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PHYSIOLOGICAL RESPONSES OF SOYBEAN SEEDS (*GLYCINE MAX* L. MERR.) TO METAL POLLUTANTS

BY

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This thesis is dedicated to Professor Peter William Linder, supervisor, mentor, friend and to my parents.

Knowledge navigates the ocean and is perpetually on voyages of discovery.

I. Disraeli. Curiosities of Literature, Characteristics of Bayle.

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ABSTRACT

Seeds, especially cereals and legumes are a vital component of the human diet and as a result of elevated levels of environmental pollution, seed-bearing crop plants are grown increasingly on contaminated soils. Although several studies have looked at seeds as potential sources of metals that may enter the food chain, very little research has been carried out to examine the effect of such toxicants on the physiology of these plant parts. This study examines the effect of two metal pollutants, namely Cd and Ni, on the development and functioning of soybean seeds. Cadmium was chosen because it is considered to be the most serious of the metal pollutants, is highly toxic to mammals and easily enters the food chain. Nickel, is relatively mobile within plants compared to other metal pollutants and also represents a potential threat to the environment.

Soybean plants (*cv* Crawford) were grown to maturity in a circulating nutrient solution system, which in the case of treatment plants, was amended with either Cd or Ni. From the results of preliminary trials in which the effect of metal pollutant concentration on plant growth and pod production were examined, nutrient solution concentrations of 0.05 mg Cd/litre or 1 mg Ni/litre were used for routine cultivation of the plants (termed metal-*treatment* plants). Seeds were harvested at four (initially five) different growth stages and the effect of the metal pollutants on size and other developmental parameters investigated. Accumulation and distribution of Cd, Ni and other elements within the seeds was examined. Firstly, by using inductively coupled plasma–atomic emission spectroscopy (ICP-AES) and secondly, at a finer resolution utilising a nuclear microprobe, coupled with proton induced X-ray emission (PIXE). The anatomy and ultra-structure of metal pollutant-treatment seeds was compared with that of control seeds using light as well as transmission electron microscopy (LM and TEM). Possible structural

aberrations caused by the presence of Cd or Ni were identified. In another set of experiments, seeds were germinated in solutions of different concentrations of Cd or Ni (termed metal-*germinated* seeds). The LC_{50} and EC_{50} values for germination and radicle extension respectively, were derived. The effect of metal pollutants on seedling establishment was also examined by cultivating the plants further in nutrient solution containing different concentrations of the metal pollutants. Other seedlings (termed *recovery* seedlings), subsequent to germination in the metal pollutant, were transferred to the standard control nutrient solution. Uptake of metal pollutant, concentration of photosynthetic pigments and photosynthetic functioning were examined in metal-germinated, recovery and control seedlings. In the final section of the study, chemical speciation in the nutrient solution used for cultivation of metal-treatment plants was modelled using the speciation software MINTEQA2. Percentage bioavailability of the metal pollutants as well as of four nutritionally important metals, Fe, Mn, Mg and Zn was examined. Furthermore, computer simulations were also carried out to model the effect of pH and increasing metal pollutant concentration, on the bioavailability of the above-mentioned metals.

Addition of even low concentrations of Cd or Ni to the nutrient solution resulted in reduction in root biomass and pod (and hence seed) production. This effect increased with metal pollutant concentration. Cadmium appeared to be more phytotoxic than Ni and lower concentrations of the former were required elicit an equivalent response. Visual toxicity symptoms noted, included red pigmentation in the petioles, chlorosis of the trifoliate leaves followed by the appearance of necrotic areas. In addition, Ni-toxicity symptoms included terminal deformed pods, as well as red spots in the inter-veinal areas of leaves. Both Cd and Ni accelerated plant senescence. Leaf abscission was promoted and in the case of the older growth stages, the rate of pod development was increased relative to that of control pods. Nonetheless, the presence of metal pollutants did not appear to enhance pod abscission during the developmental period examined.

In metal-treatment plants, pollutant loads in roots were much higher than in shoots. Cadmium levels in the seeds harvested from these plants were extremely low (approximately 1μg/g.dm) indicating that the metal is excluded from these tissues to a great extent. Nickel was more mobile than Cd, reaching higher levels than the latter in all plant parts and a concentration of approximately 50 μg/g.dm in mature treatment seeds. Pods did not appear to exclude entry of metal pollutants into the seeds and contained similar concentrations as the seeds in the case of Cd and lower concentrations in the case of Ni. Seed concentration of both metal pollutants (when expressed as μg/g.dm) was highest in the youngest growth stages and then decreased with age.

Cadmium was found to decrease mean seed size relative to control seeds, but had no effect on the number of seeds per pod. Nickel on the other hand, exerted no effect on size but did reduce the average number of seeds contained in each pod. As a result of reduced mass, the presence of Cd in the nutrient solution reduced the lipid, starch and total N content of seeds harvested from soybean plants grown in such a medium. No significant effect on the quantity of storage reserves could be detected in Ni-treatment seeds. Mature seeds harvested from Cd-treatment plants had lower Fe and Mn, but higher Zn and Mg contents than control seeds. Nickel-treatment seeds also exhibited reduced Fe, Mn and elevated Zn contents, but Mg levels were also reduced. Shifts in seed concentrations of the nutritionally important metals noted above, were also found in pods, most notably a reduction in Fe content. Despite the presence of metal pollutants within the seeds, the extent of germination in metal pollutant-treatment seeds was not impaired compared to control seeds. The rate of germination however, was depressed slightly in both metal treatments.

Examination of metal distribution within seeds using ICP-AES revealed that Cd was localised mainly in the testa and cotyledons, with very little in the axis. Nickel was mainly concentrated in the axis and least in the cotyledons. Cadmium levels in metal-treatment seeds were too low for distribution maps to

be made using PIXE and only point analyses were carried out. Overall, these results agreed with those obtained from ICP analysis. Nickel, which accumulated to higher levels within seeds, was mapped successfully using PIXE. The embryo axis appeared to contain the highest concentrations of Ni, particularly in the apical meristem and cortex, but was virtually absent from the root cap area and the central stele. Interesting elemental maps were also obtained for S, Fe and Mn (supplied in the nutrient solution at normal physiological concentrations). Levels of Ni in control seeds were extremely low and could not be mapped.

The LC_{50} and EC_{50} values for germination and radicle elongation respectively, in the presence of exogenous metal pollutant, were found to be lower for Cd than Ni. This is consistent with the higher phytotoxicity of the former element. Radicle elongation was found to be more sensitive to the presence of exogenous metal pollutants than seed germination. The major effect on seedling establishment was reduction in growth, particularly of the lateral roots. As in the case of mature plants, pollutant loads in the roots of seedlings were higher than in shoots. Recovery seedlings appeared relatively healthy after a period of exposure to metal pollutants, up to a critical concentration of metal pollutant. Nonetheless, although little reduction in the concentration of photosynthetic pigments or the efficiency of photosynthetic functioning was recorded, two weeks after exposure to the metal pollutants, root biomass was still reduced relative to that of control seedlings. The total chlorophyll content of metal pollutant-germinated seedlings decreased at low concentrations of the metal pollutants, but then increased at higher concentrations. It is suggested that this is the result of the combined effects of inhibition of photosynthetic pigment synthesis, coupled to reduced leaf expansion.

Metal pollutant-treatment and control seeds did not differ from each other in external appearance nor at the LM level. Slight ultra-structural variations were noted using TEM however, including the presence of vesicles in the nucleoplasm of Cd-treatment cotyledon cells, an increase in the number of crystalloid inclusions in protein bodies (possibly phytate) as well as an increased number of

starch grains in the radicle tip cells of Ni-treatment seeds. Further research is needed to confirm these results.

Significant ultra-structural changes in metal pollutant-germinated seedlings were noted compared to the controls. From examination of the ultra-structure of such seedlings, both Cd and Ni appeared to affect nuclear functioning, proteolysis, as well as starch grain formation. Cadmium elicited a response at lower concentrations than Ni. It is stressed that these are not necessarily the principal toxic actions of the metals however, as marked structural changes were apparent only at high concentrations. Aberrations to cytoplasm adjacent to the cell wall were also noted in cells from seedlings germinated in the presence of Ni.

Computer speciation simulations using MINTEQA2 predicted that in the respective treatment solutions, 87% of Cd, but only 49% of Ni, was in a form suitable for plant uptake. Shifts in seed contents of Mg, Mn and Zn, in response to amendment of the nutrient solution with metal pollutants, could not be explained by changes in chemical speciation in the growth medium. The decrease in Fe content in Ni-treatment seeds on the other hand, may possibly be a consequence of decreased bioavailability of this ion in the nutrient solution. pH was found to exert an effect on the speciation profile of metal pollutants, as well as on that of nutrients. The most marked effect was noted on Ni^{+2}. The proportion of metal in this form (the bioavailable form) decreased from 49% to 3% when pH was increased from 6.0 to 7.0.

Although plants are able to limit entry of metal pollutants into seeds to some extent, they do still enter these tissues and it is important that the effects on functioning of such plant parts be examined. This study reports preliminary findings on this aspect. Much work remains to be done however particularly with regard to the effect of metal pollutants on the quality of storage reserves, especially proteins. Furthermore, this work should be extended to the seeds of other important crop plants.

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ABBREVIATIONS

BD – below detection
CEC – cation exchange capacity
Chl_a – chlorophyll a
Chl_{a+b} – total chlorophyll
Chl_b – chlorophyll b
cv – cultivar
C_{x+c} – total carotenoids
DAF – days after flowering
EDTA - ethylene-diamine-tetra-acetate
EDX - energy dispersive x-ray analysis
ER – endoplasmic reticulum
FIAM - free-ion activity model
GI – germination index
GV – germination value
ICP-AES – inductively coupled plasma-atomic emission spectroscopy
K_{sp} - solubility constant
LM – light microscopy
Log k – stability constant
MDG – mean daily germination
MDL – mean detection level
n – sample size
ND – not determined
NIST - National Institute of Standards
PAS – periodic acid-Schiff's base
PIXE – proton/particle induced x-ray emission
PLS body - protein-lipid-sugar body
PM – physiological maturity
PPM – parts per million
PSII - photosystem II
PV – peak value
RER – rough endoplasmic reticulum
SD – standard deviation
SOD – superoxide dismutase
TEM – transmission electron microscopy
US EPA – United States Environmental Protection Agency
WHO – World Health Organization

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

One of the greatest challenges facing South Africa lies in providing an adequate, nutritious diet for all socio-economic groups. This is especially important when it is considered that according to the latest estimations, 42% of the population is under the age of 20 years (Statistics South Africa 1998). The high nutritive value of legumes in general and soybeans in particular is well known (Gupta 1983; Vitale and Bollini 1995). Depending on the cultivar and growing conditions, soybeans can contain between 38-45% protein, 18-22% oil, and 10% carbohydrate when expressed on a dry mass basis (Hartwig 1973; Poehlman 1979; Kondo, Sugimoto and Saio 1986). Although proteins from this source tend to be low in methionine and other sulphur containing amino acids, the lysine content is considerably higher than that of cereals (Nicholson, Harrison, Masefield *et al.* 1971; Mossé and Pernollet 1983). As part of a mixed diet therefore, soybeans offer an excellent, economical source of protein (Gupta 1983). In fact according to Odendaal, Smith and Smith (1984), the most important international nutritional research centres consider the soybean to be a solution to the protein/energy problem of the world.

Levels of heavy metal pollution in the environment today are considerably higher than pre-industrial revolution times or even since the beginning of this century (Friedland 1990; Bishnoi, Dua, Gupta *et al.* 1993). Increasing demand for consumer goods, use of automobiles, burning of fossil fuels and disposal of toxic wastes has resulted in enhanced burdens of pollutants, including metals. The US Environmental protection Agency (US EPA) estimated that in 1989 alone, 285 million kg of heavy metals was released into the environment of the United States (Nellessen and Fletcher 1993). Such activities result in elevated pollutant levels in soils, as well as in all other parts of the environment (Kieffer 1991). Thus the chance of crops being grown on contaminated soils is increasing.

Since plants play an essential role in the environment as primary producers and extract nutrients from the soil, the principal route for metals entering into the food chain is via plants (Reilly 1991; Nellessen and Fletcher 1992). High levels of metal pollutants in agricultural soils in particular, are of major concern because, besides frequently resulting in reduced crop yields (Rauser 1978), the consumption of such crops may be hazardous for both humans and animals (Steyn, van der Watt, Claasens 1996; Pierzynski and Schwab 1993).

Despite extensive research on metal pollutants and plants (Marschner 1983), little of this work has been directed towards possible effects on seed development (Siegel and Siegel 1985; Fargasova 1994). A database survey on papers concerned with uptake, accumulation and translocation of heavy metals by vascular plants, revealed that fewer than 11% of the almost 25 000 listed papers studied the effect of metals on reproductive parts (Nellessen and Fletcher 1993). Research that has been carried out has largely been restricted to analyses of total metal content within seeds and the associated risk to consumers. Little could be found concerning the effects metal pollutants may possibly exert on metabolic and developmental processes occurring within these plant parts. Since seeds are responsible for the propagation of future plant generations as well as for nutrition, it is of vital importance to know what effect, if any metal pollutants may have on their functioning. In addition, knowledge of the effect trace metals may exert on seed development and seedling establishment could possibly lead to development of cultivars more able to withstand such stresses in the field (Salt, Pickering, Prince *et al.* 1997).

1.2 LITERATURE REVIEW: SOYBEANS

The soybean is a multi-functional crop, since in addition to providing food for humans and livestock, they also provide the starting material for many industrial uses (Cowan 1973). Oils extracted from the seeds are used to make edible fat products such as margarine and cooking oils, as well as lecithin. Industrial non-food uses of soybean oil include paints, varnishes, insecticides, soap and plastics (Nicholson *et al.* 1971, Cowan 1973). Although soybeans have been used in the east for centuries, it is only relatively recently that processed soybean protein has become important for human consumption in the West (Scott and Aldrich 1970; Poehlman 1979). According to Smit (1989) this crop represents one of the most underestimated options for South African agriculture, since because of its nitrogen fixing capacities, soybeans have low fertilisation requirements and can have a beneficial effect on the soil (Odendaal *et al.* 1984).

Soybeans are considered to have originated in north-eastern China (Plitmann and Kislev 1989).. This crop is known to have been cultivated for several thousand years before the birth of Christ, although it is only in the last 60 years that it has become important to western farmers (Vaughan 1970; Probst and Judd 1973). The soybean is a member of the family Fabaceae, genus *Glycine*, sub-genus *Soja*. The cultivated form is *Glycine max* (L.) Merrill. whilst the most likely progenitor is *G. soja* (formerly *G. ussuriensis*) a wild, annual vine (Poehlman 1979). The crop is currently grown chiefly in the Far East, USA and South America. The USA now produces 50% of the world crop and it is that country's most important agricultural export (Kromer 1973; Poehlman 1979).

