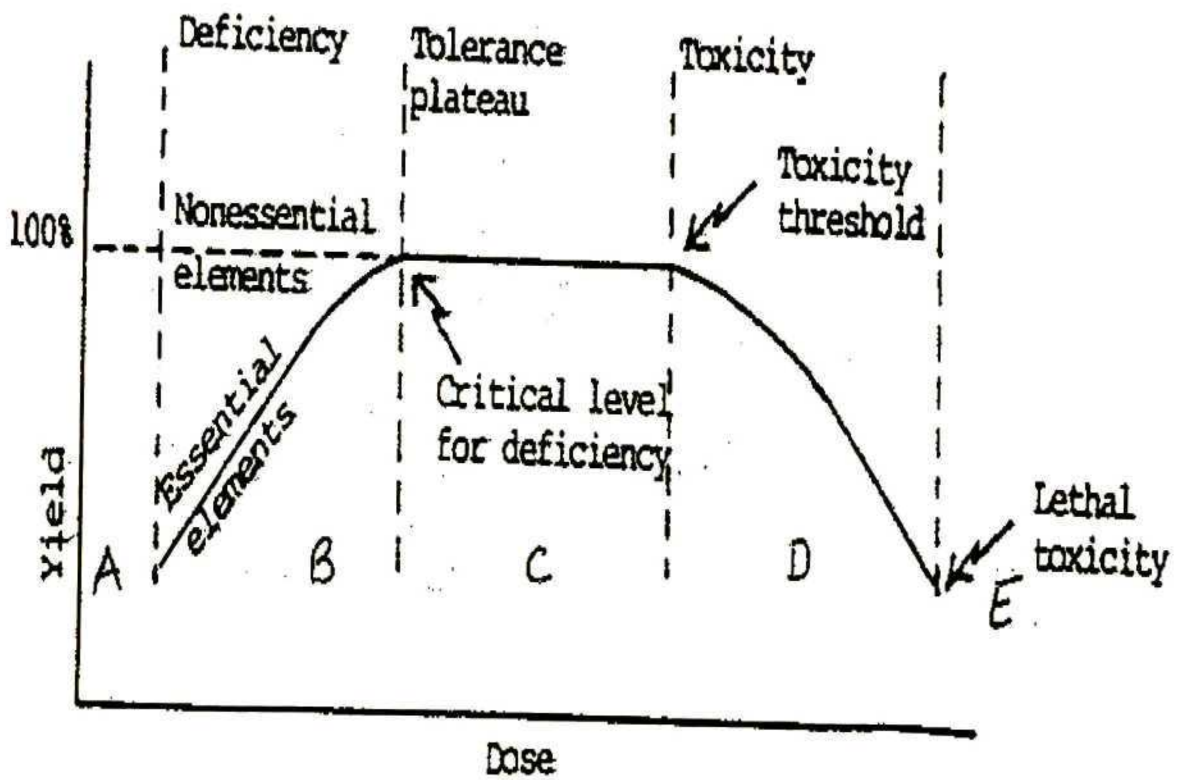


Figure 1.1. Generalized dose response curve (modified from Beckett, 1984)

PARTICULATE UPTAKE

Uptake of particles may either be in the form of particulate matter which may be trapped between the cells. Although it is currently impossible to quantify the proportion of an element that is in the form of particles, several lines of evidence suggest that particles may represent an important component of the total element content of lichens or bryophytes. Richardson and Nieboer (1980) showed that the ash content of lichens increase close to sources of particulate emissions. Secondly, because Fe and Ti are present in insoluble forms in non-calcareous rocks, the approximately constant Fe:Ti ratio obtained in cryptogams over a large concentration range suggests that particle entrapment is fairly common (Nieboer *et al.* 1982). Many workers have also shown that aerially dispersed heavy metals consist mostly of particulate material from which elements are not immediately soluble (Brown, 1984). Although not fully analysed, it is probable that the plant morphology may be an important factor in particulate-trapping efficiency. The extent to which solubilization of the elements of particles can occur, thus rendering them available for ionic uptake is little understood.

IONIC UPTAKE

When bryophytes are supplied with soluble cations or anions, these may be subsequently be recovered from three cellular

locations in the plant, namely, between the cells, external to the plasma membrane or contained within the regions circumscribed by the plasma membrane (Brown, 1984). Uptake to extracellular site is by physicochemical processes, and the material is mostly bound to the cell wall by virtue of its greater bulk. Binding to the exterior surface of the plasma membrane may occur, and may be of particular significance in understanding the toxicity of certain elements. In contrast, the uptake to intracellular locations is the result of biological processes controlling the passage of ions across the bounding lipoprotein membrane.

Extracellular uptake

Extracellular uptake is dominated by ion exchange phenomena which are rapid and passive, being only slightly affected by temperature and independent of changes in metabolism induced by illumination or darkening, reduced oxygen tension or the addition of metabolic inhibitors (Brown, 1984). This is an exchange reaction, and the process can be treated as the replacement of one positively charged cation bound to an immobile, extracellular negatively charged anionic exchange site by another cation. Richardson (1995) states that in lichens, the entering ions displaces hydrogen ions or ions of other more weakly bound metals from the binding site on or in the cell walls. The degree of replacement depends on the affinity of the metal for the cell wall and the concentration of the displacing ion. Thus the amount of metal taken up from

a range of solutions, each containing a different metal at a given concentration, would be in the order class A < borderline < class B, because class B elements have the highest affinity for the cell wall.

Where the anionic sites have been saturated by a particular cation, quantitative displacement can be shown to occur in 1:1 ratio between divalent cations and a 1:2 ratio with divalent:monovalent cations. For example, if exchange sites are saturated with Zn ions and the plant is later incubated in Ni ions,the ratio of Ni taken up to Zn replaced is 1:1. The rapid release of protons (H) on addition of salt solutions, measured by a decrease in pH, must be included when charge balancing is considered and it probably represents the proportion of anionic sites not occupied by other cations.

Total metal binding is determined by the number of available exchange sites. Most binding is assumed to be to carboxylic acid groups and sulphur containing groups in protein in the wall. The outer surface of the lipoprotein cell membrane may also be involved. The fact that cations bound to the cell wall are exchangeable has been used as a method to determine the quantity of an element that is inside the cell (Brown, 1984). This technique is strictly only applicable to cations taken up in the laboratory. This is because it is always uncertain how much of an element remaining after displacement of cell wall-bound cations is really intracellular and how much is in the form of insoluble particles. The technique cannot even be used

to calculate the amount of field-acquired metals bound to the cell wall of plants, because the extent to which metal present in particles is removed by displacing agents is unknown. Because the number and the nature of exchange sites is dependent of species, tissue age and growth conditions, investigators need to verify this information for their particular circumstances, rather than extrapolate from other studies (Brown & Bates, 1990).

The large extracellular cation exchange capacity of bryophytes may also provide the basis for explaining metal uptake but does not provide the information regarding the survival of the plant. Although it may be numerically smaller than the extracellular uptake, intracellular uptake is probably of major importance for plant survival. Elements located within the cell potentially have an immediate impact on metabolism and may be of more biological importance to the bryophyte than elements held on external sites.

Intracellular uptake

Intracellular uptake requires passage across the limiting plasma membrane of the cell, using transport sites with various degrees of selectivity (Brown & Bates, 1990). The entry is determined by the affinity of an element for the appropriate transport site, the presence of other possibly competitive elements and the turn-over rate of the site. Unlike extracellular binding, the intracellular uptake of cations into

the cell is an energy requiring process. Although solutes may pass through a channel in a free energy gradient (passive), this energy may have been created by other processes such as that contributing to the membrane potential (Flowers & Yeo, 1992). The pumping of protons across the membranes consumes adenosine triphosphate (ATP) and generates a difference in electrical potential across the membrane. In all cases, work is done to move ions from a region of lower to higher chemical potential where the transport process is said to be active. There are two levels of active transport. Primary active transport involves the direct use of metabolic energy to pump the solutes across the membrane. In secondary active transport, the free energy gradient generated by the primary transport process is harnessed to move other solutes through the membrane via the carrier or channel proteins.

To determine the quality of an element present within a plant cell, the amounts present in other cellular locations must be known. Brown & Buck (1979) used a technique of sequentially eluting bryophytes. This involved discharging most cations from extracellular exchange sites by treatment with 1000 ppm Ni and recovery of residual material by digestion in concentrated HNO_3. The total digest has been shown to contain material bound within the cell, but may also include ions which were formerly soluble within the cell but which, due to their high affinity for new exposed sites, became bound during the process of destroying the cell membranes (Brown, 1982).

The rate of intracellular ion uptake in a plant is not directly proportional to the concentration of that ion in the solution bathing the plant, but typically displays "bi-phasic" kinetics (Flowers & Yeo, 1992). Possibly, two distinct transporting systems are involved. The first system saturates at about 0.1 mM, and has a high affinity for ions. The second system saturates at about 100 mM, and has a very much lower affinity for ions. Within the concentration range 0 to 0.3 mM, a double reciprocal plot of uptake against concentration yields a straight line. Uptake systems that behave this way are referred to as displaying Michaelis-Menten kinetics. The double reciprocal transformation allows the calculation of the kinetic constants K_m and V_{max}. K_m is the concentration of an ion which must be supplied for half of the maximum uptake rate to occur and is a measure of the affinity of the transporting systems for that ion. V_{max} is the maximum possible uptake rate and is a measure of number of transporting systems.

Plants have frequently been found to discriminate quite effectively between physiological cations. Non-physiological elements such as Cd, may enter on non-specific carriers and accumulate due to the absence of intracellular control processes (Brown & Bates, 1990). In general, plants are unable to discriminate between borderline elements and class B metals. It has been suggested that in lichens these are taken up by systems normally transporting Mg (Beckett & Brown, 1984a). In bryophytes the system transporting Ca seems to be likely to transport the class B metals (Wells & Brown, 1990).

THE EFFECTS OF METALS ON BRYOPHYTE PHYSIOLOGY

There are possibly two ways in which plants may be exposed to heavy metals (Puckett & Burton, 1981). These may either be

(1) Short term exposure to a high concentration of a heavy metal. This has an acute effect resulting in death of the tissue or plant;

(2) Long term exposure to a low concentration of a heavy metal. This has a chronic effect, decreasing the growth rate and yield.

Metals have been postulated to exert toxic effects on plants by: (1) blocking the functional group of a biomolecule; (2) displacing an essential element from a biomolecule; or (3) modifying the active conformation of a biomolecule (Nieboer & Richardson, 1980). The high affinity of class B elements for nitrogen- and sulphur containing ligands probably makes them toxic to plants.

Most of laboratory studies on heavy metal uptake in aqueous solutions are short term experiments (Beckett, 1984). A problem with this kind of experiment is that the concentration of metals used is much higher than those in polluted field solutions. The viability of the plants can be determined in various way, eg. K^+ loss, an indication of membrane damage. High concentrations are needed to obtain clear evidence of

damage. Unfortunately, high quantities will disrupt any physiological process of the plant. This makes it difficult to determine how important the toxic effects noted in the laboratory are in field conditions. Secondly, it is not clear which of the observed effects are most important in determining the threshold levels of metals that are toxic to these plants (Sidhu & Brown, 1996).

A number of studies have assessed the effects of metals on various aspects of physiology and growth. Physiological traits that have been measured include photosynthesis and respiration, chlorophyll content and spore germination (Shaw, 1990a). Bryophytes subjected to atmospheric pollution are generally infertile, and most of them do not form sporophytes. In most investigations, the bryophytes are collected from sites polluted by other toxic compounds in addition to metals. Thus it is difficult to determine to what extent the effects are from heavy metal toxicity only.

HEAVY METAL TOLERANCE IN PLANTS

Some areas of the world have soils that are naturally enriched with heavy metals. These include areas with heavy metal deposits or mine tailings. Some species of plants that colonize such soils also occur over large geographic areas and populations on metalliferous soils may consist of locally adapted races. Conversely, the so-called copper mosses include about seven species of bryophytes that are more or less

restricted to substrates with higher than average concentrations of copper and/or other metals (Shaw 1990a). It should be noted that the term "copper mosses" include both mosses and liverworts and it is not clear that copper *per se* is the critical element determining their distribution and abundance. The copper mosses are resistant to copper added in the laboratory. In general, both areas of natural metal enrichment and areas contaminated by mining or smelting activities contain species which are also found in unpolluted sites.

In higher plants it is known that populations of plants growing in metal contaminated sites can be genetically distinct metal tolerant ecotypes. No attempts have been made to verify that metal tolerance can be inherited in bryophytes although it has already been demonstrated that bryophytes sampled from polluted sites display increased resistance of metals added in the laboratory (Briggs, 1972; Brown & House, 1978; Lepp & Hockenhull, 1983; Wells & Brown, 1995).

It is possible to make several generalizations about the characteristics of metal tolerance in plants. First, tolerance is usually specific to the metal that is present at an elevated concentration. That is if plants are collected from habitats which contain high levels of Zn, then plants sampled from the site will be more tolerant to Zn. However, there are examples of "co-tolerance" that is tolerance to one element may be accompanied by the tolerance to another element present in only

trace amounts. For example, Shaw (1987 & 1994) noted that pretreatment of *Funaria hygrometrica* with Zn increased tolerance to Cd. It has not been shown whether metal-tolerant populations of bryophytes possess tolerance mechanisms specific for the metal experienced in the environment. It is not certain to what extent the concentration of a metal must be elevated before selection to tolerance occurs. Rather than collect plants from soils containing "high" concentrations of one element and "low" concentrations of another, it would be better to induce tolerance using one element and monitor any change in tolerance in another in order to critically test co-tolerance in plants.

Second, metal tolerance occurs throughout the plant kingdom. Because mine sites possess restricted floras compared with surrounding areas, it is unlikely that all species can evolve metal-tolerant ecotypes.

Third, metal-tolerant ecotypes of some species appear to have a greater requirement for metals than non-tolerant plants (Bradshaw & McNeilly in Beckett, 1984). This may possibly be because tolerance mechanisms may complex metals, making them unavailable for their normal function in growth and development.

Finally, there are indications that there might be a biological cost associated with metal tolerance. Jules & Shaw (1994) working on *Ceratodon purpureus* showed that smelter plants

produced fewer stems and fewer gametangia on the control than on the contaminated treatment. The results were similar to those originally observed by Shaw (1990b) in the metal-tolerant *Funaria hygrometrica*. In this case metal-tolerant plants formed stems more slowly on normal treatment that non-tolerant individuals. In angiosperms, plants tolerant of metals are found to be competitively inferior on uncontaminated soils and thus occur in low frequencies in the absence of metal contamination (Bradshaw, 1976).

THE MECHANISMS OF METAL TOLERANCE

Several mechanisms have been proposed to explain how plants can tolerate metal pollution. Shaw (1990a) suggested a possible mechanism of "death zones". Death zones occur where low and high concentrations caused little mortality but intermediate levels were lethal. He suggested that a layer, impermeable to metals, is formed on the cell wall when plants are immersed in concentrated metal solutions. When subjected to low concentrations, metal ions are taken up without harm to the cell, but at very high concentrations, the rapid formation for this impermeable layer inhibit further uptake. Intermediate concentrations are too low to cause that formation of the impermeable layer but too high to be tolerated if absorbed internally.

Rates of intracellular metal uptake can be important in determining the tolerance of plants to heavy metals. Wells &

Brown (1987) found that in the moss *Rhytidiadelphus squarrosus*, one population accumulated greater quantities of Cd than the second population at concentration greater than 1 mM. Wells & Brown (1987) also found that the population which accumulated greater quantities was also more sensitive to Cd, this being measured by the reduction of CO_2 uptake. From this they concluded that greater sensitivity of the population could be related to its greater intracellular Cd uptake. Thus the lesser the intracellular Cd uptake, the lesser the plant would be susceptible to Cd toxicity. Wells & Brown (1995) found that in apical segments of the moss *R. squarrosus*, the metal-contaminated population consistently showed a lower decline in photosynthetic rate per unit intracellular Cd uptake than the uncontaminated population

Essential and non essential metals exist at low to high concentrations depending on natural and man-made disturbances. In a fluctuating environment, the plants may experience shifting internal concentrations of these bioreactive metals. Therefore, it is beneficial for plants to have mechanisms that (a) maintain internal concentrations of essential metals between deficient and toxic limits and (b) keep non essential metals below their toxicity thresholds. The phytochelatins sequester metals and thereby accomplish cellular metal homeostasis and detoxification (Rauser, 1995). These molecules are rich in cysteine that provide thiols for binding the metals such as copper, zinc, cadmium, lead and mercury.

Metal homeostasis requires intracellular complexation of metals when there is a cellular surplus and later release of metals to metal requiring apoprotein and perhaps to final storage sites within cells. Grill *et al.* (1988) observed that when various cultured plant cells were transferred to a variety of fresh media containing micronutrients of copper and zinc, the concentrations of phytochelatins increased and the medium was depleted of copper and zinc. The phytochelatin concentrations were maximum just prior to the stationery phase and coincided with total disappearance of the metals from the medium. This observation further supports the involvement of phytochelatins in homeostasis. The stationary phase cells then gradually lost phytochelatins presumably to peptide degradation. The phytochelatins probably behave as transient metabolites with considerable turnover. The extent to which cellular copper and zinc actually occurred as a metal-phytochelatin complex during the culture cycle was, however, not evaluated.