The cultivated soybean is an erect, bushy annual, which depending on the cultivar and growing conditions, may vary from 0.3 - 2.0 m in height and may be densely or sparsely branched (Poehlman 1979). The first true leaves (the primary leaves) are unifoliate, paired and occur in the first node above the cotyledons. Subsequent leaves are alternate and trifoliate (Scott and Aldrich 1970). The leaves, stems, sepals and pods are hirsute, the hairs being either white or light brown in colour

(Bernard and Weiss 1973, Carlson 1973). Flowers are either white or purple, borne on axillary or terminal racemes of up to 35 flowers per cluster and are self-pollinating (Smit 1987). They are also very small (6-7mm in length) and numerous. The flowering period in soybeans is relatively long, up to 6 weeks from the appearance of the first flower to the last. Since there is no sharply defined transition from flowering to pod formation, especially on indeterminate plants, it is normal to find both new and withered flowers as well as pods of different development stages on the same plant (Scott and Aldrich 1970). Pods contain from 1 to five seeds, depending on the cultivar (Hartwig 1973). Several authors have reported that the soybean with its numerous cultivars is an extremely diverse species, showing a wide range of morphological attributes (Poehlman 1979, Smit 1987), although it is considered to have a narrow genetic base (McDonald, *pers.comm. 1999)*. According to Poehlman (1979), soybeans, depending on the variety can have either a determinate or indeterminate growth form. In the case of indeterminate types, the flowers appear before stem elongation ceases, the flowers being borne only on axillary racemes. Flowers borne by determinate varieties appear both on axillary and terminal racemes, stem elongation ceasing with differentiation of the terminal bud. Another important characteristic of these plants, is that whilst they are short-day plants, varieties differ in their day-length requirements for initiation of flowering (Smit 1987).

1.3 LITERATURE REVIEW: METAL POLLUTANTS IN THE ENVIRONMENT

1.3.1 Use of the term "heavy metal"

The term "heavy metal" is used extensively in the literature, from papers on biology, to those covering toxicology and environmental chemistry. This expression is vague however, generally meaning metals with atomic numbers greater than 20 (Phipps 1981). Some authors restrict the term to second and third row transition metals only including Sn and Pb, others to all transition metals as well as Be, Li, Na, Al, K and Mg (Borovik 1990; Dallas and Day 1993). The term "heavy metal" also carries a connotation of toxicity or pollution and many authors reserve this term for metals such as Cd, Co, Ti, Cr, Hg, that are not normally found in biological tissues and that frequently exert a harmful effect. For these reasons, several authors have tried to introduce more exact terminology (Nieboer and Richardson 1980; Woolhouse 1983; Phipps 1981). Such efforts to discourage the use of this expression have been largely ineffective however (Punz and Sieghardt 1993) and because the vast majority of papers still employ this term, usually without defining its meaning, it is often difficult to know which elements are specifically included or excluded. In this study therefore, the term "heavy metal" will be used only when unavoidable.

In a similar vein, according to Punz and Sieghardt (1993), expression of concentration values on a molar basis accommodates the differing atomic masses of the various metal pollutants. Nonetheless, these authors go on to state that expressing concentration as mass per unit volume (e.g. mg/litre or ppm - parts per million) is easier to calculate and since this terminology is used extensively in the literature, comparison of values is facilitated. In this project therefore, the concentration of metals will be expressed as mg/litre rather than on a molar basis.

1.3.2 Essential, beneficial and toxic elements

With regard to plant nutrition, metals may be classed as essential, non-essential or beneficial (Punz and Sieghardt 1993; Brune, Urbach and Dietz 1995). An essential element is defined as one whose presence is mandatory in order for a plant to complete its life cycle (Noggle and Fritz 1976; Brown, Welch and Cary 1987). Metals in this group include Cu, Fe, Mn, Mo, and Zn (Berrow and Burridge 1991). The majority of metals belong to the class of non-essential elements i.e. those for which (as yet) no role in plant metabolism has been observed and include Pb, Cd, Cr and Hg (Punz and Sieghardt 1993). Some authors include another class, namely beneficial elements. These are elements whose presence seems to enhance growth in certain plant species. Examples of metals in this class include Ni, Co, Se, and V (Marschner 1986; Punz and Sieghardt 1993).

All metals tend to be toxic to organisms at one level or other (Breckle 1991). Some, for example Cu and Zn, only when supplied in excess, and others such as Pb, Cd and Hg at relatively low concentrations (Sandman and Boger 1983; Schmidt, Bartels, Tittel *et al.* 1997). This concept is summarised in Fig. 1.1, which shows a generalised dose response curve for the effect of increasing metal availability on plant performance (yield or growth). Note that the response of plants to essential elements shows an initial increase in the concentration range where this element is deficient, whereas such an increase is absent in the case of non-essential elements. The nature, direction and magnitude of the response of plants to a metal (i.e. the shape of the dose-response curve and value of the toxicity threshold) will vary and is dependent on several factors. These include the sensitivity of the plant species, the intensity (concentration and duration) of the exposure, the identity of the metal concerned and well as the form in which it is supplied (Baker and Walker 1989). These factors are discussed further in section 1.3.6.

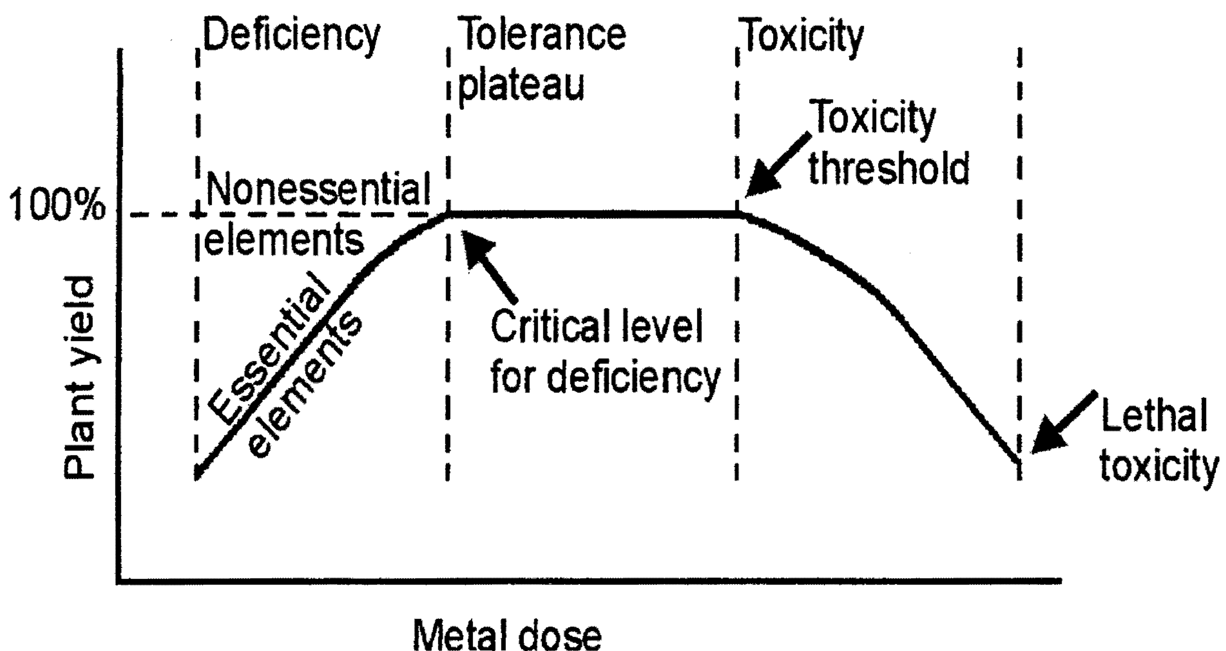


***Fig. 1.1** Generalized dose-response curve, showing the effect of increasing metal availability on plant yield. Note the difference in response between essential and non-essential metals. (Adapted from Berry and Wallace 1981).*

1.3.3 Metal pollutants and soils

1.3.3.1 The impact of metal pollution

Metal loads in plants can originate from the soil or from aerial deposition as a result of atmospheric emissions (Gupta 1992; Eklund 1995). Those pollutants, deposited onto plant surfaces may either remain as a residue, or be taken up directly into the aerial plant parts (Voutsa, Grimanis and Samara 1996). Heavy metals in the air also enter the soil dissolved in rain or snow, or as dust and from there can enter the plant via the roots (Haghiri 1973). The relative importance of aerial versus root uptake of metal pollutants into plants is not clear (Eklund 1995). Jones (1991) claims that crops are subject to atmospheric input that in many cases dominate over input from the soil. The extent of foliar uptake is likely to vary with many factors including plant species, surface topography, age and growth stage, as well as the speciation of the metals involved (Berrow and Burridge 1991). Haghiri (1973) found that root uptake of

^{115}Cd, was significantly more efficient than via foliar uptake. In the present study, foliar uptake was not investigated and therefore this literature review will be restricted to consideration of uptake from the growth medium only.

Comparison with archived material has shown that the soils of all industrialised nations are contaminated with certain heavy metals above their pre-industrial levels (Jones 1991; Tjell and Christensen 1992). The increase in levels of such metals as Cd, Hg, Pb in European agricultural soils since the turn of the century, is estimated to be in the order of 10-15% (McBride 1995). In some areas the problem is considerably more serious, due to localised high impact pollution, originating from nearby mining or industrial activity (Farago, Cole, Xiao *et al.* 1992; Eklund 1995). Some studies have shown an increase of 40% in the Pb content of forest floors over the past 20 years (Friedland 1990). Metal pollution in agricultural soils has seriously affected food production in some areas (Vogeli-Lange and Wagner 1996), such as in the Tri-state Pb and Zn mining region of USA (Pierzynski and Schwab 1993), south-western Poland (Dudka, Piotrowska and Terelak 1996) and southern Bulgaria (Stefanov, Seizova, Pandev *et al.* 1995). According to Asami (1984), (cited by Reilly 1991) the release of Cd from non-ferrous metal mines and smelters, has resulted in 9.5% of paddy soils, 3.2% of upland soils and 7.5% of orchard soils in Japan being severely contaminated with Cd. Considering the above, it is of interest that several authors report declining metal input in Europe over the past few years, resulting primarily from improved legislation and decreased atmospheric emissions. These authors however, consider that inputs still exceed losses, since the residence time of most metal pollutants in the soil is extremely long (Jones 1991; Tjell and Christensen 1992).

Many investigations have demonstrated that increasing levels of metal pollutants in soils result in enhanced plant uptake (Khan and Khan 1983; Kumar, Dushenkov, Motto *et al.* 1995; Lozano-Rodriguez, Hernandez, Bonay *et al.* 1997). Despite this overall trend however, uptake of metals into plants is difficult to predict (Chaney and Hornick 1978). As a result, increased soil pollution levels over the last few decades,

have sometimes (Tjell and Christensen 1992) but not always (Jones 1991) been reflected in elevated metal pollutant loads in crops.

There is increasing evidence that microbial processes in the soil may be especially sensitive to heavy metals and that metal concentration limits may be set too high to protect these processes (Giller and McGrath 1989). This may have serious implications, especially for N-fixation and efficient cycling of nutrients (Heckman, Angle and Chaney 1987a). Results from research into these aspects are conflicting however and generalisations regarding the effect of metal pollution on soil micro-organisms and myccorhiza-plant interactions, difficult to make since many factors influence the response (Angle, McGrath, Chaudri *et al.* 1993; Punz and Sieghardt 1993; McIlveen and Negusanti 1994).

1.3.3.2 Metals involved in soil pollution

There are several metal pollutants that are considered to be of potential threat to environmental systems. The relative importance of these elements varies, depending on which part of the environment is considered. The most important metal pollutants found in soils include, Cr, Cu, Cd, Hg, Ni, Zn, and Pb (Marschner 1983; Friedland 1990). Due to their distinct chemistry and characteristics, each represents a different hazard (Woolhouse 1983). It should be stressed that the chemical behaviour of any element in the environment, including in the soil, depends on the nature of its compounds and species as they occur in the environmental system concerned. Physiological, ecological and toxicological effects of a metal are frequently specific and are dependent on the chemical species rather than the total metal content (Morgan and Stumm 1991).

1.3.3.3 Modes of entry into the soil

Various routes exist whereby metal pollutants can enter into the soil and thence into plants. These include either directly, from application of contaminated sewage sludge or fertilizer to agricultural soils, or indirectly, from atmospheric emissions and contaminated ground water. The relative importance of each route has been debated (Jones 1991; Chaney 1991), and will differ, depending on the extent and type of

pollution in a given area. This aspect is discussed in more detail under each specific metal pollutant (section 1.3.4.4 and 1.3.5.4).

1.3.4 Cadmium

1.3.4.1 Chemical characteristics and toxicity mechanisms

Cadmium (atomic number 48, atomic mass 112.4) belongs to the same group of the periodic table as Zn and exhibits similar chemistry (McKenna, Chaney and Williams 1993; Deighton and Goodman 1995). The oxidation state in all compounds is +2. Because the size of the Cd^{+2} ion is very similar to that of the Ca^{+2} ion, some similarity can be seen between the two chemical species (Stoeppler 1991). The divalent form of Cd is considered to be a Class B metal cation (Phipps 1981), or placed in the Borderline Class with relatively high Class B characteristics (Nieboer and Richardson 1980). Irrespective of which classification system is used, Cd^{+2} tends to be a soft Lewis acid and binds preferentially to donor atoms in the order S>N>O (Phipps 1981; Morgan and Stumm 1991). This is reflected in the fact that in biological systems, Cd is often found bound to S-containing molecules (Girling and Peterson 1981; Reilly 1991; Deighton and Goodman 1995). According to Sandman and Boger (1983), the toxicity of Cd (as well as Hg and Pb), is due to its strong affinity for acidic and thiol groups of proteins and nucleotides, thus interfering with the functioning of these compounds. These authors also claim that Cd competes with other metals such as Zn within the cell. Replacement of Zn in enzymes by the metal pollutant can lead to disruption of metabolic processes.

1.3.4.2 Hazards and toxicity

Cadmium is a non-essential element in plants (Verkleij and Schat 1990) and is recognized as one of the most potentially hazardous of all metal pollutants since it is extremely toxic to humans and other mammals (Rascio, Dallavecchia, Ferretti *et al.*1993; Cieslinski, Neilsen and Hogue 1996a). Whilst acute Cd poisoning in humans occurs, this metal also accumulates in mammalian kidneys, and has a

biological half-life of more than ten years. As a result, low levels in the diet over many years may lead to chronic toxicity symptoms (Quaife 1981; Reilly 1991; Stoeppler 1991). The situation is complicated by the fact that concentrations of other metals such as Zn, Cu, Fe and Se in food can influence safe dietary levels (Page, Bingham and Chang 1981). Addition of Zn to the diet can protect against Cd toxicity (Stoeppler 1991). Maximum ingestion levels for humans as recommended by the World Health Organisation (WHO) are 400-500 μg Cd per week and background levels from food usually contribute 200-250 μg Cd per week (Page *et al.* 1981). According to Tjell and Christensen (1992), a significant proportion of the world's population may already be suffering Cd effects of a sub-clinical nature, since in many parts of the world critical levels have been reached and the highest human exposure to this metal is in the most densely populated regions.

The first documented case of chronic Cd poisoning occurred in Japan in the late 1960s, where high levels of this element in rice caused outbreaks of *itai-itai* disease (Reilly 1991; Dudka *et al.* 1996). The rice had originated from nearby paddy fields, irrigated with water contaminated by Cd-rich mine-tailings from an adjacent zinc smelter (Page *et al.* 1981). It was this incidence of metal poisoning that initiated the intense research on metal-related environmental pollution that is still in progress today (Marschner 1983). It is of especial relevance to this study since the causative agent was Cd-contaminated seed.

The fact that this metal is fairly readily taken up by plants and translocated to aerial organs facilitates its entry into the food chain (Rauser and Meuwly 1995; Salim, Al-Subu and Ismail 1995a and b). An important aspect of Cd-toxicity is that plants can accumulate this element to high levels within edible portions before there are any obvious effects on growth (Chaney and Hornick 1978). This is in contrast to contamination with many other metals. For example, excessive uptake of Zn causes crop death thus preventing consumption by higher trophic levels (Giller and McGrath 1989).

1.3.4.3 Uses of cadmium

Although natural levels of Cd in the environment are generally low, anthropogenic activities can drastically increase these levels (Woolhouse 1983). Cadmium is obtained as a by-product of Zn, since for the purpose of commercial mining it occurs with Zn-containing ores (Lund 1981). Activities involved in the processing of raw Zn, such as mining, smelting, and galvanising therefore, often result in the presence of Cd as an impurity. Use of Cd is a relatively new phenomenon and as a result Cd pollution has increased rapidly in the past few decades as more uses for the metal are discovered (Alloway 1990). Forty-five percent of the total Cd production is used as corrosion-resistant plating, 15% is used as a pigment in paints, plastics and ceramics. Other uses include the production of alloys, plastic stabilizers, batteries, solar energy cells and in the manufacture of control rods for nuclear reactors (Lund 1981; Reilly 1991). It is expected however, that a decrease in consumption should occur in the next decade as restrictions and substitutions come into force (Stoeppler 1991).

1.3.4.4 Cadmium pollution and the soil

Alloway (1990), summarising the work of several authors lists the relative importance of Cd inputs into the soil. He states that for individual western countries the relative contributions of Cd from the major anthropogenic sources have been estimated to be: phosphate fertilisers 54-58%, atmospheric deposition 39-41% and sewage sludge 2-5%. These different modes of entry into the soil are discussed below.

i) Use of phosphate fertilizers

Phosphate fertilizers are an important source of metal pollution in soils (Jones 1991). Although P fertilizers contain trace amounts of Cr, Ni, Zn, the major contaminant is Cd (Hapke 1991; Gavi, Basta and Raun 1997; Grant and Bailey 1997). The amount of this metal in the final product depends on the source of the phosphatidic rock used, as well as the manufacturing process itself (Chaney and Hornick 1978; Marschner 1983). Phosphate fertilizers manufactured from phosphorite mined in the western USA, contained an average of 174mg/kg Cd and raised the soil Cd levels significantly in a 36-year field trial (Alloway 1990). According to this same author,

the relatively high Cd content of most phosphorites used for the manufacture of fertilizers, have resulted in P fertilizers becoming the most ubiquitous source of Cd contamination in agricultural soils. To counteract this problem, in some countries, such as Switzerland, legislation has been introduced to limit Cd levels in this class of soil amendments (Ewers 1991).

ii) Atmospheric emissions

Cadmium emissions in the atmosphere originate from smelting and refining of Zn, Pb, Cu and Ni, incineration of refuse and combustion of fossil fuel (Seaward and Richardson 1990; Stoeppler 1991). Other sources include, attrition of car tyres (Huang, Bazzaz and Vanderhoef 1974; Marschner 1983) as well as manufacturing and industrial processes (Reilly 1991). According to Berrow and Burridge (1991), Cd is associated with the very small particles (below 2 μm in diameter) that are typical of combustion sources and would be susceptible to foliar absorption.

iii) Use of sewage sludge

One aspect of soil pollution that has received extensive interest in the past few years is the application of sewage sludge to agricultural lands. This practice is an efficient waste disposal method and also improves soil condition, since sewage sludge contains high levels of valuable plant nutrients such as N and P, as well as high organic matter contents (Higgins 1984; Gardiner, Miller, Badamchian *et al.* 1995). Although sludge from rural areas is safe, that from urbanised areas can have very high metal contents (Wolnik, Fricke, Capar 1983). The composition of sewage sludge varies widely (Singh and Narwal 1984; Heckman, Angle and Chaney 1987b). Zinc, Cd, Cu, and Ni are the most common toxic components (Hani 1991), Mn, Fe, Cr, Pb, have also been reported (Valdares, Gal, Mingelgrin *et al.* 1983; Higgins 1984; Bojakowska and Kochany 1985). Factors governing uptake appear to be complex (Chang, Page, Warneke 1982; Wallace and Wallace 1994) and will be discussed in section 1.3.6.

There has been considerable controversy concerning application of sewage sludge to the same soil over extended periods of time. Whereas nutrients added to the soil

from the sewage sludge will be fairly rapidly removed by plant uptake and leaching, metals persist in the soils for a long time and pose a long-term phytotoxic threat. Because of possible changes in soil pH as well as other properties that influence metal bioavailability, it is difficult to predict long-term effects from sludge application (Heckman *et al.* 1987 a and b). It is well established that the organic fraction binds heavy metals, thereby hindering plant uptake to a large extent (Hani 1991). Chaney and Ryan ((1993) cited by McBride 1995) propose that a residual organic fraction will persist as long as the metal pollutants in the soil, thus protecting plants from excessive uptake. McBride (1995) on the other hand has rejected this claim and proposed the aptly termed "sludge time bomb hypothesis". He postulates that slow mineralization of organic matter in sludge may release metals into a more soluble, bioavailable form, resulting in elevated plant uptake. One of the obvious solutions to disposal of sewage waste, would be either to remove metal pollutants from the sludge before applying to land, or alternatively to reduce levels in industrial effluents at source (Chaney and Hornick 1978). Unfortunately both of these alternatives are extremely expensive (Giller and McGrath 1989).

1.3.5 Nickel

1.3.5.1 Chemical characteristics and toxicity mechanisms

Nickel has an atomic mass of 58.7 and an atomic number of 28. Several oxidation states can occur, but only Ni^{+2} is stable over the wide range of pH and redox conditions found in the soil environment (McGrath and Smith 1990; Reilly 1991). The Ni^{+2} ion is classed as a Borderline metal cation and has less Class B characteristics than Cd^{+2}. This element therefore reacts with a wider range of ligands and exhibits more complicated chemistry than Cd (Nieboer and Richardson 1980; Phipps 1981; Woolhouse 1983).

It has been postulated that the mutagenic and carcinogenic activity of Ni^{+2} may be due to the substitution of Mg^{+2} bound to the phosphate moieties of DNA and nucleotides. Replacement of Zn^{+2} by Ni^{+2} in enzymes also results in loss of activity

(Nieboer and Richardson 1980). According to Blakeley (1981), when substituted for a divalent metal ion in a metalloprotein or metalloenzme, Ni^{+2} generally adopts the ligands and co-ordination geometry of the original ion. If the normal functioning of the divalent metal ion in a biological molecule is to provide a positive charge, then Ni^{+2} can often replace ions of similar size such as Mg^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, Cu^{+2} and Zn^{+2}, with minimal effect on the functioning of the metalloprotein. Alternatively, if specific chemistry such as oxidation-reduction or oxygenation of the metal ion is an essential part of the function of the metalloprotein, then replacement of the normal metal ion by Ni^{+2} ion will generally result in protein deactivation. In addition, stable Ni^{+3} -complexes can form under certain biological conditions (Cross *et al.* 1985 - cited by Sunderman and Oskarsson 1991) and it is thought that valence shifts between Ni^{+2} and Ni^{+3} may be responsible for Ni-induced free-radical reactions and lipid peroxidation.

1.3.5.2 Hazards and toxicity

Although a serious environmental pollutant (Dallas and Day 1993; Sajwan, Ornes, Youngblood *et al.* 1996), Ni is considerably less toxic to living organisms than Cd (Rauser 1978; Nieboer and Richardson 1980). Unlike Cd, Ni does not appear to accumulate in mammals and only 3-6% of dietary Ni is retained in the body. It also appears to be an essential element in humans (Kieffer 1991). Nevertheless, cancer of the respiratory tract and dermatitis frequently occur in workers in Ni refineries and there has been growing interest in possible allergenic effects caused by high levels of Ni in the diet (Reilly 1991; Sunderman and Oskarsson 1991).

It has been postulated that Ni may be an essential micronutrient in plants under certain circumstances (Bollard 1983; Woolhouse 1983; Breckle 1991). The metallo-enzyme urease, has been shown to contain Ni as an integral component (Dixon, Gazzola, Watters *et al.* 1975). This enzyme catalyses the degradation of urea to carbon dioxide and ammonia. Thus plants grown on a urea-based medium that is deficient in Ni, show toxicity symptoms due to an accumulation of urea in the leaves (Eskew, Welch and Norvell 1984). This requirement for Ni, when grown utilizing urea as the sole N-source, was originally demonstrated in legumes (jack bean) but

has now been shown in a wider range of plants (Gerendas and Sattelmacher 1997). According to Marschner (1986), Ni is also essential for plants in which ureides (e.g. allantoin) play a significant role in nitrogen metabolism. In many legume species, including soybeans, ureides are an important form of soluble nitrogen during translocation from the root nodules to the shoots and seeds. Indeed, Eskew, Welch and Cary (1983) have reported that under conditions of severe Ni deficiency, soybean showed necrosis due to urea accumulation, whatever the source of N (fixed N, NO_3, or NH_4). In conventional experiments however, Ni is not likely to be limiting, as it is a common contaminant of water and chemical reagents (Brown *et al.* 1987).

1.3.5.3 Uses of nickel

World wide, up to 900 000 tons of Ni are produced annually (Sunderman and Oskarsson 1991). The Sudbury region of Ontario produces about 70% of the world's Ni, although considerable reserves do occur in other parts including South Africa (Cooley 1981). Nickel is used mainly for the production of corrosion-resistant metal alloys, particularly stainless steel. This versatile element is also utilised in the production of Ni-Cd batteries, electroplating and welding, and as a pigment for paints, fabric and ceramics. Nickel catalysts are also employed for organic syntheses, petroleum refining, and hydrogenation of edible fats (Cooley, 1981; Reilly 1991; Sunderman and Oskarsson 1991).

1.3.5.4 Nickel pollution and the soil

Background levels of Ni in soils from non-contaminated regions are usually in the region of 40 mg/kg but are extremely variable (McGrath and Smith 1990; Reeves, Baker, Borhidi *et al.* 1996). Some native soils however, specifically ultramafic (serpentine) soils, have high indigenous levels of this element (L'Huillier and Edighoffer 1996; Steyn *et al.* 1996; Kramer, Smith, Wenzel *et al.* 1997). Plant species and populations have evolved that are able to survive in nickeliferous soils (Peterson 1983; McGrath and Smith 1990). Nonetheless, in the vast majority of plant species including most economically important crops, Ni retards plant growth at relatively low concentrations (Bollard 1983; L'Huillier and Edighoffer 1996; Reeves *et al.* 1996).

Nickel from anthropogenic sources enters the soil via pathways similar to those of Cd. Namely, as a result of mining, burning of fossil fuels, fertilizer application (McIlveen and Negusanti 1994) and industrial activities (McGrath and Smith 1990). Although Ni contamination from fertilizers is low, Ni from sewage sludge has been found to accumulate in plants and this element has to be considered as a serious soil pollutant (McGrath and Smith 1990).

1.3.6 Factors affecting uptake and accumulation of metal pollutants in plants

1.3.6.1 Factors related to the growth medium

Numerous papers have illustrated that increasing metal content in the growth medium (whether soil, vermiculite or nutrient solution) result in enhanced levels in roots (Patel, Wallace and Romney 1977; Bishnoi, Chugh, and Sawnhey 1993). This has been shown for Cd in soybean, as well as in other plants (Smith, Brennan and Greenhalgh 1985; Costa and Morel 1994; Jalil, Selles and Clarke 1994). Similar results have been obtained for Ni (Pandolfini, Gabbrielli and Ciscato 1996; Yang, Baligar, Martens *et al.* 1996). Due to the many inter-related factors that regulate metal uptake into plant roots however, this trend is not always directly proportional to metal concentration and its magnitude is difficult to predict (Chaney and Hornick 1978; Baker and Walker 1989). To complicate matters further, occasional studies, (Dunemann, Von Wiren, Schulz *et al.* 1991) have not shown a positive correlation between soil metal content and plant uptake. Factors involved in metal uptake include, soil characteristics and composition, the chemical speciation of the metal, as well as the plant species. The main points pertinent to uptake of Cd and Ni are discussed below.

i) Metal speciation

Metal cations bind to exchange sites on soil constituents such as clay colloids, organic matter (e.g. fulvic and humic acids) as well as mineral (Fe, Mn and Al) hydrous oxides (Schmitt and Sticher 1991; Mench, Didier, Loffler *et al.* 1994). The strength of this binding depends on the nature of the components involved and will

determine what proportion of the total metal content is bound (Chaney 1991). It is generally accepted that with respect to potentially toxic ions, including Cd^{+2} and Ni^{+2}, plant uptake is related to the activity of the free metal ion, M^{n+} (Chaney 1991; Morgan and Stumm 1991; Ritchie and Sposito 1995). Although recent research (Lorenz, Hamon, Holm *et al.* 1997), indicates that this may not be a valid assumption for all metals in all soils. The question of bioavailability is discussed further in Chapter 7. Metal cations that bind very strongly to soil components will not be accessible to plants for uptake and thus different metals show different bioavailablities. For example, even though Pb is ubiquitous in soils due to widespread pollution from automobiles, most plants show low levels of Pb uptake (MacNicol and Beckett 1985; Hooda, McNulty, Alloway *et al.* 1997), since this element is bound tightly to soil particles (Kumar *et al.* 1995; Ewers and Schlipkoter 1991). In contrast, Cd (Mench *et al.* 1994) and Ni (Schmitt and Sticher 1991) are relatively mobile within acid or neutral soils, although movement is somewhat restricted at pH > 6.7. The anomalous results obtained by Dunemann *et al.* (1991), who demonstrated that short-term uptake of Ni by oat plants from three different soils, was not determined by the total Ni concentration in those soils, can be explained by the fact that Ni was tightly bound in one of the soils and hence bioavailability was low. Thus, not only total metal content determines the extent of plant uptake, but also the chemical form or speciation (Xian 1989; Hirsch and Banin 1990). It follows therefore that the chemical form in which the metal ion is supplied in the growth medium, is also critical to uptake (Alloway 1990). Girling and Peterson (1981) compared uptake from nutrient solution amended with five different inorganic Cd compounds. Root Cd levels were seven times higher when the metal was supplied in the form of the sulphate ion than as CdS. Similarly, uptake of Cd when supplied as an inorganic salt can also differ widely from when supplied in an organically bound form, such as in sewage sludge (Valdares *et al.* 1983). Amendments to soils and nutrient solutions, such as addition of chelates or fertilizer, can all affect the speciation of the heavy metal in question and thus plant uptake (Chaney and Hornick 1978).