Therefore, in higher plants phytochelatins are commonly implicated in heavy metal tolerance, although it is not clear to what extent they account for differential tolerance between plant populations (Wells & Brown, 1995). It seems that heavy metals are the primary inducers of phytochelatin production in plants but that increased levels of phytochelatins within the plant may confer both specific and non-specific tolerance to metals, according to species and genotype.

INTRODUCTION TO THE PRESENT STUDY

The main aim of the present study was to characterize the uptake kinetics of Cd in the liverwort *Dumortiera hirsuta*. To date, no information is available on the uptake of Cd by liverworts under laboratory conditions. Where possible the results obtained were compared to those found by Beckett & Brown (1984a) in the lichen *Peltigera* and Brown & Beckett (1985) and Wells & Brown (1987) in the moss *Rhytidiadelphus squarrosus*. In addition, the effect of temperature on uptake kinetics and possible induction of heavy metal tolerance was investigated using the moss *Atrichum androgynum* as a model system.

CHAPTER TWO

MATERIALS AND METHODS

PLANT MATERIAL

The thalloid liverwort *Dumortiera hirsuta* (SW) Nees. was collected from rocky boulders forming a waterfall at Ferncliffe, Pietermaritzburg, KwaZulu Natal Province, Republic of South Africa (29° 31' South, 30° 20' East). The material was washed in tap water to remove debris and stored moist in a culture room under continuous fluorescent lighting (75 µmoles m^{-2} s^{-1} at 22 ± 1 °C). The plant material was re-washed in deionized distilled water before use. Representative material was fixed in gluteraldehyde, embedded in resin and 1 µM sections cut. Sections were stained with Ladd's multiple stain and observed under a light microscope.

The moss *Atrichum androgynum* (CM) Jaeg. was collected from the canopy floor of Doreen Clarke Nature Reserve, Pietermaritzburg (24° 39' South, 30° 17' East) and was stored as mentioned above.

METAL UPTAKE AND DETERMINATION OF CELLULAR LOCATION

Sufficient disks (10 mm diameter) were cut for each experiment, and for each treatment randomly selected samples comprising 25 disks were incubated in 1 litre of 100 µM $CdSO_4$ at 20 ± 1 °C,

under sodium lamp lighting at 125 µmoles m^{-2} s^{-1} (light saturating for photosynthesis). Compressed air was continually bubbled through the treatment solutions to prevent the formation of unstirred layers. After 1 h the disks were filtered from the solution, divided into 5 replicates of 5 disks and the cellular location of the Cd determined using the method of Brown and Buck (1979) (Figure 2.1). This involved washing the material in deionized distilled water (10 ml) for 0.5 h to displace intercellular Cd. Extracellular Cd was displaced by shaking the material with 10 ml of 20 mM $NiCl_2$ for 0.5 h twice. The material was then shaken in 10 ml of 1 M HNO_3 for 1 h to displace intracellular Cd, and finally washed in deionized distilled water for 0.5 h. The material was oven-dried at 80 °C and weighed. For moss *Atrichum androgynum*, 25 (2 cm) apical segments were used per treatment. The weights of each replicate were determined before plants were shaken in 1 M HNO_3 to displace intracellular Cd.

CATION ANALYSIS

All elements were determined by atomic absorption spectrophotometry in an air/acetylene flame. Flame analyses were performed on a Varian Techtron Model AA 1200.

To minimise analytical errors, La was added to standards and solutions containing Ca and Mg to prevent formation of refractory compounds. CsCl was added to standards and solutions containing K to avoid ionization.

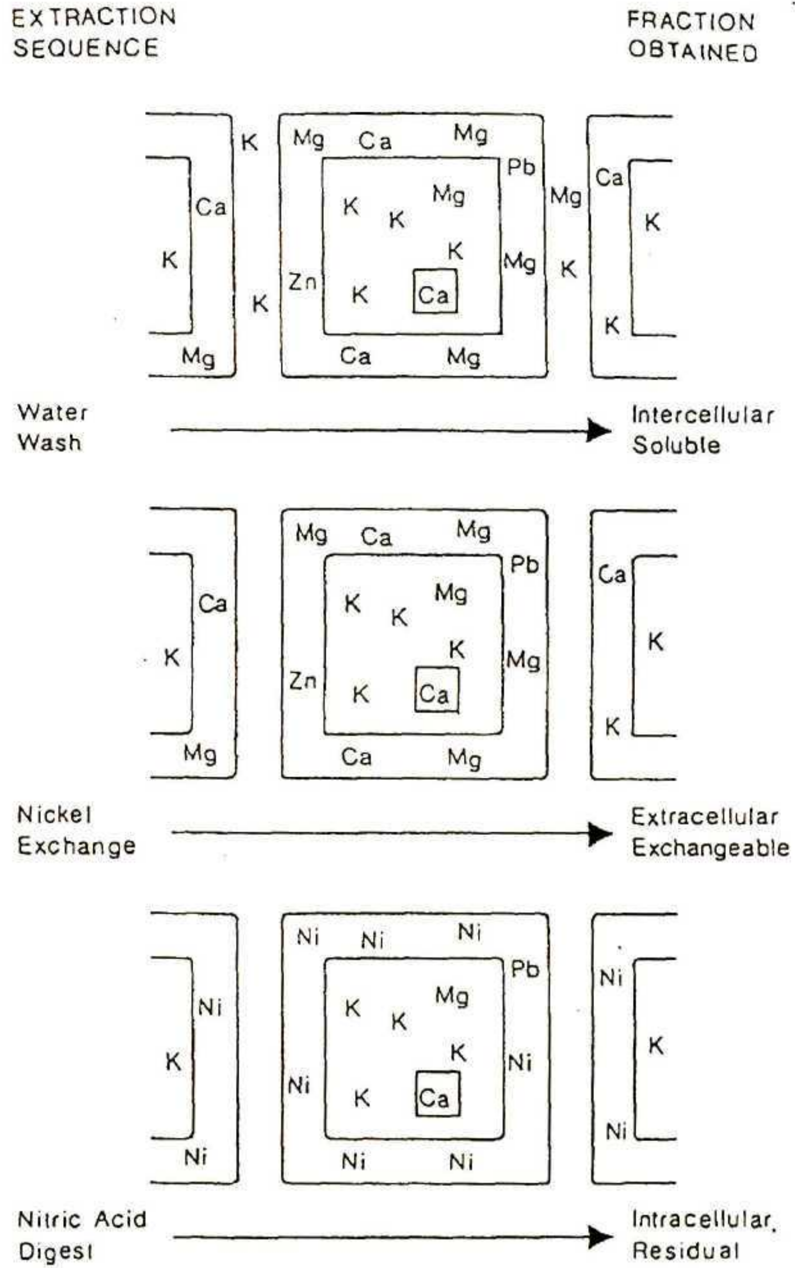


Figure 2.1. Diagrammatic representation of sequential elution technique (from Beckett, 1984).

The concentration of Cd required for half the maximum uptake rate (K_m) and the maximum uptake rate (V_{max}) were calculated using the method of Wilkinson (1961). The effect of dark storage on uptake was investigated by measuring uptake in plants stored on moist filter paper under continuous lighting (75 µmoles m^{-2} s^{-1}) or in the dark at 22 $\pm$ 1 °C for 20 days.

THE EFFECT OF K PRETREATMENT ON Cd UPTAKE BY *D. hirsuta*

The effect of K pretreatment on Cd uptake was tested by incubating thallus disks in 1 litre of deionized distilled water or 80 mM KNO_3. Disks were stored in the light on moist filter paper overnight as above, and Cd uptake was measured in K pretreated and untreated plants. The Ca and Mg displaced by KNO_3 pretreatment were taken as the amounts of Ca and Mg in nickel fraction (extracellular) when plants were not pretreated less the amounts of Ca and Mg when plants have been pretreated with KNO_3. These amounts of Ca and Mg were then added to the uptake solutions of replicate water and K pretreated plants, and Cd uptake measured.

THE EFFECT OF PLANT COLLECTION SITE ON Cd UPTAKE BY *D. hirsuta*

It was noted that some variation in uptake rates existed between individual collections. The site where plants were collected was taken as one of the possible factors that would cause the variation.

In this experiments, plant material was collected from three sites thus: (1) some distance from the stream, (2) the bottom of a waterfall and (3) the top of the waterfall. The plant material from the sites was fixed and examined under the JEOL JEM-100 CX transmission electron microscope to see if differences in thickness of the cuticle existed between the collections.

DETERMINATION OF PHOTOSYNTHETIC AND RESPIRATION RATES

The rates of photosynthesis and respiration were measured using infra red gas analysis as described by Brown *et al.* (1981). This involved putting samples of plant material in bottles. The bottles were flushed with air from outside the building to bring CO_2 concentration in the bottles to ambient. To create a positive air pressure inside the bottles, 2.5 ml of air was initially injected. About 1 ml of air from the bottle was extracted and the initial concentration of CO_2 inside the bottle analysed using an ADC Mark II bench top Infra Gas analyser attached to a plotter. The bottles were submersed in a waterbath kept at 20 °c under continous lighting at 75 µmoles m^{-2} s^{-1}. After 10 minutes, the bottles were removed from the waterbath and the concentration of CO_2 determined. The difference between this and the initial concentration CO_2 gives the amount of CO_2 used in net photosynthesis. To measure respiration, the bottles were then wrapped in tin foil and put in a waterbath for 10 minutes after which concentration of CO_2

was determined. Gas from a cylinder containing a known concentration of carbon dioxide was used to calibrate the infra red gas analysed. Gross photosynthesis was taken as the sum of net photosynthesis and respiration.

TOXICITY OF Cd TO *D. hirsuta* AND *A. androgynum*

In preliminary experiments, the toxicity of Cd to *D. hirsuta* and *A. androgynum* was tested by measuring the effect of Cd on K leakage and photosynthesis. In *D. hirsuta*, disks (25 of 10 mm diameter) of liverwort thallus were incubated in 1 litre of deionized distilled water, 100 µM or 300 µM $CdSO_4$ at 20 $\pm$ 1 °C, under sodium lamp lighting at 125 µmoles m^{-2} s^{-1}. After 1 hour, the disks were filtered from the solution, divided into five replicates of five disks and gross photosynthesis determined using the method of Brown *et al.* (1981). The extra- and intracellular concentrations of Cd and intracellular concentrations of K were determined. In addition, replicate samples of liverwort tissue were treated as above, and left for 24 hours on moist filter paper under continuous fluorescent lighting at 22 $\pm$ 1 °C. Gross photosynthesis, extra- and intracellular concentrations of Cd and intracellular concentrations of K were determined.

In *A. androgynum*, 25 apical segments (2 cm) were used in treatments as outlined above and gross photosynthesis, extra- and intracellular concentrations of Cd and intracellular

concentrations of K were determined immediately and 24 hours later. In addition, the moss segments were incubated in deionized distilled water and 300 µM $CdSO_4$. Gross photosynthesis, extra- and intracellular Cd concentrations and intracellular concentrations of K were determined immediately, 48 hours and 96 hours later.

TEMPERATURE ACCLIMATION IN *A. androgynum*

To test for effect of environmental temperature on Cd uptake, moss segments were kept at 10, 20 and 30 °C for 21 days in the dark. After this period, the segments were incubated in a range of temperatures and the Cd uptake rates determined. In addition photosynthesis and respiration of the material stored at 10 and 30 °C were also determined to see if respiration rates could be responsible for differences in uptake.

INDUCTION OF TOLERANCE

In preliminary experiments, segments of *A. androgynum* were incubated in $CdSO_4$ solutions ranging from 1 to 5 µM for 1 hour and stored for 21 days under moist and light conditions at 20 °C. After storage gross photosynthesis and intracellular concentrations of K and Cd were determined. $CdSO_4$ supplied at 1 µM did not induce toxic symptoms in plants and this concentration was used in a subsequent experiment to test if Cd tolerance can be induced in *Atrichum*. This experiment tested if

incubating the plant material with low concentrations of Cd decreased the sensitivity of photosynthesis to Cd in the moss. Moss segments were incubated in 1 µM $CdSO_4$ for 1 hour. The segments were then filtered from the solution and washed in deionized distilled water for 30 minutes. They were then placed on moist filter paper at 22 ± 1 °C under continuous fluorescent lighting (75 µmoles m^{-2} s^{-1}) and maintained under these conditions for 21 days. Gross photosynthesis, extra- and intracellular concentrations of Cd and intracellular concentrations of K for half of the material were then determined. The other half was incubated in 2.5 µM $CdSO_4$ for 7 days at 22 °C and 75 µmoles m^{-2} s^{-1}. The subsequent gross photosynthesis, extra- and intracellular concentrations of Cd and intracellular concentrations of K were determined.

CHAPTER THREE

EFFECT OF Cd ON PHOTOSYNTHESIS AND POTASSIUM LOSS IN THE LIVERWORT *D. hirsuta* AND THE MOSS *A. androgynum*.

INTRODUCTION

In the present study, the toxicity of cadmium on the liverwort *D. hirsuta* and the moss *A. androgynum* was tested by measuring the inhibition of photosynthesis. Potassium leakage was also used as a measure of membrane damage. This was done to see if Cd was toxic to plants over the 1 hour the uptake experiments described in this thesis were carried over.

RESULTS

Incubation of *D. hirsuta* in 100 and 300 μM $CdSO_4$ for 1 hour did not significantly affect gross photosynthesis (Table 3.1). However, after 24 h of storage following treatment, 300 μM $CdSO_4$ reduced photosynthesis. Intracellular K concentration was slightly affected by exposure to $CdSO_4$ for 1 hour but both Cd concentrations reduced intracellular K concentrations after 24 hours. During storage, extracellular Cd moved across the cell wall and consequently intracellular Cd concentrations increased while the extracellular concentrations decreased. In *A. androgynum*, gross photosynthesis was not affected and intracellular K was slightly affected when the moss was

Table 3.1. The toxicity of Cd to *Dumortiera hirsuta*. Plants were incubated in $CdSO_4$ solutions for 1 h and Cd uptake, intracellular K concentration and gross photosynthetic rate measured immediately or after storage for 24 h. Figures in each column followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

Cd Concent-ration (μM)	Time of measure-ment after exposure to Cd (h)	Intra-cellular Cd uptake (μmoles g^{-1})	Extra-cellular Cd uptake (μmoles g^{-1})	Intra-cellular K after uptake and sequen-tial elution (μmoles g^{-1})	Gross photosyn-thetic rate (μmoles CO_2 g^{-1} min^{-1})
0	0	0	0	1560 b	2.87 bc
0	24	0	0	1597 b	3.31 c
100	0	11.39 a	30.51 b	1321 b	3.05 bc
100	24	25.55 c	17.63 a	830 a	2.45 b
300	0	19.04 b	32.09 b	1439 b	2.50 b
300	24	33.93 d	18.44 a	935 a	1.56 a

subjected to similar conditions (Table 3.2). Toxicity symptoms were only evident when plants were stored for 48 and 96 hours following pretreatment with 300 μM $CdSO_4$ (Table 3.3). *D. hirsuta* accumulated more intracellular Cd from the cell wall than *A. androgynum* during 24 hours following incubation in 300 μM $CdSO_4$.

DISCUSSION

Cd inhibited photosynthesis in *D. hirsuta* more that in *A. androgynum*. Several authors have shown that the substitutions of the central atom of chlorophyll, magnesium, by heavy metals (mercury, copper, cadmium, nickel, zinc and lead) *in vivo* is an important damage mechanism in stressed plants (Gross *et al.*, 1970; Küpper *et al.*, 1996 Puckett, 1976;). These workers noticed *in vitro* that heavy metals chlorophylls (hms-chls) have remarkably lower fluorescence quantum yields in comparison with Mg-chl or do not exhibit fluorescence at all. Hms-chls also exhibit fluorescence at different wavelengths than Mg-chl. Because the excitation states which are transferred from the antenna pigments to the reaction centres in the chloroplasts are the same ones responsible for fluorescence (Karukstis in Küpper *et al.*, 1996) hms-chls are not adapted to light harvesting. Wanatabe *et al.* (1985) also found that Mg-chla has the highest ability of electron releasing from the singlet excited state, compared to other hms-chls. This ability makes only Mg-chl suitable for effective electron transfer in

Table 3.2. The toxicity of Cd to *Atrichum androgynum*. Plants were incubated in $CdSO_4$ solutions for 1 h and Cd uptake, intracellular K concentration and gross photosynthetic rate measured immediately or after storage for 24 h. Figures in each column followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

Cd Concentration (μM)	Time of measurement after exposure to Cd (h)	Intracellular Cd uptake (μmoles g^{-1})	Extracellular Cd uptake (μmoles g^{-1})	Intracellular K after uptake and sequential elution (μmoles g^{-1})	Gross photosynthetic rate (μmoles CO_2 g^{-1} min^{-1})
0	0	0	0	410 a	1.68 a
0	24	0	0	335 a	1.74 a
50	0	2.11 a	22.75 a	339 a	1.65 a
50	24	3.73 a	20.22 a	331 a	1.76 a
300	0	7.27 b	51.80 c	336 a	1.61 a
300	24	8.88 b	33.01 b	328 a	1.69 a

Table 3.3. The toxicity of Cd to *Atrichum androgynum*. Plants were incubated in $CdSO_4$ solutions for 1 h and Cd uptake, intracellular K concentration and gross photosynthetic rate measured after storage for 48 h and 96 h. Figures in each column followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

Cd Concentration (μM)	Time of measurement after exposure to Cd (h)	Intracellular Cd uptake (μmoles g^{-1})	Extracellular Cd uptake (μmoles g^{-1})	Intracellular K after uptake and sequential elution (μmoles g^{-1})	Gross photosynthetic rate (μmoles CO_2 g^{-1} min^{-1})
0	0	0	0	450 a	1.60 b
0	48	0	0	442 a	1.58 b
0	96	0	0	463 a	1.57 b
300	0	7.12 a	43.91 b	435 a	1.60 b
300	48	9.46 a	30.23 b	417 a	0.33 a
300	96	14.51 b	22.07 a	423 a	0.21 a

reaction centres.