ii) pH and other factors

Any factor that shifts the equilibrium in favour of increased Cd^{+2} activity in the soil solution (or growth medium) can lead to enhanced uptake into plants (Chaney and Hornick 1978). In the case of Cd, the most important factors governing uptake are considered to be pH and total Cd concentration (Page *et al.* 1981). It is well documented that for Cd and Ni, as well as for most metal ions, decreasing pH of the soil solution generally leads to an increase in uptake (Mizuno 1968; Singh and Narwal 1984; Hirsch and Banin 1990; Punz and Sieghardt 1993). This is most likely due to competition at soil binding sites between protons and metal cations, resulting in reduced adsorption of the metal. Such a hypothesis is supported by the work of Schnitzer and Skinner (1967), who found that the amount of metal bound to fulvic acid (a ubiquitous organic soil component) increased at higher pH values. As a result of chemical speciation and because of the absence of soil particles to bind excess protons, similar results have not always been obtained for nutrient solution systems however (Zhu and Alva 1993). It should be noted that several other soil-related factors such as is the cation exchange capacity (CEC), soil redox potential, temperature, texture and moisture content (Chaney and Hornick 1978; Schmitt and Sticher 1991; Ernst 1996) all influence metal availability. These will not be discussed further however as they are not relevant to nutrient solution systems used to cultivate plants in the present study.

iii) Root exudates and soil microflora

It has become increasingly apparent that higher plants, bacteria and fungi, are able to modify the growth medium immediately adjacent to roots (Rovira and Davey 1974; Lorenz *et al.* 1997). Plant roots secrete a wide range of compounds from protons and hydrogen carbonate ions, amino acids, organic acids, simple phenolics to more complex molecules such as isoflavenoids (Curl and Truelove 1986; D'Arcy Lameta and Jay 1987). Such plant exudates are able to change the speciation of metal ions in the growth medium (Linder, Voye' and Cocks 1990) and thus alter the rate and extent of uptake into plants. In the case of nutrients, enhanced mobilization may be due to the direct effect of root exudates, or indirectly due to activation of micro-organisms which in turn affect soil pH, redox potential or secrete chelating agents

(Chaney 1991). There are several non-specific mechanisms utilised by plants in the uptake of nutrients, including metal ions. These include root-induced changes in pH as a result of preferential cation uptake, as well as release of organic acids by the roots (Marschner 1986). Research into this aspect of metal uptake has focused primarily on uptake of Fe from Fe-deficient soils since this element is often limiting to plant growth (Chaney 1991). Compared to nutrients however, little is known concerning the role of plant exudates in metal pollutant uptake. According to Ernst (1996), the impact of these plant-borne organic compounds on the uptake of metals from contaminated soils is great. Lorenz *et al.* (1997) found that soil solution from the rhizosphere contained higher levels of DOC (dissolved organic carbon) than the bulk soil solution. They ascribed this to the release of organic root exudates and resulting increase in microbial activity. Increasing concentrations of DOC during plant growth were correlated with a decrease in the proportion of free Cd^{+2} and Zn^{+2} in the rhizobial soil solution, due to complexation. Mench and Martin (1991) on the other hand, found that an increase in Cd solubility in the rhizosphere of apical root zones due to root exudates, was likely to be an important cause of the relatively high Cd accumulation in *Nicotiana* spp. compared to *Zea mays.* There appears to be a paucity of research covering this aspect of metal pollutant biotoxicity. Even less is know concerning the importance of plant exudates in plant uptake from nutrient solutions. Perhaps this is not surprising since according to Hodge, Grayston and Ord (1996), the qualitative and quantitative measurement of root exudates is fraught with difficulties.

1.3.6.2 Plant-related factors

From the soil or nutrient solution, metal ions diffuse into the root apoplast where the interfibrillar and intermicellar pore sizes, as well as the cation exchange capacity (CEC) of the root walls, can have a discriminating effect on metal ion uptake (Marschner 1986). As in the case of nutrients, metal pollutants have to pass through the plasma membrane into the symplast and may be subjected to control at this point (Ouariti, Gouia and Ghorbal 1997). The most obvious way for plants to tolerate the presence of metal pollutants in the growth medium, would be exclusion at the root level, either by alteration of membrane permeability or increased exudation of metal-

chelating substances. Despite this however, compared to lower plants, there are few convincing reports of restriction of metal pollutant uptake in metal-tolerant races of vascular plants (Baker and Walker 1990; Verkleij, Lolkema, De Neeling *et al.* 1991). In fact, there is much evidence to suggest that plants growing on a metalliferous growth medium contain the element, or elements in question and hence it can be said that such plants cannot prevent metal uptake, they can only restrict it (Peterson 1983).

i) Excluder, accumulator and hyper-accumulator plants

There is a general tendency for immobilization of metals within root tissues (Baker and Walker 1990; Garate, Ramos, Manzanares *et al.* 1993) and root metal levels are usually (but not always) the highest of all plant parts. Translocation to the aboveground portions of the plant is dependent on many factors and the accumulation patterns of these organs have come under close scrutiny since they can indicate mechanisms of coping with metal-induced stress (Baker and Walker 1990). Peterson (1983) has classified tolerant plants into excluders, index plants and accumulators according to the extent of metal uptake from the roots into the shoots. In the case of excluder plants, metal concentrations in aerial plant parts are maintained at constant and/or low levels over a wide range of soil concentrations (Punz and Sieghardt 1993). Thus the ratio of leaf/root concentration is low due to restricted transport to the shoots (Baker and Walker 1990). Restricted translocation to the shoot is often found in tolerant species or ecotypes but this is by no means universal. Hendry, Baker and Ewart (1992), examined Cd uptake in tolerant and non-tolerant clones of the grass *Holcus lanatus,* and found that transport of Cd to the shoots was restricted in the tolerant clone. They postulated that shoots are more sensitive to metal toxicity than roots, because of potential free radical generation during photosynthesis and therefore require protection (section 1.3.7.2). In contrast, research conducted by Mattioni, Gabbrielli, Vangronsveld *et al.* (1997) showed that accumulation of both Ni and Cd in the shoots of *Silene italica* was greater in the Ni-tolerant clone compared to the non-tolerant clone, indicating that another tolerance mechanism other than restricted shoot transport was in operation. The physiological basis for differential transport has not been closely examined (Peterson 1983), but

control is likely to be operating at the endodermis (Baker and Walker 1990). Beyond a critical metal load the mechanism breaks down however and unrestricted transport results (Punz and Sieghardt 1993). In the case of index (indicator plants), uptake and transport of metals to the shoot are regulated so that internal concentration reflects external levels (Punz and Sieghardt 1993), thus index species provide an estimation of soil metal concentration (Peterson 1983). Different plant species growing in the same soil will show varying foliar concentrations, some species being more efficient in this respect than others. According to Peterson (1983) an accumulator can be defined as "a species whose metal concentration exceeds that present in the soil or, alternatively as "a species whose metal concentration exceeds the normal values for metal concentrations in plants on a particular soil." Hyper-accumulators are a specialised group of accumulators in that metals reach very high levels in the above ground plant parts (Baker, McGrath, Sidoli *et al.* 1994). Whilst several species of Ni hyper-accumulator plants have been reported (Reeves *et al.* 1996), hyper-accumulation of Cd (>100 µg/g.dm) is rare (Baker *et al.* 1994; Pollard and Baker 1997) and may only occur in cases of co-accumulation with Zn (Vazquez, Barcelo, Poschenrieder *et al.* 1992).

ii) Monocotyledonous versus dicotyledonous plants

Within the above framework it can be seen that plants growing in the same growth medium, vary widely from one species to another in their ability to extract metals, whether nutrients or pollutants (Haghiri 1973; MacNicol and Beckett 1985; Inouhe, Ninomiya, Tohoyama *et al.* 1994). Bingham, Page, Mahler *et al.* (1975) investigated Cd uptake in crop plants and found a wide range in susceptibility, since accumulation is also dependent on the uptake ability of the specific plant species. Interesting differences have been reported regarding translocation in shoots of monocotyledons and dicotyledons. Experiments conducted by Inouhe *et al.* (1994) demonstrated that not only did the monocotyledonous species (maize, oat, barley and rice) take up more Cd than the dicotyledons (cucumber, tomato, lettuce) more was exported to the shoots. Despite this, yield was reduced less in all monocotyledonous species than the dicotyledons. Overall, monocotyledonous species in the experiment appeared to

be more tolerant and this was linked by the authors to increased phytochelatin production in the roots.

iii) Sensitivity of different plant species

Within a given species, major variations even between cultivars have been reported (Marschner 1983). Florijn and Van Beusichem (1993) investigated uptake of Cd^{+2} from soil or nutrient solution, in 19 inbred lines of maize. Although uptake of the metal pollutant was very similar between all lines, wide differences were found in the internal distribution. One group retained most of the Cd in the roots, translocating little to the shoots, whereas in another group, root to shoot concentrations were similar. Intra-specific variation in Cd uptake has been reported in soybean (Boggess, Willavize and Koeppe 1978; Marchiol, Leita, Martin *et al.* 1996). Differences between cultivars in Ni uptake have also been recorded (L'Huillier, d'Auzac, Durand *et al.* 1996; Pandolfini *et al.* 1996).

Different plant species vary in the extent of toxicity symptoms exhibited at a given concentration of metal pollutant in the tissues. This is implied in the work of Inouhe *et al.* (1994) described in the preceding section. Even though monocotyledonous plants contained higher levels of metal pollutant, yield was less depressed than in the case of dicotyledonous plants. According to MacNicol and Beckett (1985), studies on young plants have shown that uptake of Zn, Cu, Ni or Cd as well as plant yield are influence by environmental conditions and nutrient status. The upper critical levels (i.e. the lowest tissue concentration of an element at which its toxic effects reduces the production of dry matter) for a given plant species and plant organ are independent of these factors however. Using reports from the literature, these authors have calculated upper critical levels for different metal pollutants in a range of plant species. The upper critical level for Cd in soybean leaves was approximately 6 µg/g.dm whilst an equivalent value was not given for Ni. From these data, the most sensitive plant species can be identified, as well as the most toxic metal (Harrington, Roberts and Nickless 1996). Accordingly these can be ranked Cd>Cu = Ni>Zn (MacNicol and Beckett 1985). Tissue culture has shown that differential responses to heavy metals between tolerant and non-tolerant plants can

be demonstrated at the cellular and subcellular levels (Baker and Walker 1989; Kramer *et al.* 1997). Reasons for the inherent sensitivity of one species (or cultivar) compared to another are not clear. Sensitivity may be a result of the presence of specific metabolic processes susceptible to metal pollutants, or because of an absence of tolerance or detoxifying processes (section 1.3.8).

1.3.6.3 Synergistic and antagonistic effects

It should be noted, that pollution frequently results in contamination with more than one metal at a time (Wallace and Wallace 1994) and that synergistic and antagonistic effects between metals are often apparent (Wallace and Berry 1989; Singh, Bharti and Kumar 1994; Salim *et al.* 1995b). McKenna *et al.* (1993) found that due to interactions between Zn and Cd, factors influencing the accumulation of these metals in roots and leaves of lettuce and spinach were complex. Accumulation depended on plant species, on leaf age, as well as on the relative concentrations of the two metals. Nickel competes with other cations (Ca^{+2}, Mg^{+2}, Fe^{+2} and Zn^{+2}) for uptake, so that high concentrations of Ni in the growth medium can lead to zinc or iron deficiency (Marschner 1986). This is discussed further in section 7.3.

1.3.7 Toxicity effects

1.3.7.1 General resposes to metal pollutants

Metal pollutants have been found to affect a wide range of physiological and biochemical processes in all parts of the plant (Clijsters, Van Assche and Gora 1991). Physiological processes affected include photosynthesis (Carlson, Bazzaz and Rolfe 1975; Maksymiec and Baszynski 1996; Moustakas, Ouzounidou, Eleftherio *et al.* 1996), nitrogen fixation and metabolism (Bishnoi *et al.* 1993; Hernandez, Carpena-Ruiz and Garate 1996), nutrient uptake and assimilation (Mizuno 1968; Ouariti *et al.* 1997) and protein synthesis (Shah and Dubey 1995). As a result, metal-stressed plants frequently exhibit reduced biomass (Przymusinski and

Gwozdz 1994). This list is by no means complete and a more extensive review is given in Woolhouse (1983). Responses often involve enzyme inhibition, for example Cd reduces nitrate reductase activity in *Pisum spp.* shoots (Hernandez *et al.* 1996), but in some cases stimulation occurs (Mattioni *et al.* 1997). The situation is complicated by the fact that due to inherent differential sensitivities of plants, as well as a range of toxicity mechanisms, different effects are elicited in different plant species and this is dependent on the metal pollutant concerned (Clijsters *et al.* 1991). Plant age can also affect the nature of the toxicity symptoms (McIlveen and Negusanti, 1994). It is extremely difficult to distinguish between primary and secondary toxicity effects (Rauser 1978; Woolhouse 1983). Due to the complex, inter-related metabolic processes of higher plants, a detrimental event occurring in one process is likely to have serious repercussions on others. With the possible exception of Cu, the primary molecular toxicity mechanisms of metal pollutants are still not known with any certainty (De Vos and Schat 1991).

1.3.7.2 General toxicity mechanisms

Possible toxicity mechanisms can be postulated on the basis of the chemical reactivity of the metals. Firstly, the affinity of metal pollutant cations with high Class B -type character such as Cd^{+2}, for thiol groups was mentioned earlier (Nieboer and Richardson 1980). Secondly, substitution between bivalent cations is also a very likely toxicity mechanism (Clijsters *et al.* 1991). Thirdly, the ability of transition metals such as Fe and Cu, to undergo redox reactions makes them effective catalysts of the Haber-Weiss reaction resulting in hydroxyl radical generation (De Vos and Schat 1991). Nickel has already been mentioned in this regard (section 1.3.5.1). Many authors have postulated that one of the primary toxicity mechanisms, of at least some metal pollutants, is membrane damage due to lipid peroxidation (Lozano-Rodriguez *et al.* 1997). It should also be noted however, that whilst it is well known that metal pollutants cause free radical damage, lipid peroxidation is only one of the potential consequences (McDonald *pers. Comm.* 1999). Differential effects of heavy metal ions in bringing about oxidative stress (Gallego, Benavides and Tomaro 1996) and membrane damage in cells of tolerant and non-tolerant plants have been observed (Baker and Walker 1989). Although it has been well documented that

excessive toxic metal loads can result in oxidative stress and alterations in enzyme activities, this is a general effect caused by several environmental stresses not just metal pollutants (Clijsters *et al.* 1991; Koricheva, Roy, Vranjic *et al.* 1997). De Vos and Schat (1991) caution that as yet, only in the case of Cu is there some evidence that lipid peroxidation has to be considered as an important primary effect of toxic levels of this metal. They state further that it is difficult to estimate the significance of oxidative stress in metal toxicity phenomena in higher plants, since too little research has been conducted in this area. It should be noted that Cu-induced membrane damage in roots of *Silene cucubalus* is thought to be brought about by at least two different mechanisms; namely binding to thiol-proteins in the membrane as well as lipid peroxidation (De Vos, Schat, Vooijs *et al.* 1989).

1.3.7.3 Toxicity symptoms elicited by cadmium

Prasad (1995a) has reviewed Cd-toxicity effects in vascular plants and gives an extensive list of those reported in the literature. These effects range from reduction in growth, photosynthesis and respiration to alterations in chloroplast structure and water relations. Woolhouse (1983) states "it is not clear whether the wide range of reported effects represents the primary mechanisms of Cd^{+2} or merely part of what may prove to be an almost endless catalogue of secondary effects". Only selected toxicity responses are discussed therefore.

Cadmium toxicity in plants is often correlated with chlorosis and wilting which might be related to reduced Fe uptake and to disruption of water relations (Marschner 1983). The toxic effects of Cd in roots of corn and soybean were ameliorated by additions of Zn or Fe-citrate to the nutrient growth solutions (Malone, Miller and Koeppe 1978). This effect is not unique to Cd however, other metals including Ni have been cited as causing Fe-deficient chlorosis (Bollard 1983).

Oxidative stress brought about by Cd has been reported by several authors (Somashekaraiah, Padmaja and Prasad 1992; Lozano-Rodriguez *et al.* 1997). Cadmium was found to cause lipid peroxidation in the shoots (but not roots) of Cd-sensitive *Holcus lanatus* clones (Hendry *et al.* 1992). The molecular mechanisms

are poorly understood since Cd^{+2} is not a redox metal and direct generation of hydroxyl radicals is unlikely (Gallego *et al.* 1996). There are several indirect ways however, in which Cd could promote oxidative stress in plant cells. Firstly, blockage of electron transport chains in cellular membranes might lead to incomplete reduction of molecular oxygen and subsequent increased formation of oxygen free radicals (De Vos and Schat 1991). Secondly, one of the major responses of plant cells to Cd (and some other metal pollutants) is the production of phytochelatins (discussed further on) which bind via sulphydral groups to the metal. Phytochelatins are synthesised from glutathione, an important anti-oxidant in plants. Hence, increased Cd loads in the cell could possibly result in reduced protection from free radicals due to depleted glutathione levels (De Vos and Schat 1991). Addition of glutathione has been found to overcome the effect of Cd-stress in rice seedlings (Chen and Kao 1995a) and *Chlorella* spp (Kaplan, Heimer, Abeliovich *et al.* 1995). Whether this is due to lowering of free Cd as a result of increased phytochelatin synthesis, or a consequence of enhanced anti-oxidant activity due the elevated glutathione levels, is not clear. Finally, due to competition for membrane carriers or via other mechanisms, high extra-cellular levels of one metal might lead to a decrease in another involved in the radical defence mechanism (De Vos and Schat 1991). These authors quote the work of Cardinaels, Put, Van Assche *et al.* (1984) who suggested that the lowered activity of Cu/Zn SOD (superoxide dismutase) in Cd-stressed bean plants resulted from decreased uptake of Zn. Somashekaraiah *et al.* (1992) also noted that Cd resulted in a significant decrease in the activities of the anti-oxidant enzymes, catalase and SOD.

1.3.7.4 Toxicity symptoms elicited by nickel

Toxicity effects of Ni in plants, have been reviewed by Mishra and Kar (1974) and more recently by McIlveen and Negusanti (1994). A range of responses have been reported, varying from reduction in growth of leaves and roots (L'Huillier *et al.* 1996), depressed photosynthetic rates (Carlson *et al.* 1975) increased rates of respiration (McIlveen and Negusanti 1994) and enhanced carbohydrate accumulation in leaves (Rauser 1978; Moya, Ros and Picazo 1995), to enzyme inhibition (Mishra and Kar 1974) and activation (Pandolfini *et al.* 1996; Mattioni *et al.* 1997). In addition, effects

on seed germination, unique to Ni, have been reported (Mishra and Kar 1974) and will be discussed in section 5.3.

1.3.8 Tolerance mechanisms

There is extensive literature concerning metal-tolerance in higher plants and reviews include those of Peterson (1983a), Peterson (1993b) and Baker and Walker (1990). Since soybeans are considered to be relatively sensitive to metal pollutants (Bingham *et al.* 1975; Lutrick, Robertson and Cornell 1982), this topic will only be considered briefly. The fact that considerable variation between soybean cultivars with regard to susceptibility to metal toxicity exists however (Marchiol *et al.* 1996), indicates that tolerance mechanisms are not completely absent from this species.

It is generally accepted that tolerance to metal stress is based on mechanisms that maintain a low free metal concentration in the cell (Brune, Urbach and Dietz 1994). Tolerance mechanisms for many plant species challenged with heavy metals have still not been fully elucidated (Harrington *et al.* 1996; Harmens, Den Hartog, Ten Bookum *et al.* 1993a). This is partly a consequence of the fact that heavy metal tolerance results from many different strategies, which vary from plant to plant as well as between ecotypes (Baker and Walker 1990; Schultze and Hutchinson 1991; Punz and Sieghardt 1993). Metal tolerant species or ecotypes have evolved independently in several families and species, so it is perhaps understandable that a selection of tolerance strategies should be involved (Peterson 1993). It should also be borne in mind that tolerance strategies for excesses of essential metals may well differ from those of non-essential metals (Verkleij *et al.* 1991).

Two tolerance (or avoidance) mechanisms in higher plants have already been mentioned, namely exclusion from roots, as well as restriction of metals within roots resulting in low metal enrichment in aboveground parts. Another strategy involves translocation of excessive metals to the leaves, which are then shed at the end of the season (Punz and Sieghardt 1993). Possible tolerance mechanisms at the

tissue or cellular level include accumulation of Cd and Zn in the epidermal cells of metal-stressed barley leaves (Brune *et al.* 1994 and 1995) and restriction of toxic metals to cell walls (Verkleij and Schat 1990). High levels of the free metal ion in the cytoplasm may be avoided by the operation of cellular detoxification strategies. For example, metals may be bound to organic acids, such as citrate or malate and sequestered in the vacuoles (Schultze and Hutchinson 1991). Other strategies for maintenance of low metal concentrations include exudation into the apoplast, precipitation of the metal as insoluble salts as well as detoxification by complexation or binding (Brune *et al.* 1994).

Several potential metal-chelating compounds have been isolated in plants (Casterline and Barnett 1982; Rauser 1984), of which the most important is phytochelatin (Rauser 1990). Phytochelatins were first isolated by Grill and co-workers from cell suspensions of *Rauvolfia serpentina* and were found to be small polypeptides of the formula (γ-glutamyl-cysteinyl) $_n$-glycine (Grill, Winnacker and Zenk 1985). It has subsequently been found that the value of n varies from 2-11, and that in some plants, such as legumes, the terminal glycine is replace by β-alanine and (Rauser 1990). These compounds are produced in a wide range of plants in response to excessive metal loads (Harmens *et al.* 1993a and b; Gupta, Rai, Tripathi *et al* 1995; Galli, Schuepp and Brunold 1996). Phytochelatins are synthesised from glutathione (homo-glutathione in the case of some plants such as soybeans) and are not a direct product of protein synthesis (Grill *et al.* 1985; Gupta *et al.* 1995). Glutathione, as discussed earlier, is an important antioxidant in plant tissues. This area has been a field of intensive research in the last 15 years but the exact role of these compounds in metal tolerance is still not certain. Some workers contend that enhanced phytochelatin production does confer metal tolerance (Howden, Andersen Goldsbrough *et al.* 1995; Inouhe *et al.* 1994) whilst others contend that phytochelatins are merely a sign of metal stress and not a tolerance mechanism (Schat and Kalff 1992). It seems fairly certain though that phytochelatins do play a role in metal-homeostasis (Verkleij *et al.* 1991). Other

metal chelators have been postulated in plants including metallothioneins (Leita, De Nobili, Mondini *et al.* 1993) and organic acids (Guo and Marschner 1995).

Several of the tolerance mechanisms discussed above have been found to be operational in plants challenged with high levels of Cd. The most notable is that of Cd-induced phytochelatin, which is likely to detoxify excess Cd under certain circumstances (Rauser and Meuwly 1995). Cadmium seems to be especially efficient in inducing production of these compounds, the higher the metal load, the longer the γ-glutamyl-cysteinyl chain, which bind to the metal via the thiol groups of cysteine (Vogeli-Lange and Wagner 1996; Harmens *et al.*1993a). Other putative mechanisms for maintaining low cytosolic concentrations of this metal pollutant include Cd-containing granules in parenchyma cells of *Zea mays* (Rauser and Ackerley 1987) and compartmentation in vacuoles (Kneer and Zenk 1997).

Research efforts into tolerance strategies to Ni have been dominated by work on hyper-accumulators of this metal (Woolhouse 1983). Many of the tolerance mechanisms noted above have also been reported in response to Ni, including detoxification by binding to free L-histidine (Kramer, Cotter-Howells, Charnock *et al.* 1996). It is not clear from the literature whether Ni stress induces phytochelatins. The presence of high concentrations of Ni did not stimulate the formation of phytochelatins in barley (Brune *et al.* 1995), bean, rice, kale, or maize (Guo and Marschner 1995). But Ni did result in enhanced phytochelatin production in cell suspensions *of Rauvolfia serpentina* (Grill et al. 1987, cited by Rauser 1990).

1.3.9 Translocation of cadmium and nickel

The form in which Cd or Ni is translocated within the plant is of importance, since this governs mobility as well as the extent to which accumulation within specific organs will occur. Movement of Cd from root to shoot is thought to take place via the xylem and to be driven by transpiration from the leaves. On the other hand, movement of Cd into developing seeds is likely to be via the phloem, although little research has

been carried out into this aspect (Hart, Welch, Norvell *et al.* 1998). The chemical form in which Cd and Ni are translocated is still not known with any certainty (Chaney 1991). *In vivo* xylem exudates of soybean were found to contain two anionic Cd complexes and several iorganic forms of Cd (Cataldo, Garland Wildung 1981). Leita *et al.* (1993) have suggested that the metal is translocated as the free metal ion. According to Petit and Van de Geijen (1978; cited by Ouariti *et al.* 1997), ion exchange along fixed anionic sites in the xylem vessel walls plays an important role in the long-distance transport of Cd. On the other hand, Guo and Marschner (1995) postulated that phytochelatins may be involved in Cd translocation from roots to shoots in maize.

Studies of the form in which Ni is translocated in hyper-accumulator plants have found that this metal is frequently chelated with citric, malic and malonic acid (Woolhouse 1983; Baker and Walker 1990). According to Chaney (1991) the Ni-chelator in the majority of crop species however, is not citrate. Identification of this compound is likely to be complicated by the fact that the number and distribution of specific Ni-containing complexes varies with plant age, and appears to be related to the availability of organic acids, amino acids and peptides being produced and transported in the xylem as the plant matures (Cataldo, McFadden, Garland *et al.* 1988).

1.4 LITERATURE REVIEW: SEED DEVELOPMENT

1.4.1 Developmental stages in orthodox seeds

As befits its status as an important crop plant, seed development in soybeans has been investigated extensively (Obendorf and Wettlaufer 1984; Spaeth and Sinclair 1984a and b), especially with regard to factors influencing final seed yield and quality (Dunphy, Hanway and Green 1979; Beaver, Cooper and Martin 1985; Egli and TeKrony 1995). Soybeans are orthodox seeds and thus can be stored at low moisture contents for prolonged periods (Ellis, Hong and Roberts 1987). Indeed, maturation drying during development is a prerequisite for vigorous seedling growth (Rosenberg and Rinne 1986). Development in orthodox seeds can be divided into three phases (four, if germination is also included), although the duration of each phase may vary depending on the species and growth conditions (Adams and Rinne 1980; 1981). The three development phases are shown in Fig. 1.2.

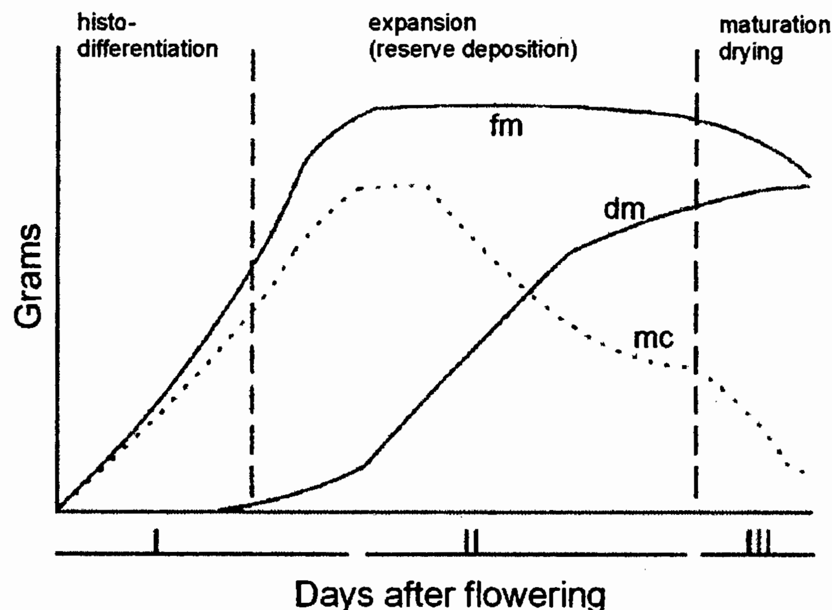


Fig. 1.2 Generalised seed development curve. Changes in fresh mass (fm), dry mass (dm) and moisture content (mc). with seed age are shown. Description of seed development phases (I, II, III) given in text. (Adapted from Bewley and Black 1994).

Phase I, initiated by pollination, is a period of prolific cell division and elongation. During this stage of development, histodifferentiation takes place, i.e. the zygote divides and differentiates into an embryo. At the sub-cellular level, organelles required for storage reserve deposition are synthesised. This stage is characterised by a rapid increase in fresh mass and high moisture content.

Phase II is characterised by a steady increase in dry mass as synthesis and deposition of storage reserves proceeds. Whilst rapid in the beginning, this slows down as the seeds approach maximum dry mass. Cells enlarge to accommodate storage reserves produced. Moisture content reaches a peak and then starts to decline as storage reserves replace water in the cells. Due to a balance between water loss and increase in dry mass, after reaching a maximum, fresh mass tends to remain more or less constant during this period.

Phase III is distinguished by a rapid decrease in moisture content accompanied by a concomitant decline in fresh mass. This is referred to as maturation drying and the seeds are eventually shed from the plant after this event. If seeds are non-dormant, and environmental conditions are suitable, germination takes place (**Phase IV**). Storage reserves laid down during development are then used for growth of the new seedling.

1.4.2 Developmental stages in soybean seeds

A review of the literature reveals that soybean seeds comply with the general pattern of orthodox seed development as described above. Seed growth curves for soybean cultivars typically show a short lag phase, followed by a linear phase of growth, extended almost until maximum seed mass (Fraser, Egli and Leggett 1982; Guldan and Brun 1985). The duration of each growth phase may differ between cultivars and as a result of differing environmental regimes (Egli 1975; Beaver *et al.* 1985). In soybean, as in most legumes, the pods reach maximum size before the seeds start to swell (Bewley and Black 1994), although some increase in pod dry mass may still

occur during seed development (Fraser *et al.* 1982). The latter authors have shown that there is a strong correlation between pod size and seed size, suggesting that testa or pod wall provides a physical limitation to seed growth. During the lag phase of growth, histodifferentiation takes place. According to Bils and Howell (1963), cell division is generally considered to be completed in the cotyledons by about 14 DAF (days after flowering), except for in the embryo axis in which mitosis may still occur. Although this may not be true for all cultivars, since Guldan and Brun (1985), found that cell division in one out of three cultivars tested (a medium seed size cultivar) showed little decrease in the rate of cotyledon cell division well into the phase of storage reserve accumulation (phase II).