The prevention of photosynthetic light-harvesting in the affected antenna pigments and inhibition of electron transfer in the reaction centres due to incorporation of various divalent heavy metals in the chlorophyll of plants, results in the inhibition of photosynthesis (Küpper *et al.*, 1996). This mechanism has a rapid and severe impact on the photosynthetic light reaction, whereas other parts of metabolism (e.g. respiration) are affected much later. Küpper *et al.* (1996) argue that the proportional relationship between toxicity and complex formation rate that damage to plants by heavy metal ions, with respect to the reactions, does not depend on thermodynamic stability of the complexes formed, but on the tendency to form chlorophyll complexes.

Because *D. hirsuta* accumulated more intracellular Cd than *A. androgynum*, the chances of substituting Mg as central atom of chlorophyll by Cd were higher and this may have resulted in higher number of Cd-chls than Mg-chls. This in turn reduced photosynthesis more in the liverwort than in the moss. Although naturally *D. hirsuta* has higher photosynthetic rates than *A. androgynum* on the dry weight basis, gross photosynthesis was more significantly reduced in the liverwort than in the moss.

Wells & Brown (1987) working on two populations of the moss *Rhytidiadelphus squarrosus* (L&S) showed that CO_2 uptake was

reduced with increasing concentrations of Cd supplied and this effect was more evident 24 hours after incubation than immediately after incubation. The results indicated that population L was more sensitive to Cd than population S. Wells & Brown (1987) also showed that, at Cd concentrations of less that 1000 μM, population L accumulated greater quantities of Cd during a 30 minutes incubation. Furthermore, during 24 hour storage of shoot apices, movement of extracellular Cd to an intracellular location occurred, and this was relatively greater in population L. The greater sensitivity of population L could therefore be related to its greater intracellular Cd uptake. Similar considerations probably apply to the present study. Although the liverwort and the moss differ greatly in morphology, higher reductions of photosynthesis in *D. hirsuta* can be attributed to higher intracellular uptake of Cd.

Brown and Slingsby (1972) concluded that a proportion of the total potassium of lichen thallus is bound to the cell wall while the remaining fractions are present inside the cell either as freely diffusible ions which *in vivo* are retained by permeability barriers or as firmly bound ions in an insoluble form. Brown & Slingsby (1972) showed that low concentrations of nickel induced only slight losses of potassium from the lichen *Cladonia rangiformis* but there was an abrupt increase in potassium loss at higher concentrations. Puckett (1976) also observed a similar pattern of potassium loss by the lichen *Umbilicaria muhlenbergii* induced by nickel, as well as by

cobalt, cadmium and lead. In the present study, loss of intracellular potassium in *D. hirsuta* was observed and this was more evident when intracellular K was measured 24 hours than immediately after treatment with $CdSO_4$. Puckett (1976) argued that potassium loss measured at low metal-ion concentrations is probably a result of displacement by ion exchange from anionic groups on the thallus surface. The subsequent abrupt increase in the K loss at higher metal concentrations may be due to the detrimental changes in membrane permeability. However, no significant loss of intracellular K was observed in *A. androgynum*.

CONCLUSION

It was interesting that Cd moved from the cell wall to the cell interior much faster in *D. hirsuta* than in *A. androgynum*. This observation was not followed up in the present investigation but reasons for the differences need further investigations. Preliminary experiments thus showed that the effect of Cd on photosynthesis and intracellular K concentrations in both *D. hirsuta* and *A. androgynum* were not significant when measured immediately after treatment. From these results it was concluded that concentrations of Cd used were not toxic during the 1 hour the uptake experiments were carried on.

CHAPTER FOUR

FACTORS AFFECTING Cd UPTAKE IN THE LIVERWORT *Dumortiera hirsuta*.

INTRODUCTION

Acquisition of elements by bryophytes has received increased attention in recent years because of improved analytical techniques and the relevance of the work in pollution monitoring (Brown, 1984). Although terrestrial bryophytes acquire elements through both dry and wet deposits and from the substratum, the exact proportions originating from each source are unknown (see Chapter 1 for a detailed discussion of this subject). Many workers argue that because bryophytes lack well-developed cuticles and elaborate rooting system, most mineral uptake is from the atmosphere and not the substratum (Brown, 1984). In plants the uptake of metals supplied in an ionic or weakly complexed form occur to two distinct cellular locations, namely on the wall and inside the cell. Uptake of metals on to the cell wall is rapid, passive and exchangeable, while uptake into the cell is considerably slower and, appears to be dependent upon metabolism.

While some laboratory investigations of heavy metal uptake by bryophytes measured only total uptake, Brown and his co-workers (e.g. Brown & Beckett, 1985; Wells & Brown, 1987; Wells &

Brown, 1990; Brown & Sidhu, 1992; Wells *et al.* 1995) measured the intracellular uptake of Cd by the grassland moss *Rhytidiadelphus squarrosus*. Although it may be numerically smaller than extracellular uptake, intracellular uptake is probably of major importance for plant survival. Elements located within the cell potentially have an immediate impact on metabolism, and may be of more biological importance to the bryophyte than elements held on external sites.

In the present study, intra- and extracellular uptake of the metal Cd were investigated in the liverwort *Dumortiera hirsuta*. Determination of intracellular uptake requires prior removal of cell wall-bound cations using a displacing agent. In these set of experiments, a modification of the method of Brown & Buck (1979) was employed using 20 mM $NiCl_2$ to displace extracellular cations in *D. hirsuta*. The method has been discussed in Chapter two.

In these experiments Cd uptake was measured. While Cd is not an essential trace element in plants, it has been found that Zn-tolerant plants are also Cd-tolerant (Beckett & Brown, 1983) and Zn competitively inhibits Cd uptake. This suggests that Cd and Zn are taken up by the same transporting system. Thus measuring Cd uptake provides a model system for studying the uptake of other divalent cations. Secondly, Cd concentrations are very low in untreated plants and hence Cd amounts taken up during experimental periods can be accurately measured.

Experiments were conducted to determine the kinetics of Cd uptake, competition for uptake by other cations and the roles of metabolism in controlling the rate of Cd uptake. The effects of K pretreatment and plant collection site on Cd uptake were also determined.

RESULTS AND DISCUSSION

The habitat, morphology and cross section of the liverwort *D. hirsuta* are shown in Plate 4.1. This thalloid liverwort is normally 3 to 7 centimetres long and has a diameter of 1 to 4 centimetres.

Cd uptake as a function of time

Using 20 mM $NiCl_2$, it was possible to differentiate between exchangeable cell wall-bound Cd (extracellular) and Cd remaining after displacement (intracellular). The rate of intracellular Cd uptake was linear with time (Figure 4.1a), suggesting that 20 mM $NiCl_2$ had effectively displaced extracellular Cd. The calculated rate of intracellular Cd uptake was 3.0 $\pm$ 0.3 μmoles g^{-1} h^{-1}, but it varied between experiments (1 to 10 μmoles g^{-1} h^{-1}). Rates were similar or higher to those reported by Beckett & Brown (1984a) for the lichen *Peltigera membranacea* (1.8 $\pm$ 0.2 μmoles g^{-1} h^{-1}) and lower or similar to those reported by Brown & Beckett (1985) for the moss *Rhytidiadelphus squarrosus* (11.9 $\pm$ 2.3 μmoles g^{-1} h^{-1}). In

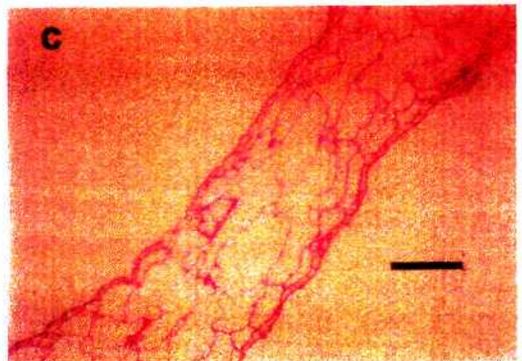


Plate 4.1. The habitat (A), morphology (B) and a vertical section through the thallus (C) of *Dumortiera hirsuta* from the Ferncliffe Nature Reserve, Pietermaritzburg. In C the bar line = 100 µm.

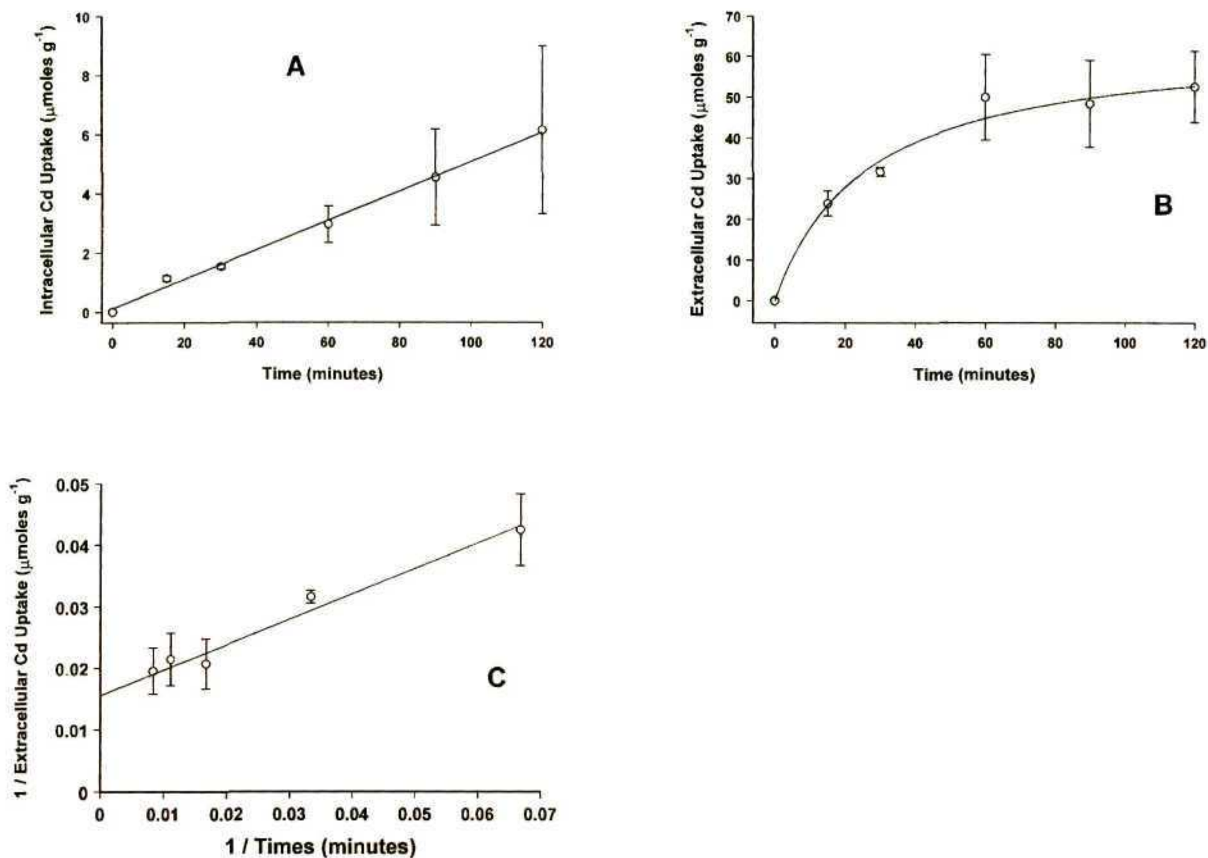


Figure 4.1. Intra- (A) and extracellular (B) Cd uptake from 100 μM $CdSO_4$ as a function of time, and double- reciprocal plot (C) of extracellular uptake in *D. hirsuta*. In this and all of following graphs error bars represent 95% confidence limits.

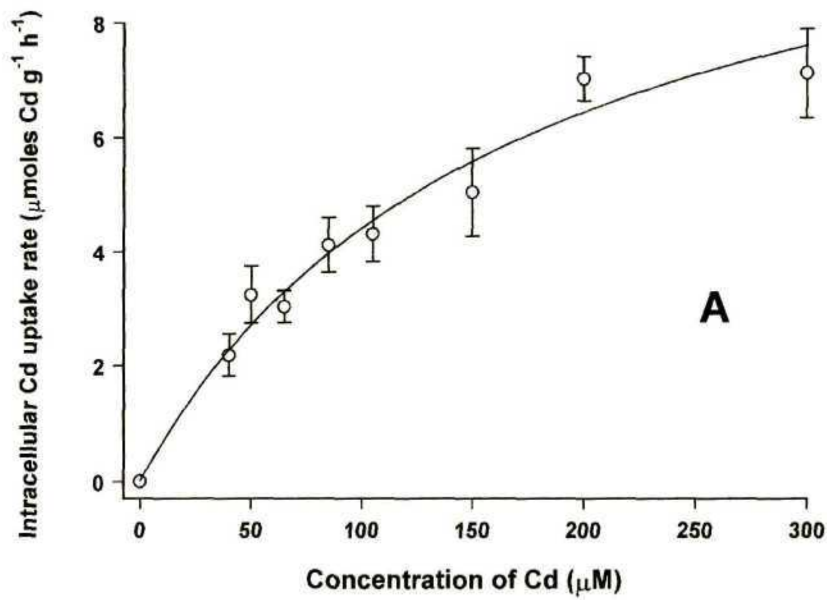
these experiments, uptake rates are expressed based on the weight of the plant material following incubation in 1 M HNO_3. This removed the protoplasm and reduced pre-acid dry weight in all collections by about 20%. This factor should be considered when uptake rates reported here are compared with results of Brown and his co-workers who usually calculated them using dry weights measured before acid treatment.

The rate of extracellular Cd uptake declined with time (Figure 4.1b). Using a double-reciprocal transformation, a straight line could be fitted to extracellular Cd uptake as function of time (figure 4.1c). The maximum extracellular Cd uptake was 64 $\pm$ 6 μmoles g^{-1} and the time required to reach half the maximum uptake was 26 $\pm$ 6 minutes. This was less than 39 minutes reported by Beckett & Brown (1984a) for the lichen *P. membranacea*, but much more than the 4 minutes reported by Brown & Beckett (1985) for the moss *Rhytidiadelphus squarrosus*. Brown & Beckett (1985) concluded that the difference between the lichen and the moss was due to the multi-layered structure of the lichen. They argued that the structure of the lichen may have increased the size of unstirred boundary layer through which metal ions must diffuse before being bound. The results of the present study possibly show that the liverwort *D. hirsuta*, which is on average eight cells thick, is intermediate in this regard between the moss *R. squarrosus* and the lichen *P. membranacea*.

After 2 hours extra- exceeded intracellular Cd uptake by close to 10 fold. This ratio was similar to that observed by Beckett & Brown (1985a) in the lichen genus *Peltigera*. Beckett & Brown (1985a) argued that the high ratio observed was due to higher cation exchange capacity of bryophytes and lichens as compared to higher plants. As for intracellular uptake, variation existed in extracellular uptakes between collections. Wells & Brown (1987) also observed similar, but less extreme variations in intra- and extracellular Cd uptakes between different populations of the moss *R. squarrosus*.

Cd uptake as a function of concentration

Intracellular Cd uptake displayed typical Michaelis-Menten kinetics when measured over a range of Cd concentrations (Figure 4.2a) and the data fitted a straight line using the double reciprocal plot (Figure 4.2b). The calculated K_m was 149 $\pm$ 19 μM and V_{max} 11.0 $\pm$ 0.8 μmoles $g^{-1} h^{-1}$. K_m was high compared with 25 μM reported by Brown & Beckett (1985) and Wells *et al.* (1995) for the moss *R. squarrosus* and 65 to 100 μM reported by Beckett & Brown (1984a) and Wells *et al.* (1995) for the lichen genus *Peltigera*. This suggests that the transporting system of *D. hirsuta* had a low affinity of Cd. The V_{max} was similar to that of the moss (10 to 15 μmoles $g^{-1} h^{-1}$) and higher than that of the lichen (2 to 4 μmoles $g^{-1} h^{-1}$). Extracellular Cd uptake also followed saturation kinetics. However, kinetic constants



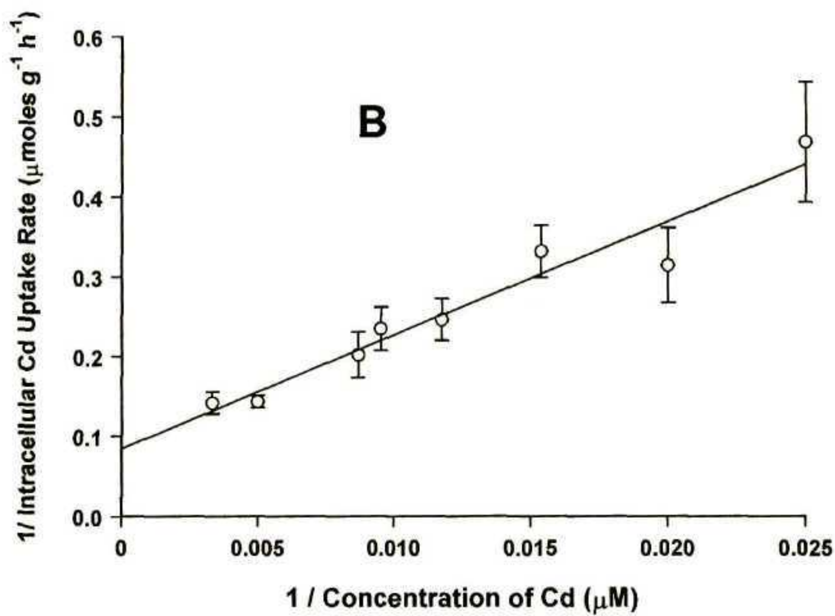


Figure 4.2. Intracellular (A) uptake rate as a function of concentration, and double-reciprocal plot (B) of intracellular Cd rate in *D. hirsuta*.

could not be calculated because uptake was not constant throughout the experimental period.