1.4.3 Storage reserve accumulation in soybean seeds

Proteins and lipids constitute the major storage reserves of soybean seeds and are deposited mainly within the cotyledons (Yazdi-Samadi, Rinne and Seif 1977). Seeds produced on a single plant late in the growing season, tend to be smaller than those produced early on (Egli, Leggett and Wood 1978). Efforts to correlate variations in seed size with position within the pod, as well as pod position on the plant, have yielded inconsistent results however (Spaeth and Sinclair 1984b). The number of cotyledonary cells is also an important factor governing seed growth and final seed mass at maturity. Cultivars with a high number of cotyledonary cells produce the biggest seeds, with the highest storage reserve content (Guldan and Brun 1985). Hymowitz, Collins, Panczer *et al* (1972) analysed 60 soybean lines and found that (on a dry mass basis), protein ranged from 33-49%, oil from 15-23% and total sugars 6-11%. The approximate balance being made up from structural carbohydrates (cellulose and hemicellulose) 17%, crude fibre 5% and ash 6% (Rubel, Rinne and Canvin 1972). Soybeans are unusual in that they contain approximately 2 parts protein to 1 part oil, compared to other oil seeds in which the ratio is usually reversed (Brim 1973). In addition to the extent of storage reserves, environmental regimes and cultivar can influence chemical composition (Hartwig 1973; Salunkhe, Sathe and Reddy 1983).

1.4.3.1 Proteins

The two most important storage proteins are conglycinin, a glycosylated 7S protein, and glycinin, a non-glycosylated 11S protein (Meinke, Chen and Beachy 1981). Tertiary and quaternary structures of these components have been well-documented (Hill and Breidenbach 1974a and 1974b; Mosse´ and Pernollet 1983; Vitale and Bollini 1995). Newly synthesised proteins are targeted via the ER (endoplasmic reticulum) and golgi apparatus to the vacuoles, which fragment and give rise to protein bodies (Vitale and Bollini 1995). The exact details of protein processing as well as formation of protein bodies are not clearly understood (Shewry, Napier and Tatham 1995). It is of interest that the protein components are not all synthesised at the same time. The 7S β sub-unit of conglycinin and 11S A-4 sub-unit of glycinin appear one to two weeks after the other protein sub-units (Meinke *et al.* 1981).

1.4.3.2 Lipids

At maturity, the total lipid content of soybean seeds comprises approximately 88% neutral lipids (triglycerides), 10% phospholipids and 2% glycolipids. Whilst the latter two lipid classes are found mainly in cell membranes, the triglycerides are stored in small organelles called oil bodies or spherosomes (Salunkhe *et al.* 1983). Oil bodies are bound by a half unit membrane and are synthesised from the ER (Bewley and Black 1994). The relative proportions of the esterified fatty acids change during seed development. Whereas palmitic, stearic and linolenic acids decrease, oleic and linoleic increase (Rubel *et al.* 1972; Dornbos and McDonald 1986). At maturity, soybean seeds contain higher proportions (79%) of unsaturated, than saturated fatty acids (Salunke *et al.* 1983).

1.4.3.3 Carbohydrates

Carbohydrate metabolism in soybean seeds is unusual (Adams, Rinne and Fjerstad 1980; Bewley and Black 1994) in that carbohydrates are transiently stored in the cotyledons as starch grains (Yazdi-Samadi *et al.* 1977). This may represent storage of excess carbohydrate until redistribution to proteins and lipids, since shortly before maturation, starch is catabolized and reallocated to different storage reserves (Adams *et al.* 1980). The starch content of mature soybean seeds is consequently

low (Bils and Howell 1963; Arora 1983). According to (Adams *et al.* 1980) this may be due to the heavy demands made on carbohydrates as precursors for protein and oil synthesis, since in pea (a species not noted for extremely high protein or lipid levels) starch accumulation continues until maturity. There also appears to be a shift in the balance between reducing and non-reducing sugars during seed development. Glucose, fructose and galactose levels are relatively high during the early stages of seed development but then decrease, whereas levels of the oligosaccharide, non-reducing sugars, sucrose, stachyose and raffinose steadily increase (East, Nakayama and Parkman 1972; Yazdi-Samadi *et al.* 1977; Lowell and Kuo 1989). Monosaccharides are either absent or present in very low concentrations at maturity (Dornbos and McDonald 1986). Shifts in carbohydrate content may possibly be in preparation for maturation drying, since stachyose has been found to play an important role in conferring desiccation tolerance in seeds (Blackman, Obendorf and Leopold 1992). The changes in free monosaccharides and sucrose levels are also consistent with the invertase control hypothesis for seed development, whereby high levels of hexose sugars promote cell division and high sucrose levels promote storage reserve accumulation (Weber, Borisjuk and Wobus 1997).

1.4.3.4 Phytate

Soybean seeds also accumulate phytate (myo-inositol 1,2,3,4,5,6-hexakis phosphate) during development (Raboy and Dickinson 1984). In addition to being the storage form of phosphorus in seeds, this compound can also bind Zn, Ca, Mg, Fe and other cations (Marschner 1986; Bewley and Black 1994). Phytate is present as discrete globoid crystals in the protein matrix of large protein bodies (Lott, Greenwood and Batten 1995), although its composition and distribution vary with species as well as tissue (Bewley and Black 1994). Phytates are an important source of mineral salts for the germinating seeds (Marschner 1986) and will be discussed further (section 4.1 and 4.4) in connection with localization of metal cations.

1.4.4 Moisture content and seed development

Physiological maturity (PM) is defined as attainment of maximum seed dry mass and in soybeans, is found to coincide with maximum seed viability and vigour (TeKrony, Egli and Phillips 1980). At this stage there is no further uptake of photosynthates into the seed due to non-functioning of the vascular system in the seed coat, brought about by maturation (TeKrony, Egli, Balles *et al* 1979). Due to the high moisture content, seeds cannot be harvested commercially at this point, but remain on the plant until harvest maturity (the first time that <14% moisture content is reached) when they are picked (TeKrony *et al.* 1980). During this period, environmental stress can lead to decreased seed quality, especially with respect to seedling vigour (Ellis *et al.* 1987).

Change in seed water status triggers the metabolic events leading to the cessation of dry matter accumulation (Egli 1990). In soybean seeds, moisture content drops steadily from approximately 85%, with development. At about 60% moisture content, seeds enter the maturation phase when dry weight accumulation ceases and rapid dehydration commences (Adams and Rinne 1980). A value of 61-58% moisture content for soybean seeds at PM has been reported by many authors (Crookston and Hill 1978; Obendorf *et al.* 1980; Ellis *et al.* 1987) and appears to be constant across cultivars and environmental conditions (TeKrony *et al.* 1979). After PM, during maturation drying, there is a rapid decrease in water content, which is primarily controlled by environmental factors (TeKrony *et al.* 1980).

Although seed germination can occur in soybean seeds as early as 35% of final dry mass, such seeds frequently do not establish as seedlings. It is only once the moisture content has dropped below a critical value of 60%, that full seedling vigour is attained (Miles, TeKrony and Egli 1988). Thus a moisture-loss treatment (to below 60%) applied either artificially in the laboratory, or naturally as during seed development *in planta*, is required in order for developing seedlings to make the switch from cell elongation required for germination, to the activities of cell division

and growth, which are required for seedling establishment (Rosenberg and Rinne 1986). According to these authors, moisture loss from soybean seeds appears to be a prerequisite for the synthesis of new proteins, which may be part of the metabolic process or processes that allow the seed to undergo the transition from germination to growth. Much of the time spent during soybean seed development is not for production of viable seed, but rather for the production of storage reserves (Adams and Rinne 1981).

1.4.5 The anatomy of developing and mature soybean seeds

1.4.5.1 The testa

The seed coat plays a critical role in the nutrition of the developing seed (Murray 1987; Weber *et al.* 1997). In order to make the discussion of pathways followed by nutrients and other chemicals relevant therefore, the anatomy of developing soybean seeds is described with special reference to the testa. Since this study is largely concerned with metal pollutant-mediated effects occurring during the later stages of development (phase II and III), histodifferentiation and early embryogeny of soybeans will not be considered in any detail. A review of soybean seed ontogeny can be found in Carlson (1973), or in Chamberlin, Horner and Palmer (1993).

Soybean seeds are attached to the pod at the placenta, which runs the length of the pod (Carlson 1973). Ovules of the soybean are camphylotrophous, i.e. they bend towards the distal portion of the ovary during development. The micropyle is thus close to the placenta and part of the outer integument becomes joined with the funiculus which connect the seed to the maternal plant (Thorne 1981). According to Chamberlin *et al.* (1993), the ovules are bitegmic and thus have an inner and outer integument. Ontogeny of the soybean embryo follows the normal pattern of development through globoid, early and late heart stages (Carlson 1973; Chamberlin, Horner and Palmer 1994). At the earliest growth stage at which seeds were investigated in this study (VIP seeds, <15 days after flowering, section 3.2.1.1), the cotyledons had developed so that they almost filled the central cavity. According

to Chamberlin *et al.* (1993), by approximately 25 days post-fertilization, the embryo is structurally mature. This equates to roughly 25 days after flowering (DAF) since fertilisation occurs on the first day of full anthesis (Peterson, Mosjidis, Dute *et al.* 1992).

A longitudinal section through the testa of soybean seeds (Fig. 1.3) reveals that the testa consists of three distinct layers, namely; the outer epidermis, middle hypodermis and inner parenchymatous layer, which have developed from the outer integument (Esau 1960; Van Staden, Manning and Kelly 1989). Situated beneath the cuticle, the epidermis is comprised of a single row of radially elongated, palisade cells with cell walls that are thickened towards the long axis of the cell (Vaughan 1970). These sclerified cells are characteristic of leguminous seeds and are also known as malpighian cells (Corner, 1976; Gunn 1981). The palisade cells of legumes commonly have a "light line" *(linea lucida)*, which is an area of the cell wall that is refractive to light.

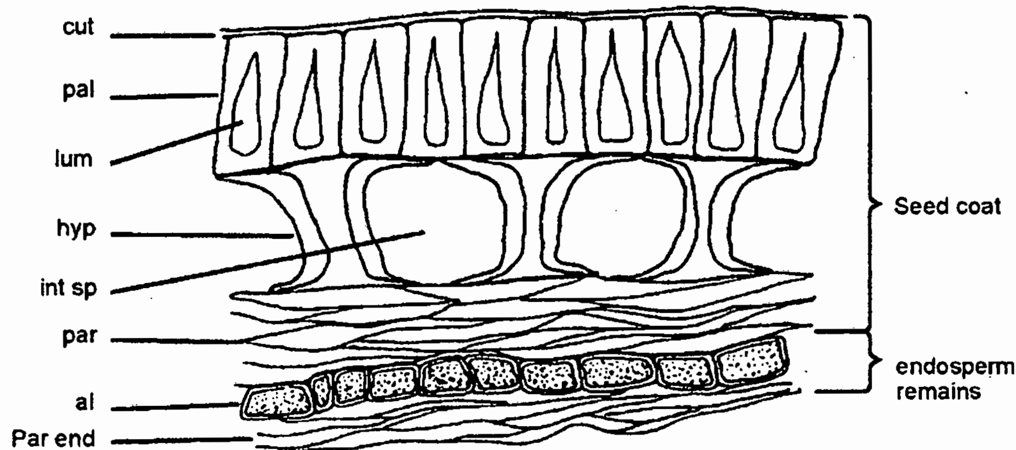


***Fig. 1.3** Section through the testa of soybean (cv "Hawkeye"). Mag. X 535 (approximate). al = aleurone cells of endosperm; cut = cuticle; hyp = hourglass cells of hypodermis; int sp = intercellular space; lum = lumen; pal = palisade cells; par = compressed parenchyma cells; par end = remains of cells of endosperm. (From Carlson 1973).*

Extensive research has been conducted into this feature as it is thought that the impermeable nature of the testa may be due to the structure of the palisade cell walls (Boesewinkel and Bouman 1995). Beneath the epidermis lies the hypodermis (sub-epidermal layer) which is differentiated by 18 DAF (Carlson 1973). This is comprised of a single layer of distinct hourglass-shaped cells with large air spaces in between. These have thickened walls in the middle portion of the cell and play an important role in providing structural support (Carlson 1973, Thorne 1981). The third layer of the testa is comprised of 6-8 layers of parenchymatous cells, which, according to Thorne (1981), in developing seeds is divided into two distinct zones. An outer zone with thick cell walls, many intercellular air spaces, and many plasmodesmata, maintaining symplastic integrity between adjoining cells. The cells of this area possess relatively dense cytoplasm and organelles characteristic of cells engaged in active carbohydrate transport and excretion. An inner parenchymatous zone of thin-walled aerenchyma is also present. These cells have very few organelles and little cytoplasm. The vascular system of the seed is found at the junction between these two types of parenchyma.

The inner surface of the seed coat, the endothelium is formed by single layer of small thick walled cells, most likely originating from the inner integument (Thorne 1981; Chamberlin *et al.* 1993). According to (Carlson 1973), by 20-30 DAF the endosperm has almost disappeared and at maturity, it is no longer functional (Bewley and Black 1994). At this stage, only a few remnants of endosperm tissue remain; namely: an outer aleurone layer, with a high protein content and underneath this, several layers of flattened parenchymatous cells (Vaughan 1970; Carlson 1973). Thorne (1981) describes the wall of the embryo sac, immediately interior to the endothelium, as consisting of a single layer of multi-cellular tubules, attached at one end to form an undulating surface, facing the seed coat, the other ends free. According to this author, these microtubules were still living weeks after the endosperm they surrounded was digested. In a more recent review of soybean seed development (Chamberlin *et al.* 1993), although an endothelium was observed, no mention of an embryo sac wall composed of multicellular tubules was made.

According to Thorne (1981) this layer deserves more study, because of a strong putative role in embryo nutrition.

In the region of the hilum, two layers of palisade cells are found (Thorne 1981). The inner layer is derived from the epidermis of the seed, the outer layer from the funiculus (Vaughan 1970). Beneath this region is the hypodermis, composed also of a single layer of characteristic hourglass-shaped cells. Interior to this, are three zones of parenchyma. The outermost layer is stellar parenchyma with large air spaces directly in contact with the air spaces of the hypodermis. A middle parenchyma layer consists of small, flattened cells with many vascular bundles. The third and innermost layer is made up of more or less typical parenchyma. A bar of non-vascular, tracheid-like cells is present down the middle of the hilum and is thought to store water at certain times during seed development (Thorne 1981).

1.4.5.2 The embryo

The cotyledons are the major storage organs of the embryo (Smith 1981) and take up most of the seed interior. Carlson (1973) describes the cotyledons as having a semicircular shape in transverse section, with a flattened adaxial surface and rounded abaxial. An epidermis of cuboid-shaped cells is present with aleurone grains and stomata. The flattened adaxial surface has 1-3 layers of palisade cells, which are less pronounced on the abaxial surface. The inside is filled with loosely arranged mesophyll cells that are filled with protein bodies and oil droplets and occasional calcium oxalate crystals. The hypocotyl-radicle of the embryo axis is slightly flattened and fits into a groove between the two cotyledons. An epicotyl or plumule is present with two well-developed primary leaves (Carlson 1973).