The effect of light on uptake

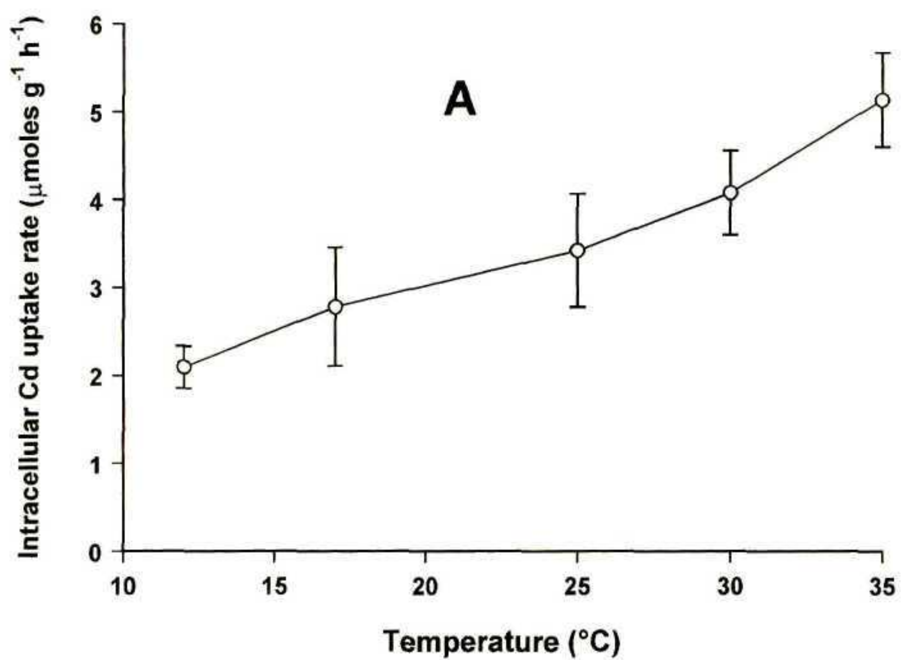
Light stimulated intracellular uptake by about 15% (Table 4.1). By contrast, Brown & Beckett (1985) reported little effect on intracellular Cd uptake in *R. squarrosus*. It is interesting that workers have often reported that light stimulates the membrane potential and enhances K uptake in other thalloid liverworts (see Brown, 1984 for review). Presumably, light stimulates proton pumping, creating a more favourable electrochemical gradient for entry of Cd into the cell. Surprisingly, storing plants in the dark for 20 days had little effect on intracellular uptake (Table 4.1). The maintenance of similar uptake rates even following long periods of dark storage indicates that either uptake not is active, or, more likely, intracellular transport receives high priority in the energy budget of the plant. Dark storage slightly increased extracellular Cd uptake.

The effect of temperature on Cd uptake

Intracellular Cd uptake increased with increasing temperature (Figure 4.3a), suggesting that uptake in at least in part an active process. Brown & Beckett (1985) obtained similar results for Cd uptake by the moss *R. squarrosus*. In *D. hirsuta*,

Table 4.1. Effect of light and dark storage for 20 days on intra- and extracellular Cd uptake from 100 μM $CdSO_4$ in ***Dumortiera hirsuta*** in the light and the dark. Within intra- and extracellular Cd uptakes, figures followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

	Treatment conditions	Storage conditions	
		Light	Dark
Intracellular Cd uptake rate (μmoles g^{-1} h^{-1})	light	1.23 b	1.28 b
	dark	1.01 ab	0.92 a
Extracellular Cd uptake after 1 h (μmoles g^{-1})	light	8.93 a	10.48 b
	dark	8.53 a	9.10 a



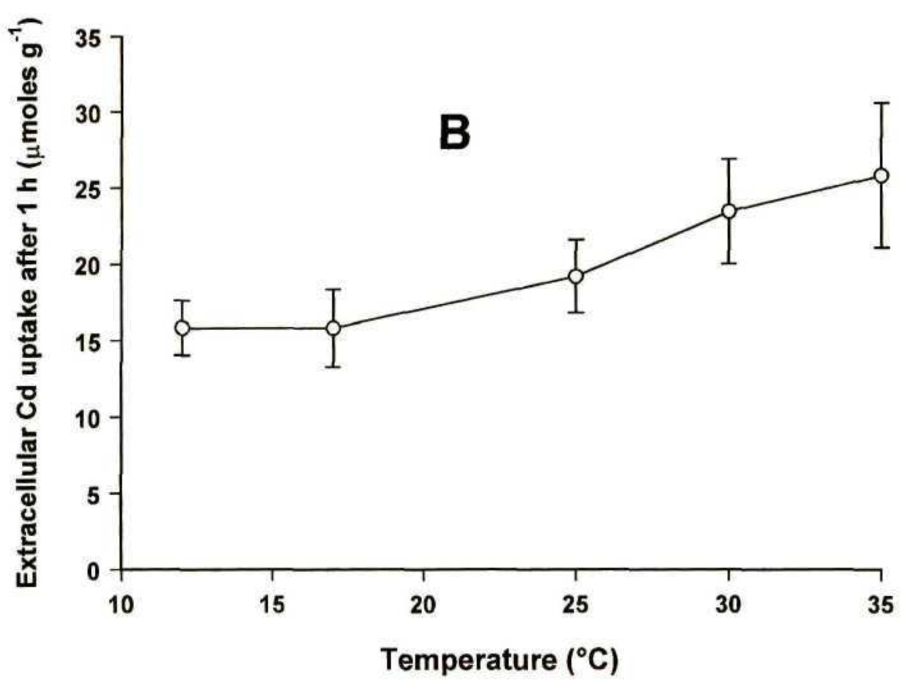


Figure 4.3. Intracellular uptake rate (A) and extracellular Cd uptake after 1 h (B) from 100 μM $CdSO_4$ as a function of temperature in *D. hirsuta*.

extracellular Cd uptake also increased with increasing temperature (Figure 4.3b), although the ratio of extra- to intracellular uptake declined from 7.5:1 at 12 °C to 5:1 at 35 °C. Again, the moss *R. squarrosus* gave similar results, and in this species, extracellular Cd increased more with increasing temperature when uptake was measured after 0.5 h than after 1 h. From this, Brown & Beckett (1985) concluded that increased extracellular Cd uptake probably represented a change in the rate of equilibration between cell wall exchange sites and the metal in solution, rather than a temperature-induced alteration in the total Cd uptake capacity. Similar considerations may also apply for the results obtained in the present study.

The dependence of Cd uptake on plant age

Intracellular Cd uptake was highest in disks cut 0 mm from the apex (Table 4.2), and declined by 28% and 35% in disks cut 10 mm and 20 mm from the apex respectively. Presumably, the growing apical regions were metabolically more active than the older parts of the thallus. Brown & Brūmelis (1996) working on the moss *Hylocomium* showed that the cellular location of elements can be considerably affected by the age of tissue and its response to desiccation stress. Brown & Brūmelis (1996) interpreted these age-related differences to differences in the quality of cell wall material, changes in the metabolic behaviour and cation requirements of the cell cytoplasm during maturation. Extracellular Cd uptake declined by 11% and 12% in

Table 4.2. Effect of thallus portion sampled on intra- and extracellular Cd uptake from 100 μM $CdSO_4$ in *Dumortiera hirsuta*. Within intra- and extracellular Cd uptakes, figures followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

	Distance from the apex (mm)		
	0	10	20
Intracellular Cd uptake rate (μmoles g^{-1} h^{-1})	1.63 b	1.14 b	0.91 a
Extracellular Cd uptake after 1 h (μmoles g^{-1})	33.70 a	29.78 a	29.64 a

disks cut 10 mm and 20 mm from the apex respectively, but the differences were not significant. When carrying out uptake experiments, replicates were prepared from randomly sampled disks from all parts of the thallus. Clearly, this contributed to the variation of the data, but limited availability of material precluded the sampling of disks from selective parts of the thallus.

The effect of competing ions on Cd uptake

In this experiment, the concentration of Cd used (300 μM) was chosen to be high enough for the liverwort to display near maximal intracellular uptake rates when supplied with Cd only (see Figure 4.2a). This allowed clear observation of the effect on uptake of competing cations, supplied at an equimolar concentration. Mg was added as sulphate, and Ca and Zn were added as nitrates. Mg could not be added as nitrate due to the high hygroscopic nature of the salt. Cd was added as the same salt as the competing ion. Supplying Cd as nitrate rather than sulphate gave higher rates of intracellular Cd uptake (Table 4.3). No obvious explanation exists for these results, but Brown & Beckett (1985) also noted that supplying *R. squarrosus* with Cd and different anions caused small differences in intracellular uptake.

Mg, Ca and Zn reduced intracellular Cd uptake to 80%, 62% and 36% respectively of the rate when Cd was supplied on its own

Table 4.3. Effect of Mg, Ca and Zn on intra- and extracellular Cd uptake in *Dumortiera hirsuta*. In the table below, - refers to Cd uptake when Cd was supplied on its own, and + when Cd was supplied with a competing ion. Within intra- and extracellular Cd uptakes, figures followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$)

		Intracellular Cd uptake rate (μmoles g^{-1} h^{-1})		Extracellular Cd uptake after 1 h (μmoles g^{-1})	
Cd Salt	Competing ion	-	+	-	+
300 μM $CdSO_4$	300 μM $MgSO_4$	16.82 c	13.43 b	27.93 c	17.27 b
300 μM $Cd(NO_3)_2$	300 μM $Ca(NO_3)_2$	22.25 d	13.72 b	29.04 c	17.49 b
300 μM $Cd(NO_3)_2$	300 μM $Zn(NO_3)_2$	22.25 d	8.03 a	29.04 c	13.57 a

(Table 4.3). This suggests that Cd transporting systems had low specificity. Wells & Brown (1990) obtained similar results for *R. squarrosus*, although in the lichen *Peltigera*, Mg was more effective at reducing intracellular Cd uptake (Beckett & Brown, 1984b; Brown & Avalos, 1993). All competing cations reduced extracellular Cd uptake, and again Zn was the strongest competitor.

The effect of K pretreatment on Cd uptake

Wells & Brown (1990) and Wells *et al.* (1995) showed that pretreating lichens and bryophytes with K can increase the subsequent rate of intracellular Cd uptake. During an experiment, Cd taken up onto the cell wall will displace existing divalent cations such as Ca and Mg into the uptake solution, where they may compete with Cd for intracellular uptake and therefore reduce uptake rates. Wells & Brown (1990) suggested that K pretreatment increases intracellular Cd uptake by removing existing divalent cations, thus preventing this competition from occurring.

In the present experiment, pretreating *D. hirsuta* with 80 mM KNO_3 significantly increased Cd uptake (Table 4.4). As outlined in Material and Methods, measuring the amount of Ca and Mg in the nickel fraction (extracellular) enabled estimation of the additional Ca and Mg present in the uptake solution of the non-K pretreated plants. Addition of these amounts of Ca and Mg to

Table 4.4. The effect of K pretreatment and amendment of uptake solutions with Ca and Mg on intra- and extracellular Cd uptake from 100 μM $CdSO_4$ in *Dumortiera hirsuta*. Ca and Mg were supplied as nitrates at 3 μM and 0.6 μM respectively (see text for details). Within intra- and extracellular Cd uptakes, figures followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.05$).

Pretreatment	Competing ion	Intracellular Cd uptake rates (μmoles g^{-1} h^{-1})	Extracellular Cd uptake after 1 h (μmoles g^{-1})
H_2O	None	9.14 b	60.60 b
80 mM KNO_3	None	13.40 c	85.56 c
H_2O	Ca + Mg	7.29 a	45.22 a
80 mM KNO_3	Ca + Mg	8.33 ab	55.95 b

the uptake solutions of K pretreated plants reduced the intracellular Cd uptake rate to that of water pretreated plants (Table 4.4). This suggests that K pretreatment does indeed stimulate intracellular Cd uptake by removing cell wall-bound divalent ions that Cd may subsequently displace during uptake experiments. These divalent ions can obviously then compete for intracellular uptake. The results of this experiment support the contention of Wells & Brown (1990) that workers should be careful when comparing bryophytes from different localities, as uptake rates may vary because of differences in ions bound to the cell wall rather than inherent differences in transporting systems.

The effect of plant collection site on Cd uptake

The aim of the present experiment was to investigate if plants from different sites had different extracellular cation contents which might affect the uptake of Cd. This was done because variation in uptake rates between individual collections was noted from previous experiments. The site where plants were collected is a possible factor that could cause variation in uptake rates. The site of collection might affect the uptake rates in two ways (a) the accumulation of the cations on the cell wall might be due to the amount and availability from the soil of the site and (b) the sites may have different water regimes which in turn might affect the water content of cells, the development of cuticles if water is limiting, cell size or the number of cells in a section of the

thallus.

Three sites were chosen: a very wet site at the top of the waterfall, a slightly dry sites further down stream, and a much dryer site by the path next to the stream. Plants were collected from these sites and Cd uptake measured as described under Materials and Methods. The results of this experiment are summarised in Table 4.5. The plant material collected from the top of a stream had the highest V_{max}. This material had the lowest dry weight per unit surface area and the highest water and cytoplasm content. In all collections, Ca concentrations were highest in the nickel fraction (extracellular) and lowest intracellularly. The opposite was observed for K and Mg. The plant material from top of a stream had the highest concentration of extracellular Ca but the material collected from the path and bottom of a stream had similar concentrations of extracellular Ca. Extracellular K and Mg were not significantly different between the three collections. Clearly, the suggestion of Wells & Brown (1990) for variation in uptake rates between different populations being due to the presence of divalent cations on cell wall can not apply here.

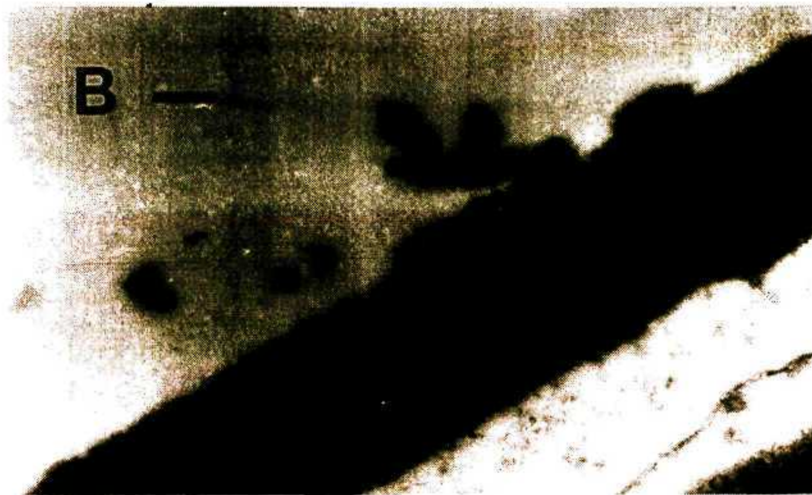
Intracellular concentrations of Ca, K and Mg were slightly higher in the bottom of stream collection but otherwise there were no significant differences between the three plant collections. However, the potassium concentration of the cytoplasm of a fully hydrated thallus from the top collection (97 µM) was significantly lower than the concentration of

Table 4.5. V_{max} and K_m for intracellular Cd uptake, weight, extra- and intracellular cation content, cuticle thickness and thallus thickness of *Dumortiera hirsuta* collected from 3 sites. 5 replicates of 5 discs were used. Plants were incubated in a range of $CdSO_4$ concentrations at 20 $\pm$ 1 °C. Figures in a column given $\pm$ 1 standard deviation. Figures in a row followed by the same letter are not significantly different using Duncan's multiple range test ($P < 0.5$).

		Path	Bottom of a stream	Top of a stream
V_{max} (μmoles g^{-1} h^{-1})		11 $\pm$ 3	18 $\pm$ 1	35 $\pm$ 2
K_m (μM)		96 $\pm$ 11	25 $\pm$ 5	62 $\pm$ 9
Weight (mg)		13 $\pm$ 8	12 $\pm$ 1	9 $\pm$ 1
Water content (g H_2O g^{-1} dry weight)		8a	16b	17b
Cytoplasm (%)		68a	69a	73a
Intracellular cation content (μmoles g^{-1})	Ca	22 $\pm$ 5	36 $\pm$ 3	24 $\pm$ 2
	K	2300 $\pm$ 20	2700 $\pm$ 30	2100 $\pm$ 40
	Mg	1900 $\pm$ 30	2200 $\pm$ 10	1700 $\pm$ 20
Extracellular cation content (μmoles g^{-1})	Ca	1700 $\pm$ 56	1700 $\pm$ 30	3000 $\pm$ 40
	K	32 $\pm$ 3	38 $\pm$ 1	30 $\pm$ 2
	Mg	7 $\pm$ 2	19 $\pm$ 1	20 $\pm$ 2
Protoplasm K concentration at full hydration (μM)		219c	139b	97a
Outer cell wall thickness (μm)		19b	13a	12a
Thallus thickness (μm)		60c	48b	41a

potassium in the bottom and path collections (139 and 219 μM respectively). This probably suggests that the top population was slightly nutrient stressed. The plants may have been expending more energy to create a more negative membrane potential to enhance nutrient uptake. Possibly this explains the much higher rate of intracellular Cd uptake found in this collection.

The plants from top of the stream had a thin cuticle layer and this was not significantly different from that of plants from the bottom of the stream (Plate 4.2). Plants collected along the path had a significantly thicker cuticle layer compared to the other two collections. Cuticle thickness might slow down the diffusion of Cd into the cell and thus reducing the rate of intracellular Cd uptake. However, although cuticle thickness in bottom and the top of the stream plants was not significantly different, V_{max} in plants from the top of the stream was almost twice that of plants collected from the bottom of the stream. V_{max} in plants collected from bottom of the stream was slightly higher than that of plants from along the path but the cuticle thickness between the two populations was significantly different. These results indicate that V_{max} was not only affected by cuticle thickness even though the thickness can affect the rate of diffusion into the cell. V_{max} was also affected by thickness of the thallus (Table 4.5). The thallus probably formed a layer through which Cd must diffuse to reach the inner cells.



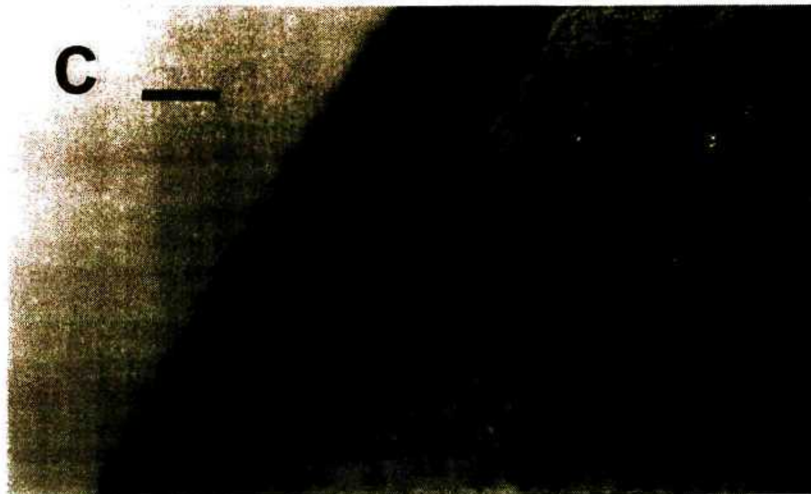


Plate 4.2. The outer cell wall of *D. hirsuta*. The plant material was collected from along a path (A), the bottom (B) and the top (C) of a stream. In this plate, the bar lines = 0.25 μM.

CONCLUSION

The results presented in this section showed that intra- and extracellular uptake rates had different kinetics. The saturation of intracellular Cd uptake with increasing concentration indicated that the uptake is carrier mediated. The effect of temperature, light and plant age on intracellular Cd uptake showed that the uptake is, at least in part, dependent on metabolism and thus active. The ability of divalent cations (Ca, Mg, Zn) to inhibit intracellular Cd uptake indicated that the system transporting Cd was not specific. K pretreatment can increase intracellular Cd uptake by removing extracellular divalent cations but variation in uptake rates between collections is not only due to the presence and nature of extracellular divalent cations. Nutrient stress is possibly one of factors that can increase intracellular Cd uptake. Cuticle thickness can affect the rate at which Cd move into the cell. However, V_{max} was found not to be simply related to cuticle or thallus thickness. Presumably, other factors are also responsible for the observed variation.

CHAPTER FIVE

FACTORS AFFECTING THE Cd UPTAKE IN THE MOSS *Atrichum androgynum*

INTRODUCTION

The main objective of this study was to characterise the uptake of heavy metal by the liverwort *D. hirsuta*. Due to a limited availability of plant material, the moss *Atrichum androgynum* was chosen for some experiments. The moss was used because first, the material could be readily collected and second, it grows in habitats similar to those of *D. hirsuta*. Most of the work described in this Chapter in many respects complements Chapter 4. The same technique was used to distinguish between intra- and extracellular metal uptake. As explained before, uptake of the element Cd was examined. The effects of time, concentration, inhibitors, temperature and storage temperature were investigated.

RESULTS AND DISCUSSION

The habitat, habit and the morphology of the moss *A. androgynum* is presented in Plate 5.1. The metabolically active part of the moss is normally between 2 to 3 centimetres long.

Plate 5.1. The habitat (A), habit (B) and morphology (C) of *Atrichum androgynum* from the Doreen Clarke Nature Reserve, Pietermaritzburg.

Cd uptake as a function of time

The rate of intracellular Cd uptake was linear with time (Figure 5.1a). However, there was a positive Y-axis intercept when intracellular Cd uptake was plotted as a function of time. Brown & Beckett (1985) working on the moss *R. squarrosus* also found in a time course study of uptake from a 100 μM Cd solution that a positive intercept on the Cd uptake axis existed. Wells & Brown (1987) found that this Y-axis intercept increased with increasing Cd concentrations. Wells & Brown (1987) argued that 20 mM $NiCl_2$ did not adequately displace the cell wall bound Cd. This suggestion probably applies to the present results. Wells & Brown (1987) suggested that to reduce the intercept, a metal which has a more class B character than Ni (for example Pb), a greater volume of 20 mM $NiCl_2$ or a higher concentration of $NiCl_2$ can be employed. In this study, 100 mM $NiCl_2$ was used instead of 20 mM $NiCl_2$, and this reduced the Y-axis intercept considerably (Figure 5.1a). This concentration was used in all of the remaining experiments. The calculated rate of intracellular Cd uptake was 3.34 ± 0.1 μmoles g^{-1} h^{-1}. The rate was similar or lower than that of *D. hirsuta* (1 to 10 μmoles g^{-1} h^{-1}) and lower than that reported by Brown & Beckett (1985) for the moss *R. squarrosus* (11.9 ± 2.3 μmoles g^{-1} h^{-1}).

Extracellular Cd uptake saturated with time (Figure 5.1b). Extracellular Cd concentrations were higher when 100 mM $NiCl_2$

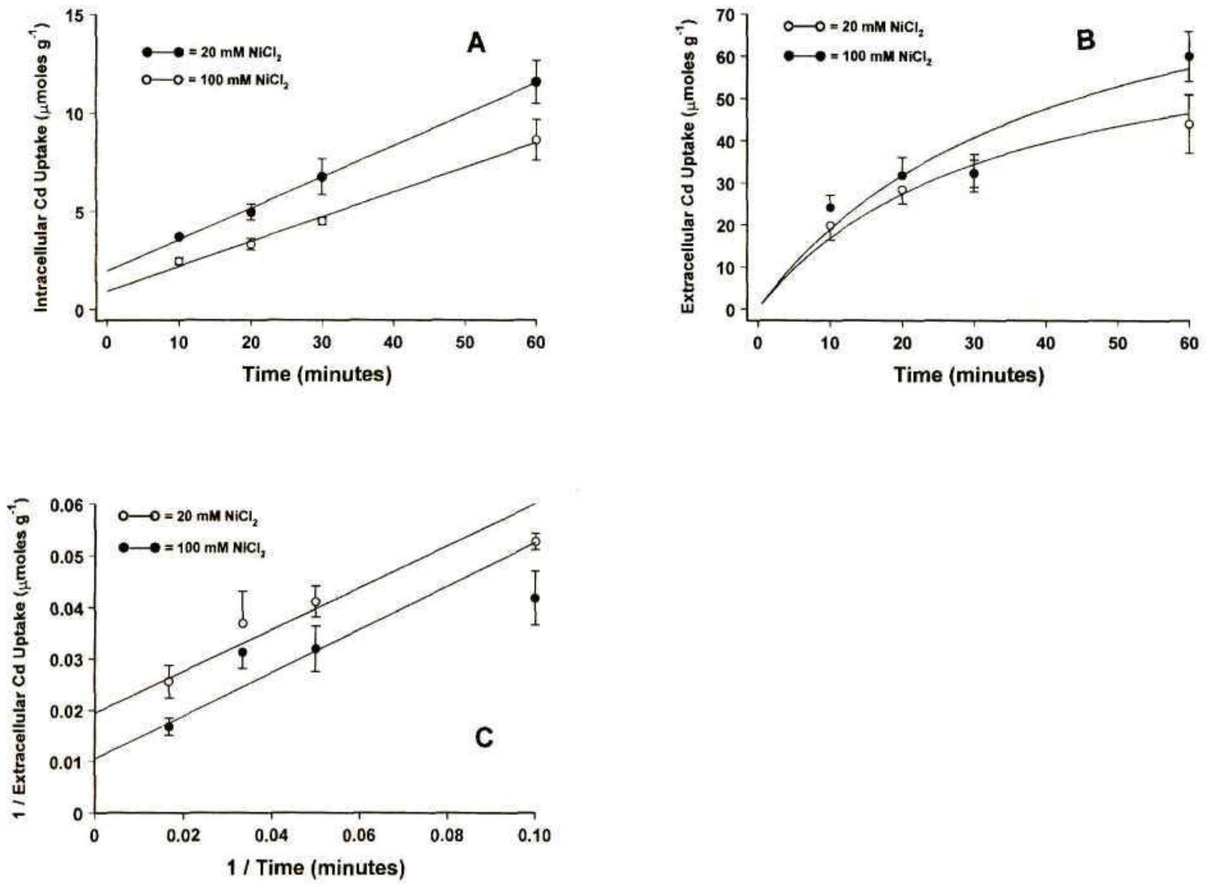


Figure 5.1. Intra- (A) and extracellular (B) Cd uptake from 100 μM $CdSO_4$ as a function of time, and double-reciprocal plot (C) of extracellular Cd uptake in *A. androgynum*.

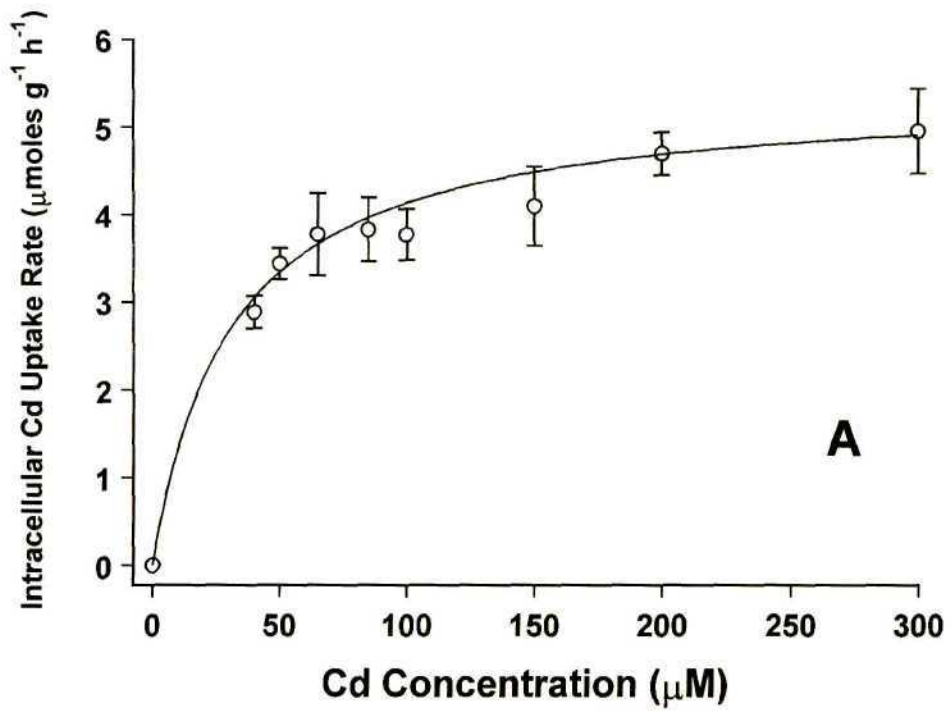
was used compared to 20 mM $NiCl_2$. This further suggested that 100 mM $NiCl_2$ was more effective in displacing cell wall bound Cd. Using a double-reciprocal transformation, a straight line could be fitted to extracellular Cd uptake as a function of time (Figure 5.1c). The calculated maximum extracellular uptake was 82.9 $\pm$ 6.9 μmoles g^{-1}, and the time required to reach half the maximum uptake was 21.6 $\pm$ 3.9 minutes.

Cd uptake as a function of concentration

When measured over a range of Cd concentrations, intracellular Cd uptake displayed typical Michaelis-Menten kinetics (Figure 5.2a). The data fitted a straight line when subjected to a double-reciprocal transformation (Figure 5.2b). The calculated K_m was 30.9 $\pm$ 4.4 μM and V_{max} was 5.3 $\pm$ 0.2 μmoles g^{-1} h^{1}. K_m and V_{max} were much lower than those of *D. hirsuta* (149 μM and 11 μmoles g^{-1} h^{-1} respectively). Extracellular uptake also followed saturation kinetics. Unfortunately, uptake was not constant throughout the experimental period and hence kinetic constants could not be calculated.

Cd uptake as a function of temperature

Both intra- and extracellular Cd uptakes increased with increasing temperature (Figure 5.3a, 5.3b). The effect of temperature on intracellular Cd uptake suggested that uptake is active. However, although in this study extracellular Cd uptake



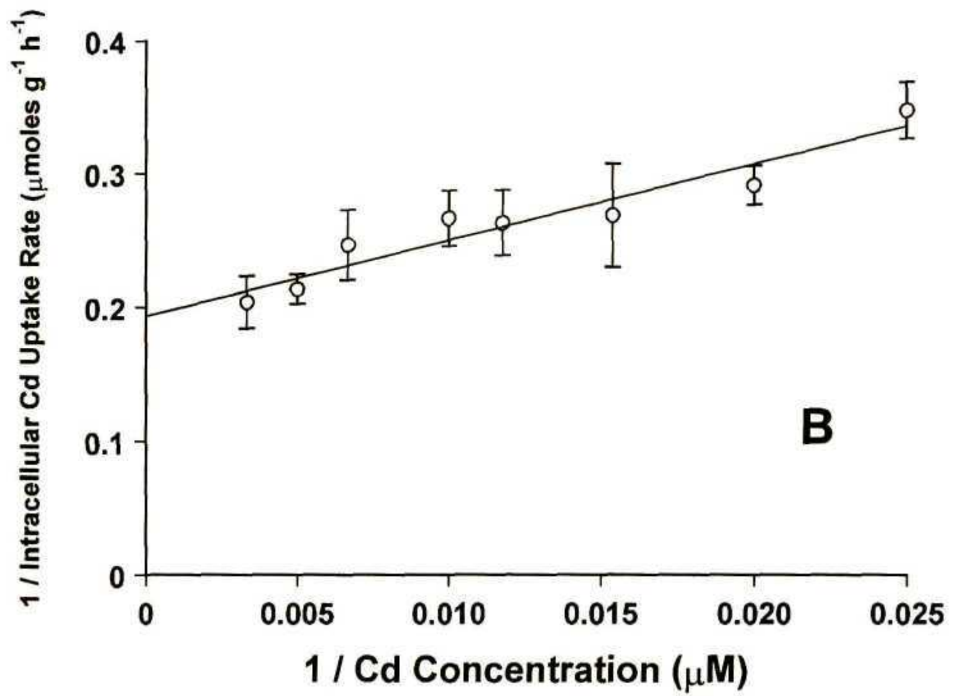
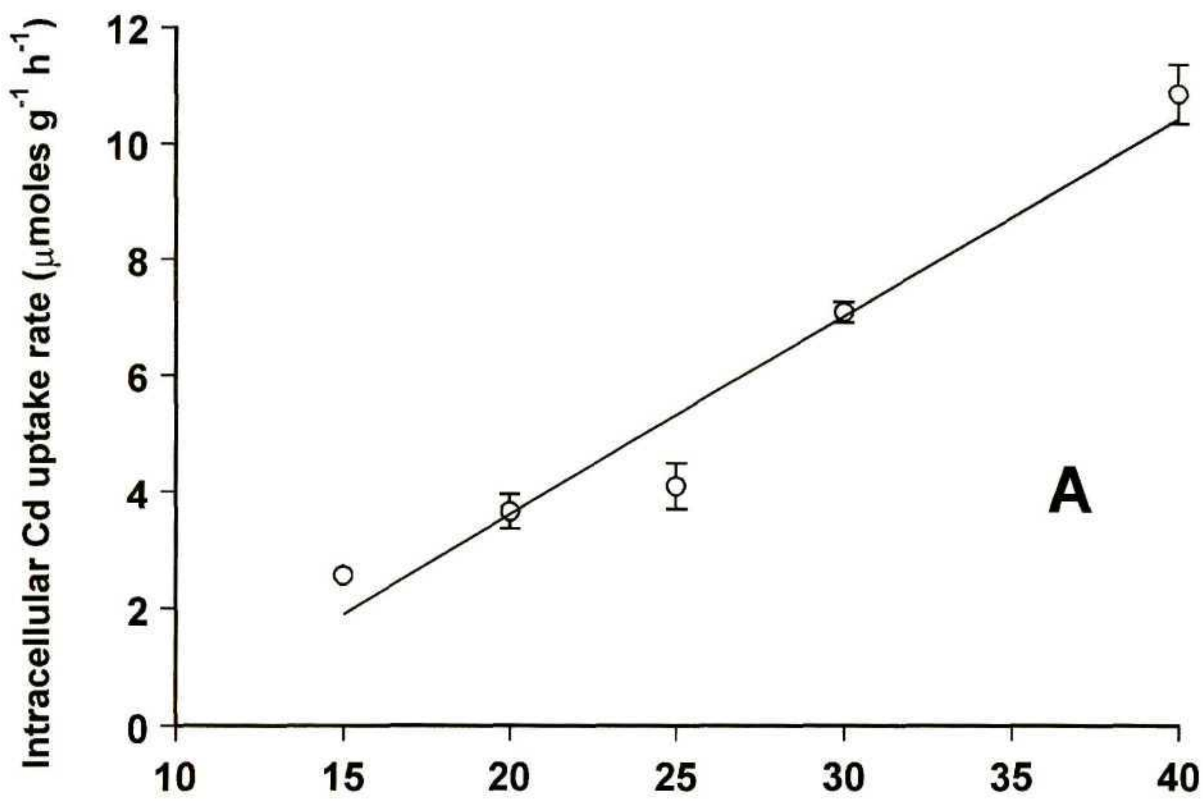


Figure 5.2. Intracellular (A) Cd uptake rate as a function of concentration, and double-reciprocal plot (B) of intracellular Cd rate in *A. androgynum*.