1.4.5.3 Uptake routes and vascular system in developing seeds

The funiculus contains a single vein and is the only vascular connection between the pod and the developing seed (Chamberlin *et al.* 1993). This vascular bundle enters the chalazal end of the hilum and then branches into three. One vascular strand ends near the hypostase at the chalazal end of the embryo sac. The two lateral vascular bundles branch off and run along the dorsal portion of the outer integument

stopping short of the micropyle (Thorne 1981). No further vascular system is present in the young ovule of soybeans until the late heart stage (approximately 25 days post-fertilization), when provascular strands in the cotyledons, branch to form a network of vasculature (Chamberlin *et al.* 1993). The entire testa then becomes vascularised except in the micropyle area over the axis (Thorne 1981). Vascular tissue in both immature ovules and as well as older seeds is comprised of mostly phloem tissue, with limited xylem (Thorne 1981; Murray 1987; Chamberlin *et al.* 1993). The vascular system is located in the parenchymatous layer of the seed coat and as seeds mature, it becomes is non-functional due to crushing of the parenchymatous layer by the enlarged embryo (TeKrony *et al.* 1979). In soybean (Thorne 1981; Chamberlin *et al.* 1993), as for other seeds (Boeswinkel and Bouman 1995; Weber *et al.* 1997) there is no vascular connection between maternal tissue and the developing embryo. Although a poorly developed vascular system is present in the embryo itself (Webster and Leopold 1977), including reticulate veination in each cotyledon (Carlson 1973).

Pathways and mechanisms involved in transport of nutrients or pollutants into seeds are poorly understood (Boesewinkel and Bouman 1995). From the point of view of quantity, sucrose is the most important solute transported into developing legume seeds (Van Staden *et al.* 1989). Little is known concerning entry of mineral salts and trace elements into seeds (Lott *et al.* 1995). Several pathways are involved in nutrient flow, depending on the stage of development and structure of the ovule (Boesewinkel and Bouman 1995). In very young soybean ovules a cuticle is present between the lateral portions of the endothelium and the embryo sac, so that labelled carbon from the three vascular traces accumulates in tissue at the micropyle and chalazal ends of the embryo sac. By the late heart-shape stage of development (approximately 25 days post-fertilization), the cuticle has disappeared and lateral uptake from the vascular bundles in the seed coat occurs (Chamberlin *et al.* 1993). Unloading of sucrose and amino acids from the phloem appears to be an active process, requiring energy (Van Staden *et al.* 1989). Plasmodesmata are numerous between the sieve cells and companion cells, as well as between the phloem and cells of the upper parenchymatous layer (Thorne 1981). There is no symplastic

connection between the maternal tissue and the embryo however (Thorne 1981). Passage of solutes from the seed coat into the embryo is therefore apoplastic, both in soybean (Thorne 1981), as well as seeds in general (Weber *et al.* 1997; Van Staden *et al.* 1989). Some degree of symplastic transport through the testa however is involved, since it has been shown clearly that the chemical composition of phloem sap arriving in the seed coat is very different to that entering the embryo (Murray 1987; Bewley and Black 1994). Thus the testa plays a role not only in transport, but also modifies, secretes and controls, nutrients and other chemicals entering the embryo.

1.5 OBJECTIVES OF THE STUDY

The objectives of this study are to address the following questions:

1. If plants are cultivated in a growth medium containing a given concentration of Cd or Ni, what proportion of metal pollutant is accumulated within the seeds?

2. What effect do Cd and Ni have on the basic physiological and biochemical processes taking place as seeds develop from late embryogenesis, through maturation to germination and seedling establishment?

3. What effect does accumulation of metal pollutants within such seeds have on the quantity and quality of storage reserves and the germinative capacity of such seeds?

4. What is the effect of exogenous applications of metal pollutants on seed germination and seedling establishment?

5. What is the chemical environment to which the plant roots are exposed in the nutrient solution? How much of the metal pollutant supplied is in a form suitable for plant uptake and what is the effect of amendment of nutrient solution with Cd or Ni on the bioavailabiltiy of other chemical species?

CHAPTER 2

PLANT GROWTH AND POD FORMATION

2.1 INTRODUCTION

As stated in the objectives, this study is largely concerned with the effects of metal pollutants on seed development and functioning. Environmental stress experienced by the mother plant during seed ontogeny however can greatly affect the final seed output (Weiner, Martinez, Muller-Scharer *et al.* 1997). This may take the form of reductions in size, yield or other seed parameters such as the quality and quantity of storage reserves (section 1.4.3). Thus in this chapter, the phenology of the plants and the effect of the two metal pollutants, Cd and Ni thereon is described. Of especial interest, is the growth and development of the pods, since these organs, in addition to supplying nutrients, are also responsible for protection of the developing seed.

It follows from the above, that in order to compare different metal treatments on seed parameters, it is necessary to control as many variables as possible during plant growth and seed development. This includes environmental variables, as well as nutritional or root-related factors. Soil is a complex and heterogeneous matrix, whose chemical composition within a given profile, varies with both time and depth (Harris 1987). This in turn affects the binding and retention of ionic species in the soil solution and hence the availability of nutrients and metal pollutants to plants. The prevailing irrigation regime as well as pH will also affect the above (Flegmann and George 1980). In addition, nutrient uptake varies in extent and specificity of ions along the length of a given root (Asher and Edwards 1983) resulting in further heterogeneity of the growth medium. To avoid the above problems, research workers have often used the technique of nutrient solution culture to grow plants under carefully controlled conditions (Imsande and Ralston 1981; Davis, Hossner and Persaud 1993). Using this approach, parameters

such as pH, ionic strength, temperature of the growth medium as well as the concentration and speciation of the macro- and micro-nutrients can be relatively easily manipulated and the effect on plant growth and function noted (Asher and Edwards 1983; Alva, Edwards, Asher *et al.* 1986).

In their review of solution culture techniques, Asher and Edwards (1983) point out that there are inherent limitations to this method. Firstly, there are major differences between solution and soil culture techniques with regard to mechanical support of the plants, root exudate-microbe interactions as well as the chemical composition of the growth medium. The chemical composition of most nutrient solutions is less complex than that of soil solution. Thus it is possible that potential essential elements in plant metabolism may be omitted from nutrient solutions. Concentrations of nutrient ions in the soil solution are relatively low. Mineral salts taken up by plants are constantly replaced from the soil matrix so that a constant but low level is maintained. In nutrient solution systems however, the provision of nutrients at such reduced concentrations requires frequent replacement of the basal medium, very large volumes of medium per plant, or sophisticated systems that can continuously measure plant uptake of chemical species and replace those species as required. The logistics of supplying the nutrient solution in large volumes or ensuring frequent replacement, as well as labour and cost can be considerable. Automated nutrient analysis and replacement systems are not cheaply or widely available. As a result, in order to avoid nutrient depletion, ions tend to be supplied in concentrations several orders of magnitude greater than present in the soil solution. In addition, because the nutrient solution is often not well buffered, large changes in pH and nutrient concentrations can occur within a short time. Despite the above limitations, it is considered that well-designed nutrient solution systems represent the preferred system for studying effects of root environment parameters on the physiology and growth of plants (Asher and Edwards 1983).

Quantitatively, N is the most important of all the macronutrients and the chemical form in which this element is supplied can have a profound effect on the pH of the nutrient solution (Hewitt 1966; Smith, Johnston and Cornforth 1983; Bugbee and Salisbury

1989). If N is supplied purely as nitrate (NO_3^-), uptake of this ion from the nutrient solution by legumes leads to an increase in pH of the medium (Imsande and Ralston 1981; Landsberg 1989). Reduction of nitrate within the plant results in the production of hydroxyl ions (OH^-) which are then exuded by the plants in order to maintain electro-neutrality. Conversely, uptake and assimilation of ammonium ions (NH_4^+) results in protons (H^+) being released by the roots and a decrease in pH of the surrounding medium (Imsande 1986). Experiments by this same author have shown that a nitrate to ammonium ratio of 4:1 or 5:1 yield relatively constant pH values in a nutrient solution used to grow soybean. In the present study, uptake of nitrate uptake was deemed to be important, not only because of its role as a macronutrient, but also because of its effect on nutrient solution pH and hence metal pollutant bioavailability.

Soybean plants have been grown by many research workers using a nutrient culture technique (Malone *et al.* 1978; Duke, Vaughan and Wauchope 1985; Alva *et al.* 1986; Marchiol *et al.* 1996), although relatively few (Haghiri 1973; Cataldo, Garland, Wildung *et al.* 1978a; Schmitt and Weaver 1984; Khan and Weaver 1989) have grown the plants through the entire life cycle, until the mature pod stage. Many studies have utilised nutrient solutions containing N in the form of NO_3^- (Rauser 1973; Cataldo *et al.* 1978b; Landsberg 1989). In some, NO_3^- was supplemented with low levels of NH_4^+, especially during the early stages of growth (Schmitt and Weaver 1984; Grusak and Pezeshgi 1994). Imsande (1986) found that whereas using nutrient solution containing a combination of both NO_3^- and NH_4^+ was beneficial for maintenance of pH homeostasis, growth was not enhanced compared to plants grown on NO_3^- alone. Preliminary experiments were carried out at the beginning of this project to compare growth of soybean plants in Long Ashton (1mM NH_4^+ plus 3mM NO_3^-) and Hoagland's (12mM NO_3^-) nutrient solution. Seedlings were grown in 1-litre jars containing nutrient solution until approximately mid-way through development of the first pods (data not reported). It was found that the second formula (Hoagland's solution) yielded the healthiest and largest plants. Differences in nutrient solution concentration (i.e. half-strength or full-strength) did not seem to have any obvious effect during the first few weeks of plant growth and so full-strength was used throughout the entire life span of the plants. In the

interest of simplicity, and to avoid the introduction of another variable, the seedlings used in this project were not inoculated with N-fixing bacteria and thus nitrate was the sole N source.

2.2 MATERIALS AND METHODS

2.2.1 Standard experimental procedures

2.2.1.1 Environmental parameters

Throughout the study, plants were grown in a controlled environment chamber with a day temperature of 25 °C and night temperature of 20 °C. A 12-hour photoperiod was employed. Relative humidity was approximately 50% and the average light intensity at full plant height, approximately 800 $\mu E/m^2/sec$.

2.2.1.2 Plant cultivation

Unless otherwise stated, plants were grown according to the general procedure detailed below. Soybean seeds (*cv.* Crawford) were obtained from the Oil and Protein Seed Centre at the Agricultural Research Council, Potchefstroom, South Africa. Twenty-five seeds of uniform size were germinated according to the standard method given elsewhere in this work (section 3.2.4.4). After 7 days the largest seedlings were transferred from the germination container to 1-litre plastic jars filled with the standard nutrient solution. The hypocotyl was encircled with a strip (approximately 20 mm x 100 mm) of dacron wool and carefully introduced into a short (approximately 10 cm) length of plastic hosepipe. The supported seedlings were then inserted into holes made in the lids, so that when placed on the jars, the roots reached into the nutrient solution. Aluminium foil was wrapped around the containers in order to exclude light. The position of the growth jars was randomized daily in order to eliminate any variation due to differences in microhabitat. The nutrient solution was aerated continuously with

compressed air supplied through 6-mm plastic tubing.

The standard nutrient solution used throughout this study was modified from that of Hoagland and Arnon (1938) as cited by Noggle and Fritz (1976). The chemical composition of the nutrient solution is given in Table 2.1. Analytical grade reagents and deionised water were used throughout. The pH of freshly prepared nutrient solution was approximately 5.02 and was adjusted to 6.0 with 0.1M NaOH before use. Nutrient solution in the starting jars were replenished (i.e. topped up to the 1 litre mark) with deionised water every day. The pH of individual plants was not adjusted daily, since it was found that pH during this phase of seedling growth changed very little and was consistently within the range of 5.8-6.2. The nutrient solution was renewed every week.

***Table 2.1** Chemical composition of the standard nutrient solution used in the study.*

Macronutrient	**Final concentration (mM)**
KH_2PO_4	1.0
$MgSO_4.7H_2O$	2.0
$CaNO_3$	4.0
KNO_3	4.0
Micronutrient	**Final concentration (μM)**
FeNaEDTA	89.9
H_3BO_3	46.0
$MnCl_2.4H_2O$	9.1
$ZnSO_4.7H_2O$	0.8
$CuSO_4.5H_2O$	0.3
$H_2MoO_4.H_2O$	0.1

After two weeks in the litre containers (3 weeks after imbibition), the seedlings were large enough to be transferred to a circulating nutrient system. Plants were selected on the basis of uniformity. The plants (still inserted into the lids) were placed over holes in plastic boards such that the roots were immersed in nutrient solution contained in 25 litre plastic tanks. Four plants were grown in each growth tank. Aerial plant parts were supported by strings, attached to the ceiling of the growth chamber. A diagram of the circulating nutrient system is shown in Fig. 2.1 (note for the sake of clarity, the system of tubing used for aeration of the nutrient solution is omitted). In addition to the upper growth tank in which the roots were immersed, nutrient solution was also contained in a lower reservoir tank (volume approximately 25 litres) and was circulated between the two. A Shiruba SP-107 aquarium air pump was used to lift the solution from the reservoir tank to the growth tank via two inlet tubes. The nutrient solution was allowed to return to the reservoir container under gravity via a single outlet. Nutrient medium entered the growth tank from the top and exited from an outlet at the bottom, thus ensuring adequate mixing. An overflow system was also fitted. The circulating system was constructed in such a manner that in the event of a power shortage or pump malfunction, the roots would not be subjected to desiccation since only one third of the growth medium could drain to the reserve tank. The total volume of nutrient solution in each system was exactly 40 litres, approximately 20 litres in each of the growth tank and reservoir tank. The overall flow rate varied depending on the relative volumes of the two containers. When the volume in the reservoir tank was at a maximum (22 litres), the water pressure and thus the flow rate into the growth tank was also at a maximum (approximately 0.5 litres/min). This decreased to a minimum of approximately 0.25 litres/min when the volume in the reservoir tank decreased to a minimum (18 litres). The average flow rate was approximately 0.33 litres/min and thus the length of time required for the entire nutrient solution to be circulated was roughly 2 hours. One cycle from maximum volume of reservoir tank and again to maximum lasted approximately 10 min.