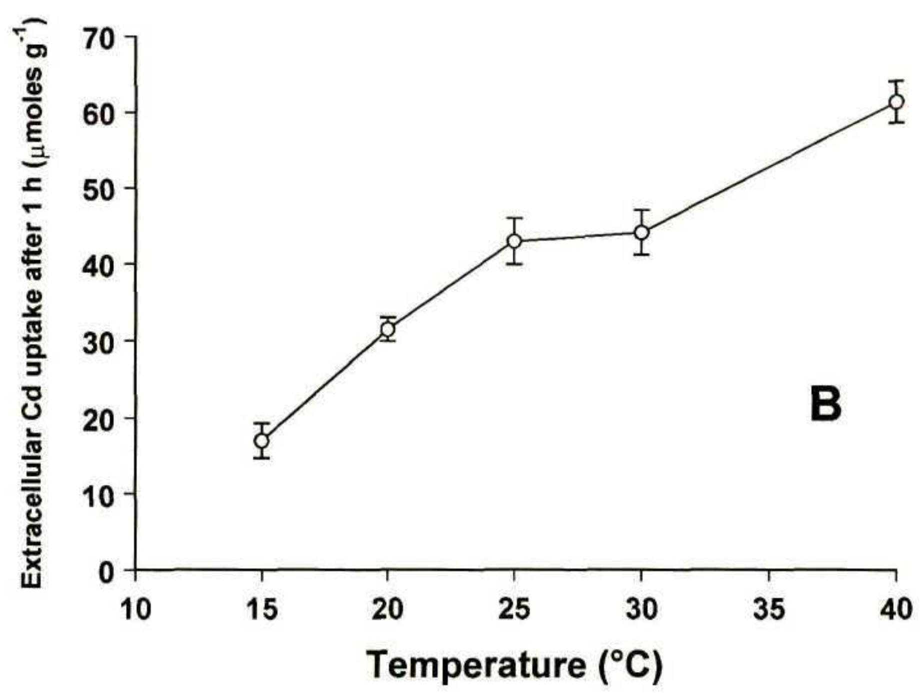


Figure 5.3. Intracellular uptake rate (A) and extracellular Cd uptake after 1 h (B) from 100 μM $CdSO_4$ as a function of temperature in *A. androgynum*.

also increased with temperature, it seems unlikely that uptake is also active. Diffusive processes can also be affected by temperature. If extracellular uptake was measured for periods greater than 1 hour, it is likely that the observed effect of temperature on extracellular Cd would be eliminated.

The effect of inhibitors on Cd uptake

Wells & Brown (1990) demonstrated the competitive inhibitory nature of calcium in the moss *R. squarrosus* on cadmium uptake. From this, Wells & Brown (1990) suggested that intracellular levels of cadmium might be controlled by the trans-membrane calcium transporter system. However, a number of inhibitors have reported to have a considerable influence on trans-membrane movement of calcium. Lanthanum has been reported to inhibit inwardly-directed channels and verapamil inhibits calcium influx into the cell (Clarkson & Lüttge, 1989; Tester, 1990). In contrast, vanadate decreases an energy-dependent efflux of calcium from the cytoplasm (Evans *et al.*, 1991). The aim of this experiment was to test if verapamil and ammonium vanadate affect the uptake of cadmium in the moss *A. androgynum*. Brown & Avalos (1993) used inhibitors to investigate whether calcium had a direct effect on intracellular cadmium uptake in the lichen genus *Peltigera*.

In the present study, cadmium was supplied at 100 μM and the inhibitors supplied at 0.5 mM concentrations. The

concentrations of inhibitors used were those found adequate by Brown & Avalos (1993). Experiments were done at different times and this caused slight variations in the uptake rates of untreated plants. Verapamil slightly reduced extracellular Cd uptake (11%) and reduced intracellular uptake by 22%. Ammonium vanadate reduced extracellular Cd uptake by 29% and increased intracellular Cd uptake by 21% (Table 5.1).

Brown & Avalos (1993) working on members of the lichen genus *Peltigera* noted that while verapamil did not affect the concentration of cadmium recovered from the cell wall, it depressed intracellular cadmium uptake. Brown & Avalos argued that the reduction in intracellular cadmium uptake observed at low concentrations of the calcium-influx inhibitor verapamil might indicate that, in lichens, calcium and cadmium are taken up by the same channels. The reductions of intracellular Cd concentrations in the presence of verapamil in the present study also suggests that Cd is transported by the same carriers as those transporting calcium in *A. androgynum*.

In agreement with results presented here, Brown & Avalos (1993) also observed that vanadate had a slight effect on extracellular cadmium uptake but intracellular cadmium uptake was enhanced. Evans *et al.*(1991) stated that the cytoplasmic concentration of soluble calcium is controlled by a variety of efflux systems from the cell interior to either the exterior, via the plasma-membrane, or various other membrane-bounded

Table 5.1. The effect of inhibitors on two plant collections of ***A. androgynum*** (see text for details). The material was incubated in 100 μM $CdSO_4$ without (-) or with (+) an inhibitor. Numbers followed by the same letter within one column are not significantly different from each other using Duncan's multiple range test ($P < 0.05$).

Inhibitor	Extracellular Cd after 1 h (μmoles g^{-1})	Intracellular Cd rate (μmoles g^{-1} h^{-1})	Intracellular K (μmoles g^{-1})
Verapamil			
(-)	**26.24 b**	**3.21 b**	**72.07 a**
(+)	**23.03 b**	**2.51 a**	**59.01 a**
Ammonium vanadate			
(-)	**27.13 b**	**2.22 a**	**76.32 a**
(+)	**19.12 a**	**2.79 a**	**58.32 a**

intracellular compartments (e.g to the vacuoles). Using this argument, Brown & Avalos (1993) suggested that increases in the intracellular concentration of calcium associated with vanadate treatment apparent in their study may be a result of this ATPase inhibitor decreasing an energy-dependent efflux of calcium from the cytoplasm. The parallel behaviour of cadmium in response to this inhibitor might imply a common out pumping mechanism to that of calcium. Possibly vanadate affects the efflux of cadmium the same way it affects the efflux of calcium from the cytoplasm. It is also possible that the failure of inhibitors to elicit identical responses from calcium and cadmium could be due to cadmium associating more strongly with intracellular acceptor sites than calcium, thereby reducing the efficiency of plasma-membrane transporting systems to decrease the cytoplasmic concentration (Brown & Avalos, 1993). Reductions of extracellular Cd uptake may be a result of competition from the ammonium ion (NH_4).

The effect of storage temperature on Cd uptake

The aim of this experiment was to investigate the effect of the temperature of the collection site of the uptake on Cd by the moss *A. androgynum*. Plants were collected and treated as described in Chapter 2. It was hoped that this experiment could explain the variations observed in uptake kinetics between individual collections.

For mosses stored at all temperatures, intracellular Cd uptake

increased with increasing temperature (Figure 5.4). The material stored at 10 °C accumulated more intracellular Cd at lower temperatures than the material stored at 20 °C. Chapin (1974) reported higher intracellular uptake of phosphate at low temperatures in cold-acclimated roots of higher plants. In addition, plants stored at 20 °C had higher uptake rates at 30 and 40 °C than plants stored at 10 °C. This could indicate that heavy metal uptake in *A. androgynum* can display temperature acclimation.

The material stored at 30 °C had the highest intracellular uptake rates at all temperatures. One possible explanation for these results is that an energy dependent Cd out pump operates in *A. androgynum*. Storing the material at 30 °C may have stimulated respiration, depleted respirable reserves and thus promoted uptake. Glime & Acton (1979) stated that the development of aquatic bryophytes and their geographic distribution are limited to cold and temperate water bodies. As the temperature of water increases, the respiration of mosses increases more rapidly than assimilation. As a consequence, high temperatures can slow down growth and influence the metal uptake rates of mosses.

To test the suggestion that storing material at 30 °C affected the energy budget of the plants, an inhibitor was used such that it would not affect the influx of Cd but would inhibit the efflux. The inhibitor used was ammonium vanadate. As discussed

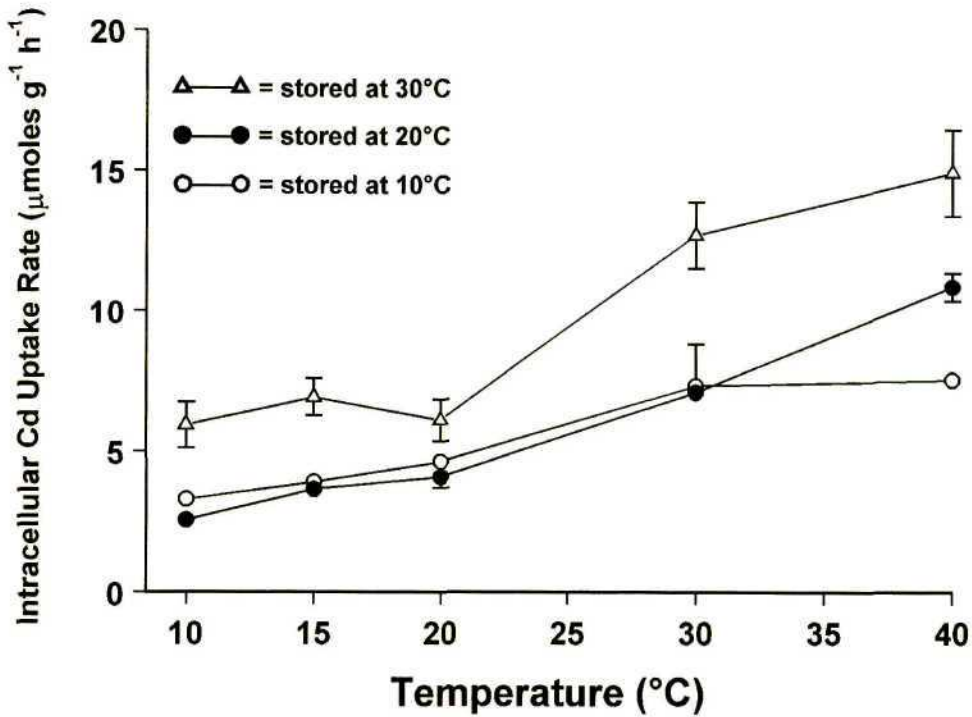


Figure 5.4. The effect of storage temperature on intracellular Cd uptake rate in *A. androgynum*. Plants were stored at 10, 20 and 30 °C under 75 μmoles m^{-2} s^{-1} for 21 days. Plants were incubated in 100 μM $CdSO_4$ over a range of temperatures.

before, this inhibitor increased intracellular uptake by 21% in material stored at 20 °C (Table 5.1). Gross photosynthesis and respiration of material were also determined.

Ammonium vanadate increased the intracellular Cd uptake by 27% in the material stored at 10 °C but had no effect on material stored at 30 °C (Table 5.2). Possibly vanadate, an ATPase inhibitor, reduced the energy-dependent efflux of cations from the cytoplasm. This inhibition of energy-dependent efflux could not occur in material stored at 30 °C because respirable reserves were already used up and hence energy was already limiting.

The material stored at 30 °C weighed 21% less and had 18% and 30% lower respiration and photosynthetic rates respectively than the material stored at 10 °C. This suggested that the machinery for photosynthesis was damaged and supports the idea that most of respirable reserves were used up. Claveri & Mouvet (1995) working on the moss *Rhynchostegium ripariodes* observed that storing material for 22 days decreased CO_2 assimilation rates and this followed the order : 29 °C < 17 °C < 7 °C. Claveri & Mouvet (1995) also observed denaturation of the chlorophyll pigments i.e. change in color from green to brown and loss of vitality in moss kept at 29 °C. Color change from green to brown was also observed in the present study in the moss kept at 30 °C.

Table 5.2. Weight, respiration, gross photosynthesis, intracellular Cd and intracellular K for the material of the moss ***Atrichum androgynum*** stored at 10 and 30 °C for 21 days. The material was incubated in 100 μM $CdSO_4$ without (-) and with (+) ammomium vanadate supplied at 0.5 mM concentration. Figures in each column followed by the same letter are not significantly different using Duncan's multiple range test (*P* < 0.05).

Storage temperature (°C)	Weight (mg)	Respiration (μmoles CO_2 min^{-1} g^{-1})	Photosynthesis (μmoles CO_2 g^{-1} min^{-1})	Intracellular Cd (μmoles g^{-1} h^{-1})		Intracellular K (μmoles g^{-1})	
10	33.2 b	43 a	300 b	(-)	2.53 a	(-)	94.82 a
				(+)	3.41 b	(+)	84.19 a
30	26.1 a	35 a	210 a	(-)	6.11 c	(-)	74.12 a
				(+)	6.87 c	(+)	68.66 a

CONCLUSION

Intracellular Cd uptake increased linearly with time and extracellular Cd uptake saturated after 30 minutes. This showed that while intracellular Cd uptake is an active process, the extracellular uptake is a passive process which involves binding to the cell wall. The dependence of intracellular uptake on temperature also showed that uptake is active. Although the extracellular Cd uptake also increased with increasing temperature, it could not be concluded that the uptake is active. If extracellular uptake was measured for periods greater than 1 hour the observed effect of temperature on extracellular Cd would probably have been eliminated.

Intracellular Cd uptake displayed saturation kinetics when measured over a range of Cd concentrations. The effect of inhibitors on Cd uptake, especially verapamil, suggests that entry of cadmium into plant cells occurs via the same channels as calcium. Ammonium vanadate, which have been reported to decrease the energy-dependent efflux of cadmium from the cytoplasm, increased intracellular Cd uptake. Material stored at 10 and 20 °C could display temperature acclimation when uptake was measured over a range of temperature. Material stored at 30 °C lost most of its respirable resources. This probably reduced the energy available to pump out intracellular Cd and thus promoted accumulation of intracellular Cd.

CHAPTER SIX

THE INDUCTION OF HEAVY METAL TOLERANCE IN THE MOSS *Atrichum androgynum*

INTRODUCTION

Plants have evolved several mechanisms to enable them to survive potentially toxic concentrations of heavy metals. They can either regulate the entry of heavy metals into the cytoplasm, exclude metals from the cytoplasm or sequester metals into harmless complex ions within the cytoplasm (see Chapter 1 for further discussion of the mechanisms of metal tolerance in plants). Results presented in Chapter 3 showed that, the inhibition of photosynthesis in both *D. hirsuta* and *A. androgynum* was directly proportional to intracellular Cd concentrations. The aim of the present experiment was to investigate if incubating plant material in low concentrations of Cd can increase tolerance to subsequent incubation in high concentrations. Beckett & Brown (1983) reported that zinc tolerance can be induced in initially non-tolerant lichens by pretreating them with low concentrations of metals. Plants were collected and treated as discussed in Chapter 2.

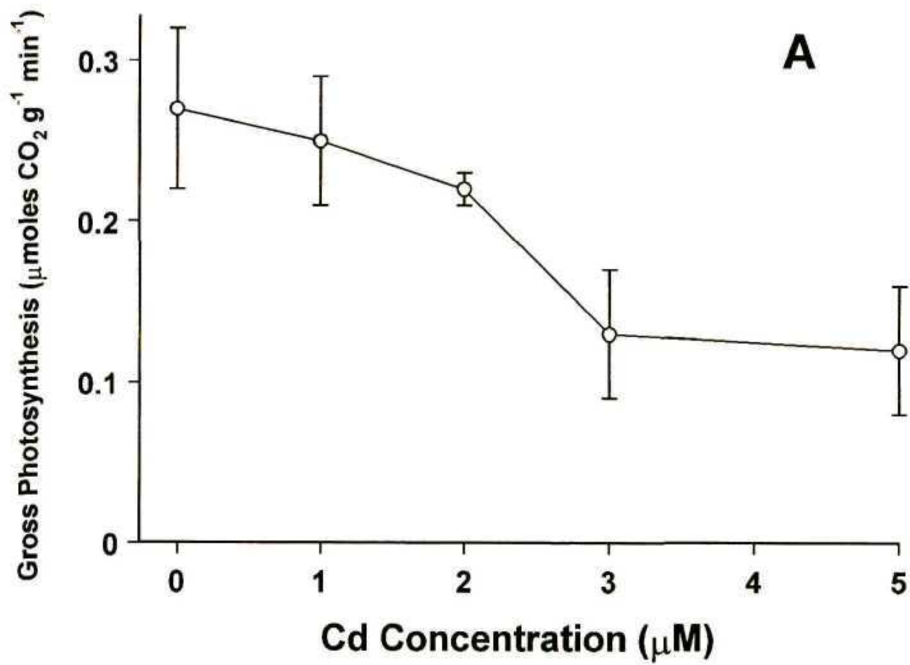
RESULTS AND DISCUSSION

After storage for 21 days plants grew by about 1 cm. Gross

photosynthesis was reduced to half in both non-treated and Cd-treated plants. There was no significant difference in gross photosynthesis between control plants and plants incubated in 1 and 2 μM $CdSO_4$ (Figure 6.1a). Gross photosynthesis in plants incubated in 3 and 5 μM $CdSO_4$ solutions was reduced by 51 and 55% respectively. Intracellular K concentrations in control plants and plants incubated in 1 μM $CdSO_4$ were similar, but 2 and 3 μM $CdSO_4$ reduced intracellular K by 11% (Figure 6.1b). Plants incubated in 5 μM Cd solution lost 29% of their intracellular K.

Plants incubated in Cd solutions showed symptoms of chlorophyll degradation (color change from green to yellow) except the tips (Plate 6.1). The color change was more severe in plants incubated in 3 and 5 μM Cd solutions than in plants incubated in 1 and 2 μM Cd solutions, and this was correlated with inhibition in gross photosynthesis. Because there were no significant differences in gross photosynthesis and intracellular K concentrations between control plants and plants incubated in 1 μM $CdSO_4$, this concentration was considered not toxic to the plants and was used in heavy metal tolerance induction experiment.

To test for heavy metal tolerance induction, plants were stressed by incubation in 2.5 μM Cd solutions following pretreatment and stored for another 7 days. Results of this



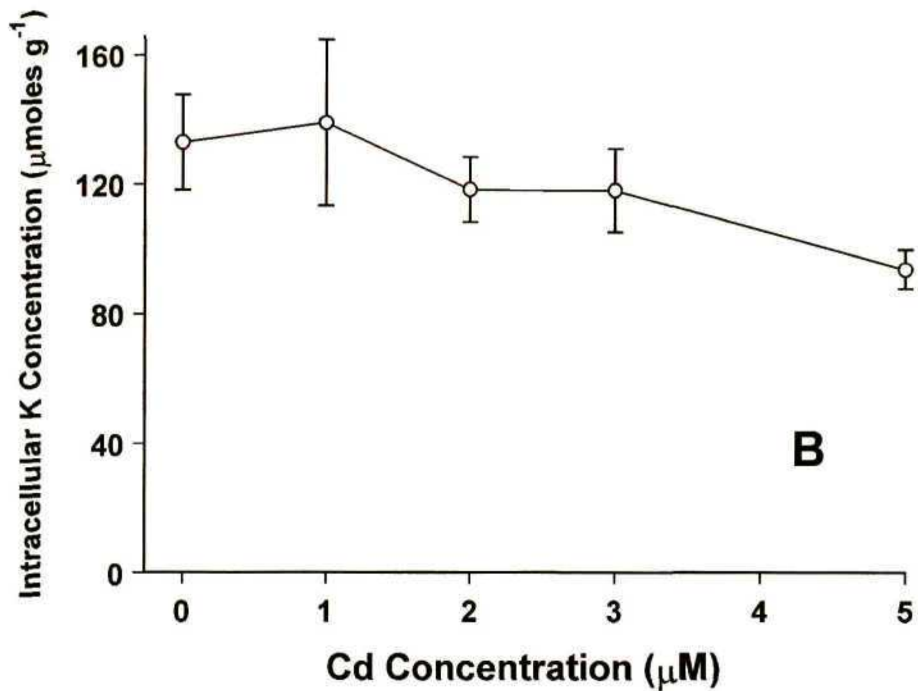


Figure 6.1. The effect of pretreatment with low concentrations of Cd on photosynthesis (A) and K loss (B) in *A. androgynum*. Plants were incubated in a range of concentrations and stored for 21 days.

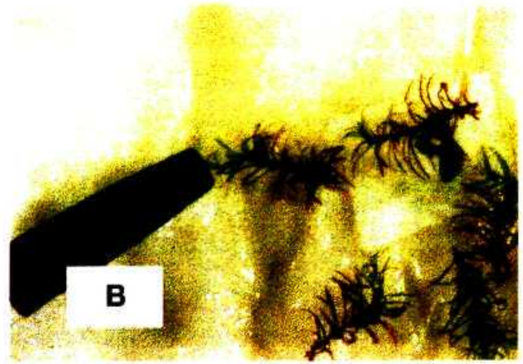


Plate 6.1. The effect of pretreatment with low concentrations of $CdSO_4$ on the physical appearance of *Atrichum androgynum* for 21 days under continuous light at 22°C. Plants were incubated in 0 µM $CdSO_4$ (A), 1 µM $CdSO_4$ (B), 2 µM $CdSO_4$ (C), 3 µM $CdSO_4$ (D), 5 µM $CdSO_4$ (E).

experiment are summarised in Table 6.1. The dry masses of control plants were increased by 17 and 29% after storage for 21 and 28 days respectively. The dry mass of the segments decreased by 8% when water treated plants were incubated in 2.5 μM Cd solution and stored for another 7 days. The mass of plants pretreated with 1 μM Cd solution was increased by 6% after storage for 21 days but decreased by 3% when treated plants were incubated in water and by 8% when plants were incubated in 2.5 μM Cd solution and stored for 7 days.

Brown & Martin (1981) state that metal tolerance mechanisms in plants often involve a considerable energy expenditure. It is estimated that tolerant plants normally have 20 to 50% lower biomass production than non-tolerant plants due to the energy required to maintain metal tolerance. In the present study, although the mass was increased after pretreatment and storage for 21 days, it was reduced by 17% when water treated plants were incubated in 2.5 μM and stored for 7 days and by 12% when Cd-treated plants were subjected to same conditions.

Intra- and extracellular Cd concentrations in Cd-pretreated plants were 0.48 ± 0.05 and 1.34 ± 0.22 μmoles g^{-1} respectively. Both intra- and extracellular Cd uptake were reduced when Cd-treated plants were incubated in either water or 2.5 μM $CdSO_4$ and stored for another 7 days. Intracellular Cd was decreased by 25% when plants were incubated in water and by 13% when

Table 6.1. The effect of pretreatment of *A. androgynum* with 1 µM $CdSO_4$ for 21 days followed by treatment with 2.5 µM $CdSO_4$ for 7 days on weight, gross photosynthesis, intracellular K and intra- and extracellular Cd uptake. Figures in a column given ± 1 standard deviation. After 28 days numbers followed by the same letter within one row are not significantly different from each other using Duncan's multiple range test ($P < 0.05$).

Cd Pretreatment concentration (µM)	0	0	1	1
Cd treatment concentration (µM)	0	2.5	0	2.5
0 days				
Weight (mg)	21.3 ± 1.5			
Gross photosynthesis (mmoles CO_2 hr^{-1} g^{-1} dry weight)	1.31 ± 0.53			
Intracellular K (µmoles g^{-1})	192 ± 10			
21 days				
Weight (mg)	24.8 ± 0.8		23.02 ± 0.96	
Gross photosynthesis (µmoles CO_2 hr^{-1} g^{-1} dry weight)	0.58 ± 0.09		0.51 ± 0.22	
Extracellular Cd (µmoles g^{-1})	0		1.33 ± 0.03	
Intracellular Cd (µmoles g^{-1})	0		0.43 ± 0.08	
Intracellular K (µmoles g^{-1})	187 ± 30		172 ± 13	
28 days				
Weight (mg)	27.76 b	22.93 a	24.25 ab	21.93 a
Gross photosynthesis (µmoles CO_2 hr^{-1} g^{-1} dry weight)	0.63 a	0.29 a	0.51 a	0.47 a
Extracellular Cd (µmoles g^{-1})	0 a	1.43 c	0.58 b	0.63 b
Intracellular Cd (µmoles g^{-1})	0 a	0.58 c	0.34 b	0.29 b
Intracellular K (µmoles g-1)	188 a	161 a	163 a	166 a

plants were incubated in 2.5 μM $CdSO_4$. Results demonstrated that removal of Cd from the cytoplasm can occur in the moss *A. androgynum*. Intra- and extracellular Cd concentrations in plants pretreated with water then incubated in 2.5μM $CdSO_4$ were 0.61 ± 0.17 and 1.39 ± 0.35 μmoles g^{-1} respectively.

Plants pretreated in Cd and stored for 7 days had lower photosynthetic rates than control plants although the differences were not significant. Intracellular K concentrations declined slightly with Cd treatment but the differences were also not significant. Beckett & Brown (1983) showed that in the laboratory, pretreatment of *P. membranacea* with low concentrations of Zn progressively decreased the inhibition of photosynthesis caused by subsequent exposure to high concentration of Zn compared with material receiving water treatment. In this study, photosynthesis in material that received water pretreatment was inhibited by 26% compared to inhibition by 7% in Cd-pretreated plants. This showed that photosynthesis in water treated plants was more sensitive to Cd than that of Cd-treated plants.

Shaw (1987) found that if the moss *Funeria hygrometrica* was pretreated with either 25 ppm zinc or 1 ppm copper, protonemal growth was less inhibited when plants were subsequently exposed to either 60 ppm zinc or 3 ppm copper. However, it should be noted that in this study pretreated plants were stored for a longer period and that tolerance was measured exclusively on

plant parts that were initiated after pretreatment. From these conditions, Shaw (1987) concluded that the significant effects he observed in his study were a result of physiological changes brought about by pretreatment and these changes were carried over to a new growth.

Supposing that the suggestion of Shaw (1987) applies to *A. androgynum*, then in the present study the effect of pretreatment could have been more pronounced if the experiment was carried on over a longer period. However, Wells & Brown (1995) demonstrated that laboratory-grown segments of the metal-contaminated population of *R. squarrosus* retained their tolerance to Cd even when levels of the metal were substantially less than those that could be detected in field-grown population. From these results, Wells & Brown (1995) concluded that Cd tolerance in the metal-contaminated population of *R. squarrosus* was a specific, genotypic response to Cd rather than a phenotypic adaptation that could be induced by metal stress in any population of the moss. If this is the case in *A. androgynum*, then pretreating non-tolerant plants for longer period may not necessarily induce Cd tolerance.

CONCLUSION

Preliminary experiments showed that intracellular K concentrations and gross photosynthesis were not significantly different between control plants and plants incubated in 1 μM

$CdSO_4$. This concentration was considered not toxic to plants and hence used to pretreat plants in order to test whether metal tolerance can be induced. In subsequent experiments involving plants pretreated with water or 1 μM $CdSO_4$, there was no significant loss in intracellular K in all treatments (>15%). Storage of plants for 21 days reduced gross photosynthesis in both non-treated and cd-treated plants. Although pretreatment with Cd slightly reduced photosynthetic rates, there was no significant difference between control and Cd-treated plants. The mass of Cd-treated plants was lower than that of water treated plants. This supported the suggestion of Brown & Martin (1981) that metal tolerance mechanisms often involve energy expenditure.

Reductions in the concentrations of intra- and extracellular Cd in Cd-treated plants suggested the existence of a mechanism to remove Cd from the cytoplasm of *A. androgynum*. Water treated plants had higher intra- and extracellular Cd uptake from 2.5 μM $CdSO_4$ than plants pretreated with 1 μM $CdSO_4$. The results thus indicated that pretreatment of plant material with low concentrations of Cd can affect uptake rates or stimulate efflux rates when plants are subsequently exposed to higher concentrations. This may be the way in which Cd pretreatment increases the tolerance of plants to subsequent high concentrations, although clearly more experiments are needed.

CHAPTER SEVEN

GENERAL CONCLUSIONS

SENSITIVITY OF *D. hirsuta* AND *A. androgynum* TO Cd

In the present study, the toxicity of Cd to the liverwort *D. hirsuta* and the moss *A. androgynum* was tested by measuring the inhibition of photosynthesis. Membrane damage was also measured using potassium leakage. The results discussed in Chapter 3 indicate that photosynthesis in *D. hirsuta* is much more sensitive to Cd than photosynthesis in *A. androgynum*. Although the incubation of either plant in 100 and 300 μM $CdSO_4$ for 1 hour did not significantly affect gross photosynthesis, storage of *D. hirsuta* for 24 hours following incubation in 300 μM Cd reduced photosynthesis significantly. Similar inhibition of photosynthesis in *A. androgynum* could only be detected 48 and 96 hours later. *D. hirsuta* but not *A. androgynum* lost intracellular K. Results show that *D. hirsuta* is more sensitive to Cd than *A. androgynum*.

Wells & Brown (1987) related sensitivity of photosynthesis of different populations of the moss *R. squarrosus* to intracellular Cd uptake. In the present study, although the liverwort and the moss differ greatly in morphology, *D. hirsuta* accumulated much more intracellular Cd than *A. androgynum* when measured both immediately after treatment and after storage.

This could explain why photosynthesis in *D. hirsuta* was more sensitive to Cd. Increased intracellular K loss in *D. hirsuta* may also indicate that intracellular Cd concentrations were too high and thus intracellular K was displaced. Puckett (1976) stated that higher metal concentrations may cause detrimental changes in membrane permeability and this can be indicated by high K loss. Reasons for differences in uptake rates of Cd by *D. hirsuta* and *A. androgynum* and for the differential sensitivity to the metal still need further investigation.

Cd UPTAKE IN *D. hirsuta* AND *A. androgynum*

Although most studies on uptake of heavy metals by plants emphasised total uptake, Brown and his co-workers realised the importance of measuring the intracellular uptake in their studies on Cd uptake in the grassland moss *R. squarrosus*. Small in comparison with the extracellular Cd uptake in the short term, Cd taken up into the cell is likely to be of more immediate relevance to metabolism.

In the present study, the kinetics of extra- and intracellular uptake of the liverwort *D. hirsuta* and the moss *A. androgynum* were investigated. Brown (1984) stated that extracellular uptake is dominated by ion exchange phenomena which are rapid, passive and only slightly affected by temperature. The results of the present study confirmed this statement. Although in the short term, extracellular Cd uptake in *A. androgynum* seemed to

be dependent on temperature, it seems likely that this effect could be eliminated if the experiment was carried over a longer period.

The kinetics of intracellular Cd uptake in *D. hirsuta* and *A. androgynum* observed in this study were to some extent similar to those observed by Brown and his co-workers in the grassland moss *R. squarrosus* and the lichen genus *Peltigera*. However, the maximum uptake rates, affinities and specificities were different between the groups. A comparison of the kinetics of Cd uptake by *D. hirsuta* and *A. androgynum* with the kinetics of Cd uptake by *R. squarrosus* and the lichen genus *Peltigera* is summarised in Table 7.1.

Although the Cd uptake rate of *D. hirsuta* varied between experiments, there was a general pattern regarding the uptake kinetics within the four representative species. The rate of intracellular uptake from 100 μM $CdSO_4$ by the moss *A. androgynum* (3.34 μmoles g^{-1} h^{-1}) was between that of *Peltigera* (1.8 μmoles g^{-1} h^{-1}) and *R. squarrosus* (11.9 μmoles g^{-1} h^{-1}). Intracellular Cd uptake rate in *D. hirsuta* varied between 1 and 10 μmoles g^{-1} h^{-1}, and was more or less similar to those of other groups. *R. squarrosus* had the highest binding capacity, and this was not very different from that of *A. androgynum*. *D. hirsuta* and *Peltigera* had lower binding capacities. The rate of equilibration between cell wall exchange sites and metal ions

Table 7.1. A comparison of the characteristics of Cd uptake by the liverwort *D. hirsuta*, the mosses *A. androgynum* and *R. squarrosus* and members of the lichen genus *Peltigera*. Figures in a column given ± 1 standard deviation.

	D. hirsuta	*A. androgynum*	+ *R. squarrosus*	* *Peltigera*
Rate of intracellular Cd uptake from 100 µM $CdSO_4$ (µmoles g^{-1} h^{-1})	1 to 10	3.3 ± 1.0	11.9 ± 2.3	1.76 ± 0.2
Time for half maximum extracellular Cd uptake from 100 µM $CdSO_4$ (min)	26 ± 6	21 ± 3	4 ± 2	39 ± 8
Maximum extracellular Cd uptake from 100 µM $CdSO_4$ (µmoles g^{-1})	64 ± 6	82 ± 6	107 ± 12	59 ± 4
K_m (concentration of Cd required for half the maximum intracellular Cd uptake rate (µM))	149 ± 19	30 ± 4	28 ± 2	65 ± 18
Vmax (maximum intracellular Cd uptake rate (µmoles g^{-1} h^{-1}))	11.0 ± 0.8	5.3 ± 0.2	14 ± 3	59 ± 4

* From Beckett & Brown (1984a)

+ From Brown & Beckett (1985)

in solution was highest in *R. squarrosus*, *D. hirsuta* and *A. androgynum* had almost identical equilibration rates and *Peltigera* had the lowest rate. The differences in equilibration rates were possibly caused by differences in morphology between the plants.

The effect of certain elements on intracellular Cd uptake rates indicated a difference in specificity of Cd uptake systems. Beckett & Brown (1984b) demonstrated that in *Peltigera*, Mg inhibited intracellular Cd that other cations. Possibly in *Peltigera* Mg and Cd are transported by the same transporting system. Wells & Brown (1990) using K pretreatment technique on *R. squarrosus* demonstrated the inhibitory nature of Ca on intracellular Cd uptake. In the present study, inhibitors reported to have a considerable influence on trans-membrane movement of calcium were used to investigate whether calcium had an effect on intracellular Cd uptake in the moss *A. androgynum*. The effects of verapamil and ammonium vanadate on intracellular Cd uptake may indicate that *A. androgynum* Cd may be in part transported by the same system transporting Ca (see Chapter 5 for a detailed discussion of this point). The strongly inhibitory effect of Zn on intracellular Cd uptake in *D. hirsuta* in comparison with other cations may indicate that in the liverwort a Zn-transporting system plays a major role in transportation of Cd. These results demonstrate that Cd does not have a specific transporting system.

Experiments were carried out to investigate the natural variation in Cd uptake kinetics in the liverwort *D. hirsuta*. Results surely suggest that variations depended on several conditions and that a specific factor could not be singled out. The age of plant had a considerable effect on intracellular Cd uptake in *D. hirsuta* (by up to 35%). Brown and Brūmelis (1996) argued that the differences in cellular location of elements can be due to the quality of cell wall material, changes in the metabolic behaviour and cation requirements of the cell cytoplasm during maturation. Possibly more energy can be used to take up nutrients in newly formed tissues than in older ones. Surely, great care must be take when sampling plant material in order to reduce the variation.

The site from which plants are collected can also be important when comparing Cd uptake rates. Wells & Brown (1987) suggested that plants can have displaceable cations on their cell wall which can compete with Cd for intracellular uptake during incubation. Wells & Brown (1990) and Wells *et al.*(1995) argued that these cations can be removed by pretreating plants with potassium. Pretreatment of plant material with K increased intracellular Cd uptake in *D. hirsuta*. In the present study, *D. hirsuta* was collected from along a path, at the bottom of a stream and at the top of a stream near a waterfall. The results showed that plants from top of stream had the highest V_{max} and those from the path the lowest. There were no significant differences in the concentration of extracellular divalent

cations between the three plant collections. These results imply that the variations in cations on cell wall cannot be the only factor that determines intracellular uptake rates.

Generally, there was no significant difference in intracellular concentrations of Ca, K and Mg between the three plant collections. However, the plants collected from the top of the stream had the lowest concentration of potassium of the protoplasm in a fully hydrated thallus, suggesting that these plants may be nutrient stressed. This may cause the plants to expend more energy to take up nutrients from solutions and can possibly explain much higher rate of intracellular Cd uptake in this collection. Cuticle and thallus thickness may contribute to the observed variations in V_{max} but other factors appear to be involved. A thicker cuticle and thallus may form layers through which Cd must diffuse to reach the inner cells. Studies involving anatomical location may explain the role cuticle and thallus thickness play in controlling the uptake of Cd by the liverwort.

The results of effect of storage temperature on Cd uptake by *A. androgynum* surely suggest that the time of the year plants are collected and temperature of the habitat at that particular time can affect uptake rates. Cold acclimated plants accumulated intracellular Cd at faster rates at low temperatures. High temperatures increase respiration more rapidly than assimilation. This limits the energy required to pump out intracellular Cd and thus promote accumulation of

intracellular Cd. To what extent seasonal variation affected uptake rates is not known. It would be interesting to investigate how temperature acclimation occurs in bryophytes. The temperature necessary to cause acclimation to occur and the time required should be investigated. The reason bryophytes seem unable to maintain a positive photosynthetic balance at 30 °C remains unclear.

At this point, it is not known which of the above mentioned factors played a role in the variations observed in uptake rates kinetics by *D. hirsuta*. Possibly, it could be necessary to know which factor becomes important and under which conditions. The conditions should be taken in to consideration when comparing uptake kinetics in plants.

THE INDUCTION OF Cd TOLERANCE IN *A. androgynum*

Plants collected from habitats which have heavy metal deposits can tolerate toxicity of such metals even when exposed to concentrations high enough to be toxic to plants from uncontaminated sites (Beckett & Brown, 1983, 1984a,b;, Shaw 1987; Wells & Brown, 1987). However, samples of *A. androgynum* used in this study were collected from habitats with no history of Cd deposits. Beckett & Brown (1983) reported that Cd-tolerance could be induced in initially non-tolerant species of lichen genus *Peltigera* by pretreating them with low concentrations of Cd. In this study, Cd-tolerance induction in

the moss *A. androgynum* was tested by incubating plants in 1 μM $CdSO_4$ for 1 hour and storing them for 21 days. This was followed by incubation in 2.5 μM $CdSO_4$ for 1 hour and storage for 7 days.

Photosynthesis in control plants was more sensitive to exposure to 2.5 μM $CdSO_4$ than photosynthesis in plants pretreated with 1 μM $CdSO_4$ for 21 days. The reasons for the increase in tolerance are not known. However, the reductions of extra- and intracellular Cd in Cd-pretreated plants when incubated in either water or 2.5 μM $CdSO_4$ suggested the existence of a mechanism to remove Cd from the cytoplasm. Presumably this is how pretreatment of plant material with low concentrations of Cd increased the tolerance of plants to subsequent high concentrations. The reductions of biomass in Cd-treated plants indicated that some energy was used to tolerate Cd. This was in agreement with the suggestion of Brown & Martin (1981) that metal tolerance mechanisms in plants often involve a considerable energy expenditure. Up to 50% of plant biomass can be lost due to the energy required to maintain metal tolerance.

Baker *et al.* (1986) stated that their study on the grass *Holcus lanatus* suggested that, at least for Cd, metal tolerance is not a genetically stable character and thus it can be affected by other phenotypic influences. In plants, phytochelatins have been suggested to sequester heavy metals in plants (see Chapter

1 for further details). The synthesis of these proteins is triggered by the presence of heavy metals. After synthesis, the proteins bind to heavy metals and render them harmless to plants. Possibly, pretreatment of the moss with low concentrations of Cd in the present study may have triggered the synthesis of these proteins but due to limited amount of time these proteins were not analysed. It could prove worthwhile to investigate if phytochelatins play any role in Cd tolerance in the moss *A. androgynum*.

However, it should be noted that phytochelatins might not be of major importance to Cd-tolerance in plants. Rauser (1986) suggested that cadmium binding protein was not the only major detoxifying agent in maize and tomato. Rauser found that maize retained a low percentage of cadmium in the root (instead of translocating it to the shoot) but had the higher fraction of root cadmium bound to the binding protein. Tomato in contrast, had a higher retention of cadmium in roots but a relatively low percentage of protein-bound Cd.

More experiments must be carried out to investigate Cd tolerance induction in *A. androgynum*. Possibly a longer period of pretreatment with a lower concentration of Cd may further increase tolerance. Shaw (1987) found that pretreatment of *F. hygrometrica* with low concentrations of either zinc or copper for longer periods had a significant effect on growth when plants were subsequently exposed to higher concentrations.

REFERENCES

BAKER, A. J. M., GRANT, C. J., MARTIN, M. H. SHAW, S. C. & WHITEBROOK J. 1986. Induction and loss of cadmium tolerance in *Holcus lanatus* L and other grasses. *New Phytologist*. 102: 575-587.

BECKETT, R. P. 1984. The effects of natural and experimental heavy metal stress on lichen physiology. PhD Thesis. University of Bristol. Bristol. UK.

BECKETT, R. P. & BROWN, D. H. 1983. Natural and experimentally-induced zinc and copper resistance in the lichen genus *Peltigera*. *Annals of Botany*. 52: 43-50.

BECKETT, R. P. & BROWN, D. H. 1984a. The control of cadmium uptake in the lichen genus *Peltigera*. *Journal of Experimental Botany*. 35: 1071-1082.

BECKETT, R. P. & BROWN, D. H. 1984b. The relationship between cadmium uptake and heavy metal tolerance in the lichen genus *Peltigera*. *New Phytologist*. 97: 301-311.

BRADSHAW, A. D. 1976. Pollution and Evolution. In: MANFIELD, I. A. (Ed). *Effects of air pollutants on plants*. Cambridge University Press. Cambridge. 139-159.

BRIGGS, D. 1972. Population differentiation in *Marchantia polymorpha* L. in various lead pollution levels. *Nature*. 238: 166-167.

BROWN, D. H. 1982. Mineral nutrition. In: SMITH A. J. E. (Ed) *Bryophyte Ecology*. Chapman & Hall. London.

BROWN, D. H. 1984. Uptake of mineral elements and their use in pollution monitoring. In: DYER, A. F. & DUCKETT, J. G. (Eds). *The Experimental Biology of Bryophytes*. Academic Press, New York.

BROWN, D. H. & AVALOS, A. 1993. The role of calcium in intracellular cadmium uptake by *Peltigera membranacea*. *Annals of Botany*. 71: 467-473.

BROWN, D. H. & BATES, J. W. 1990. Bryophytes and nutrient cycling. *Botanical Journal of the Linnean Society*. 104: 129-147.

BROWN, D. H. & BECKETT, R. P. 1985. Intracellular and extracellular uptake of cadmium by the moss *Rhytidiadelphus squarrosus*. *Annals of Botany*. 55: 179-188.

BROWN, D. H. & BRŪMELIS, G. 1996. A biomonitoring method using the cellular distribution of metals in moss. *The Science of the Total Environment*. 187: 153-161.

BROWN, D. H. & BUCK, S. W. 1979. Desiccation effects and cation distribution in bryophytes. *New Phytologist*. 82: 115-125.

BROWN, D. H. & HOUSE, L. K. 1978. Evidence of a copper tolerant ecotype of *Solenostoma crenulatum*. *Annals of Botany*. 42: 1383-1392.

BROWN, H. & MARTIN, M. H. 1981. Pretreatment effects of cadmium on the root growth of *Holcus lanatus* L. *New Phytologist*. 89: 621-629.

BROWN, D. H. & SLINGSBY, D. R. 1972. The cellular location of lead and potassium in the lichen *Cladonia rangiformis* (L.) Hoffm. *New Phytologist*. 71: 297-305.

BROWN, D. H., SNELGAR, W. P. & GREEN, T. G. A. 1981. Effects of storage conditions on lichen respiration and desiccation sensitivity. *Annals of Botany*. 48: 923-926.

CHAPIN, F. S. 1974. Morphological and physiological mechanisms of temperature compensation in phosphate absorption along a latitudinal gradient. *Ecology*. 55: 1180-1198.

CLARKSON, D. T. & LÜTTGE, W. 1989. Mineral nutrition: divalent cations, transport and compartmentation. *Progress in Botany*. 51: 93-112.

CLAVERI, B. & MOUVET, C. 1995. Temperature effects on copper uptake and CO_2 assimilation by the aquatic moss *Rhynchostegium ripariodes*. *Archives of Environmental Contamination and Toxicology*. 28: 314-320.

EVANS, D. E., BRIARS, S-A & WILLIAMS, L. E. 1991. Active calcium transport by plant cell membranes. *Journal of Experimental Botany*. 42: 288-303.

FLOWERS, T. J. & YEO, A. R. 1992. *Solutes Transport in plants*. Blackie Academic & Professional Press. Chapman & Hall. London. 48-99.

GLIME, J. M. & ACTON, D. W. 1979. Temperature effects on assimilation and respiration in the *Fontanalis duriaei*-periphyton association. *Bryologist*. 82 (3): 382-392.

GRILL, E., THUMANN, J., WINNACKER E-L & ZENK M. H. 1988. Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media. *Plant Cell Rep.* 7: 375-378.

GROSS, R. E., PUGNO, P. & DUGGER, W. M. 1970. Observation on the mechanism of copper damage in *Chlorella*. *Plant Physiology*. 46: 183-185.

JULES, E. S. & SHAW, A. J. 1994. Adaptation to metal-contaminated soils in populations of the moss, *Ceratodon*

purpureus: vegetative growth and reproductive expression. *American Journal of Botany*. 81 (6): 791-797.

KÜPPER, H., KÜPPER, F. & SPILLER, M. 1996. Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. *Journal of Experimental Botany*. 47:259-266.

LEPP, N. W. & HOCKENHULL, Y. 1983. Growth responses of Marchantia polymorpha gemmalings in relation to concentration and chemical form of applied nickel. *Bryologist*. 86: 342-346.

NIEBOER, E. & RICHARDSON, D. H. S. 1980. The replacement of the non-descript term "heavy metal" by a biologically and chemically significant classification of metal ions. *Environmental Pollution Series* B1: 3-26.

NIEBOER, E., RICHARDSON, D. H. S. BOILEAU, L. J. R., BECKETT, P. J., LAVOIE, P. & PADOVAN, D. 1982. Lichens and mosses as monitors of industrial activity associated with uranium mining in Northern Ontario, Canada -Part 3. Accumulation of iron and titanium and their natural dependence. *Environmental Pollution Series* B4: 181-192.

PUCKETT, K. J. 1976. The effects of heavy metals on some aspects on lichen physiology. *Canadian Journal of Botany*. 54: 2695-703.

PUCKETT, K. J. & BURTON, M. A. S. 1981. The effect of trace elements on lower plants. In: LEPP, N. W. (Ed). *Effects of Heavy Metal Pollution on Plants*. Applied Science Publishers, London.

RAUSER W. E. 1986. The amount of cadmium associated with Cd-binding protein in roots of *Agrostis giganitea*, maize and tomato. *Plant Science*. 43: 85-91.

RAUSER, W. E. 1995. Phytochelatins and related peptides: structure, biosynthesis, and function. *Plant Physiologica*. 109: 1141-1149.

RICHARDSON, D. H. S. 1995. Metal uptake in lichens. *Symbiosis*. 18: 119-127.

RICHARDSON, D. H. S. & NIEBOER, E. V. 1980. Surface binding and accumulation of metals in lichens. In: COOK, C. B., PAPPAS P. W. & RUDOLPH, E. D. (Eds) *Cellular Interaction in Symbiosis and Paratism*. Ohio State University Press, Columbus, Ohio.

SHAW, A. J. 1987. Effect of Environmental pretreatment on tolerance to copper and zinc in the moss *Funeria hygrometrica*. *American Journal of Botany*. 74: 1466-1475.

SHAW, A. J. 1990a. Metal tolerance in bryophytes. In: SHAW. A. J. (Ed) *Heavy Metal Tolerance in Plants: Evolutionary Aspects*.

Boca Raton, FL: CRC Press.

SHAW, A. J. 1990b. Metal tolerance and cotolerances in the moss *Funeria hygrometrica* Hedw. *Canadian Journal of Botany*. 68: 2275-2282.

SHAW, A. J. 1994. Adaptation to metals in widespread and endemic plants. *Environmental Health Perspective*. 102: 105-108.

SIDHU, M. & BROWN, D. H. 1996. A new laboratory technique for studying the effects of heavy metals on bryophyte growth. *Annals of Botany*. 78: 711-717.

TESTER, M. 1990. Plant ion channels: whole-cell and single-cell studies. *New Phytologist*. 114: 305-340.

WANATABE, T., MACHIDA, K., SUZUKI, H., KOBAYASH, M. & HONDA, K. 1985. Photoelectrochemistry of metallochlorophylls. *Cordination Chemistry Reviews*. 64: 207-224.

WELLS, J. M. & BROWN, D. H. 1987. Factors affecting the kinetics of intra- and extracellular cadmium uptake by the moss *Rhytidiadelphus squarrosus*. *New Phytologist*. 105: 123-137.

WELLS, J. M. & BROWN, D. H. 1990. Ionic control of intracellular and extracellular cadmium uptake by the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst. *New Phytologist*.

116: 541-553.

WELLS, J. M. & BROWN, D. H. 1995. Cadmium tolerance in a metal-contaminated population of the grassland moss *Rhytidiadelphus squarrosus*. *Annals of Botany*. 75: 21-29.

WELLS, J. M., BROWN, D. H. & BECKETT, R. P. 1995. Kinetic analysis of Cd uptake in Cd- tolerant and intolerant populations of the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst and the lichen *Peltigera membranacea* (Ach.) Nyl. *New Phytologist*. 129: 477-486.

WILKINSON, G. N. 1961. Statistical estimations in enzyme kinetics. *Biochemical Journal*. 80: 324-332.