Deionised water was added to each growth tank every 1 - 2 days in order to bring the total volume back to 40 litres. A Radiometer PHM80 portable pHmeter was used to determine the pH of both reservoir and growth tanks, and the mean pH calculated. The

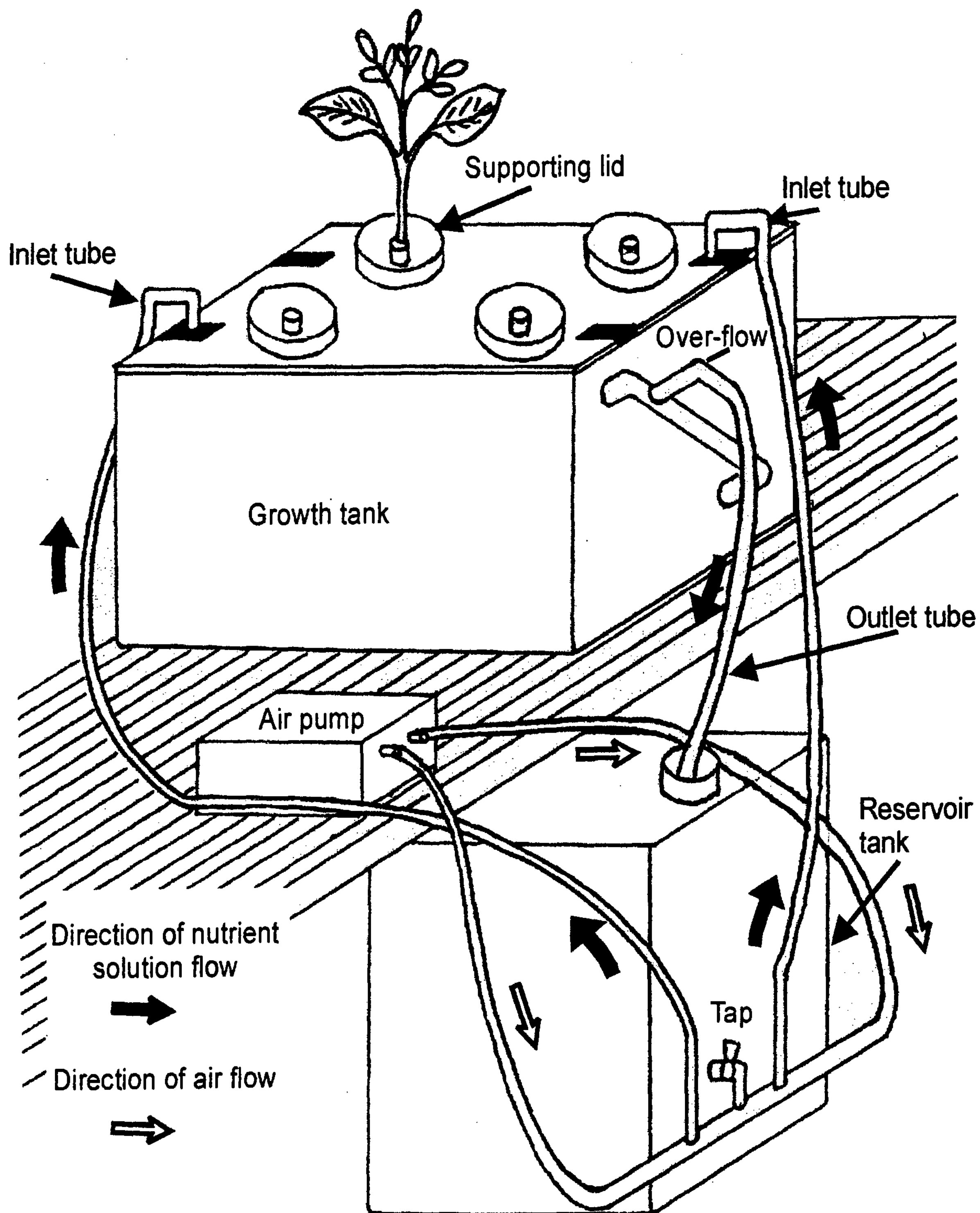


Fig. 2.1 Diagrammatic sketch of the circulating nutrient solution system used in the study. Aeration system ommitted for clarity. Total volume of system = 40 litres.

difference of this calculated value from the nominal, or starting value of 6.0 was recorded as Δ pH. The system was then adjusted to 6.0 (± 0.2) with either 0.01M H_2SO_4or 0.1M NaOH as appropriate and the nutrient solution well stirred. Every 10 days for the first three weeks and thereafter every week, the growth systems were drained, rinsed with deionised water in order to remove any microbial contamination and the nutrient solution renewed. Each growth tank was aerated continuously with compressed air, supplied by means of one plastic (6 mm) tube per plant. To prevent algal growth, light was excluded from the entire system. In addition, silicone tubing and plastic were used to avoid possible contamination of the nutrient solution (Hewitt 1966; Grusak and Pezeshgi 1994). Rubber tubing and metal components were employed only where they would not come into contact with the growth medium.

2.2.1.3 Monitoring of nitrate levels

Nitrate levels in the nutrient solution were determined using the method of Cataldo, Haroon, Schrader *et al.* (1975). The nutrient solution in each growth tank was first adjusted to a volume of 40 litres and pH of 6.0 (± 0.2) as described above. After stirring to ensure homogeneity, samples of nutrient solution were extracted during the 9th week of growth in the case of the Cd-treatment experiments and during the 7th week of growth in the case of Ni-treatment experiments. During the assay week, samples were taken on day 0 (i.e. shortly after the nutrient solution had been changed) as well as a few days later in order to assess nitrate uptake by the plants.

Nitrate standards ranging from 0.5 to 8 mM were prepared using the basal nutrient solution (from which N had been omitted). A volume of 0.8 ml of reagent A (5 g of salicylic acid in 96% sulphuric acid, freshly prepared) was added to 0.1 ml of sample or standard. The solution was then made up to a total volume of one millilitre with distilled water. The mixture was shaken well and then incubated for 20 min at room temperature. Nineteen millilitres of reagent B (2 M sodium hydroxide) were slowly added to the above, the mixture stirred again and then left to cool to room temperature. Absorbance at 410 nm was read within 60 min on a Beckman Spectrophotometer in the case of the Cd-treatment and on a Varian Cary 1E spectrophotometer in the case of Ni. Each sample

was assayed in triplicate. Optical path length was 10 mm.

2.2.1.4 Treatment with metal pollutant

Treatment plants were subjected to Cd- or Ni-stress by addition of the appropriate volumes of a $CdCl_2$ or $NiCl_2$ stock solution to the basal nutrient medium. Preliminary experiments were carried out in which the metal pollutant concentration was varied (see below). From these experiments it was decided to use a concentration of 0.05 mg/litre (0.05 ppm ≡ 0.45 μM) Cd^{+2} or 1 mg/litre (1 ppm ≡ 17 μM) Ni^{+2}, for the rest of the study. The stress treatment was initiated when the seedlings were 21 days old and transferred to the circulating growth system. Thereafter the same concentration of metal pollutant was added on renewal of the nutrient medium, until termination of the experiment. Due to space restrictions, only four growth tanks, and thus 16 plants, could be accommodated in the environmental chamber at one time. Therefore the Cd-stress experiments were first carried out, using one growth tank as the control treatment and the others as treatment tanks. The Ni-stress experiments were conducted subsequently, also with the same tank as control and three treatment tanks. Care was taken to ensure that environmental and growth parameters were constant. Before changing to Ni, the growth tanks and tubing were washed out with detergent, 10% nitric acid and finally with deionised water. Samples of water from the final rinse of each growth tank were checked for Cd pollution using the standard metal assay procedure given below. Cadmium contamination of the growth tanks was minimal and is therefore not discussed further.

2.2.1.5 Determination of metal content in plant tissue

Plant tissue was washed under running deionized water for 20 sec and then rinsed in ultra-pure (Millipore) water. After oven drying for 48-72 hr at 70°C, the material was ground to fine powder in a coffee grinder. Samples of approximately 0.5 g were weighed out into crucibles and ashed in a RKC Instruments muffle furnace for 5 hours at 500°C. On cooling, the samples were transferred quantitatively to thick walled glass boiling tubes using approximately 10 ml of concentrated HNO_3. Blanks containing no plant tissue were also included. The samples were heated to 180°C in a thermostatted

digestion block for approximately 24 hours and more concentrated HNO_3 added as required. Digestion was considered to be complete when the ash from the plant tissue had completely dissolved in the matrix solvent (0.1M HNO_3). The solution was then transferred quantitatively into a 25 ml volumetric flask using 0.1M HNO_3. Samples were analysed for total Cd or Ni content using a Jobin Yvon JY138 Ultra-trace ICP-AES (inductively coupled plasma - atomic emission spectrometer).

Metal levels in nutrient solution were determined by first filtering an aliquot of nutrient solution through Whatman's No. 2 filter paper in order to remove any plant matter. An appropriate volume of HNO_3 was then added in order to give a resulting acid concentration of 0.1 M in 25 ml of sample. The samples were assayed for Cd or Ni content using ICP-AES.

To prevent contamination, all glassware was soaked overnight, firstly in 5% Contrad and then 10% HNO_3, rinsed well in distilled water and finally in ultra-pure water.

2.2.1.6 Statistical treatment of the data

In this chapter and in the rest of this work, differences between means were tested for significance using Student's t test at the 95% confidence limit. In cases where the sample size was small, Wilcoxon's rank sum test was employed. Unless otherwise stated, all results are reported as the mean and standard deviation. The computer package Statistica (version 5.0) was employed for data analysis.

2.2.2 Effect of metal concentration on plant growth

For the initial experiments, $CdCl_2$ was added to the nutrient solution to give a resulting metal concentration of 0 (control), 0.05, 0.1 or 0.5 mg/litre. Four plants were grown in each tank according to the standard procedure. In subsequent experiments the same experimental design was employed, excepting that $NiCl_2$ was added to the nutrient solution at levels of 0 (control), 2, 5 and 10 mg/litre. Visual toxicity symptoms exhibited

by the plants were noted. At senescence, the roots of plants from each growth tank were washed in distilled water and the total volume of roots per growth tank determined. To this end, roots were immersed in a 2-litre beaker to a depth matching that of the nutrient solution system. The volume of these organs was measured as the amount of water displaced. The roots were then dried in an oven at 70 °C for 24 hours and the dry mass determined. The total number of pods produced by the plants in each concentration treatment was also recorded.

2.2.3 Plant growth and development

Growth of the soybean plants was staged according to the method of Fehr, Caviness, Burmood *et al.* (1971). Using this method, vegetative stages (V1, V2 etc.) were determined by counting the number of nodes on the main stem (beginning with the unifoliate node) that subtended a completely unrolled leaf. As recommended by the above authors, the growth stage of a group of plants was recorded as the average of the group. Appearance of flowers on any one node was denoted by the symbol R1. During the growth cycle, the number of days taken to reach each stage was recorded as were toxicity symptoms exhibited in response to metal pollutant treatment. Changes in nutrient solution parameters namely; ΔpH, water level, metal pollutant and nitrate concentration were also recorded. Pod growth and development was staged using a method modified from that of Miles *et al.* (1988). Pods were harvested at four distinct stages of development, which were determined by the size and morphology of the pod. These are described below:

Immature pods (IP) - pods dark green in colour, at least 10 mm in depth, ovules filling approximately half the locule.

Expanded pods (EP) - pods light green in colour, turgid and fully expanded (>7mm in width or fill).

Yellow pods (YP) - pods light yellow in colour and pliable.

Brown pods (BP) - pods light brown in colour and brittle.

In early experiments another growth stage category was included, namely:

Very immature pods (VIP) – pods dark green in colour, 10 mm in depth, ovules filling one quarter to one third of the locule.

2.2.4 Effect of metal pollutants on pod development and abscission

Four control plants and six treatment plants were utilised to investigate the effect of 0.05 mg/litre Cd^{+2} or 1 mg/litre Ni^{+2} on pod growth and abscission. Pods were tagged as soon as they first became visible, i.e. when they were 7 mm long and projected beyond the senescing corolla. At successive intervals thereafter, the growth parameters (length, depth, width/fill) of pods produced by control plants were recorded. Only pods containing three ovules were measured. The extent of fill was determined by measuring pod width at the widest ("fattest") point. Individual pods were checked every 3-4 days and the growth stage recorded.

2.2.5 Metal uptake into the plant

Plants were grown in nutrient solution amended with either 0.05 mg/litre Cd, or 1 mg/litre Ni. At senescence, plants were separated into roots, leaves and pods. The different plant parts were then digested and analysed for metal content according to the standard procedure described in section 2.2.1.5.

2.3 RESULTS

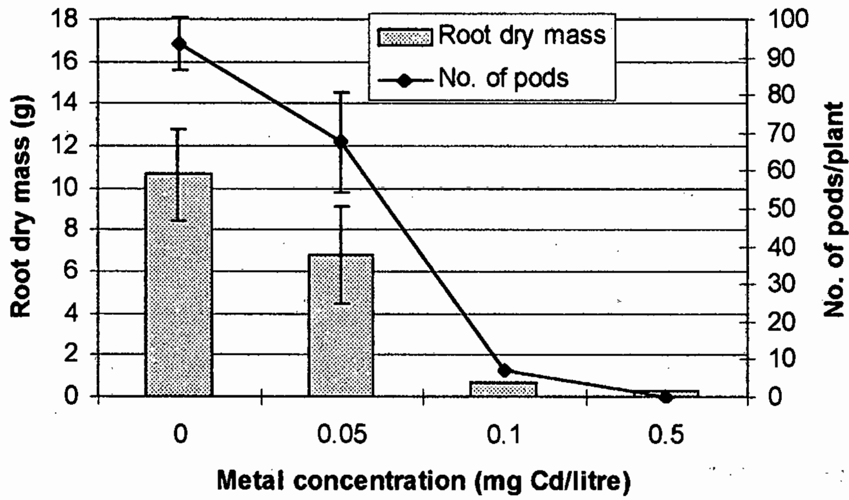
2.3.1 Effect of metal pollutant concentration

The effect of Cd and Ni concentration on the growth of soybean plants is shown in Fig. 2.2. Because four plants were grown together in close proximity it was often impossible to separate the roots or shoots. As a result, total root mass or pod yield for an entire growth tank (and thus metal concentration value) was obtained. This value was then divided by four in order to obtain the average root dry mass, or number of pods per plant. Additional data from subsequent plantings have also been included in the figure and the mean value plotted. It was felt that since each experiment was conducted under identical conditions, this approach was justified. In the case of both metals, even at low concentrations, plant growth (as expressed by root biomass), was severely inhibited relative to the controls. Plants grown in the higher metal concentrations (equal to and above 0.5 mg/litre Cd or 10 mg/litre Ni) showed severe toxicity symptoms and died before the control plants reached senescence. Control plants on the other hand, showed vigorous growth (Fig. 2.3a) and extensive, branched rooting systems.

The effect of both toxicants on pod formation was marked. Control plants produced on average 94 pods per plant. Addition of Cd or Ni however, drastically reduced this number. From these results, it was decided to use concentrations of 0.05 mg/litre Cd or 1 mg/litre Ni in future experiments. These levels exerted a demonstrable toxic effect on the plants and yet yielded enough seeds for experimentation.

The extensive root growth exhibited by control plants in particular, gave rise to concern that the volume occupied by the roots might change markedly with growth of the plant. This is turn would affect concentration values of metals and nutrients, since the nutrient solution was topped up to a calibrated mark on the growth tank. Measurement of root volumes however showed that the mean value was 361 ml (± 87.2) per growth tank for metal-stressed plants and 675 ml (± 35.6) for the controls. Thus at full size, the roots of control plants occupied no more than 1.7% of the total 40 litres of growth medium.

Cadmium



Nickel

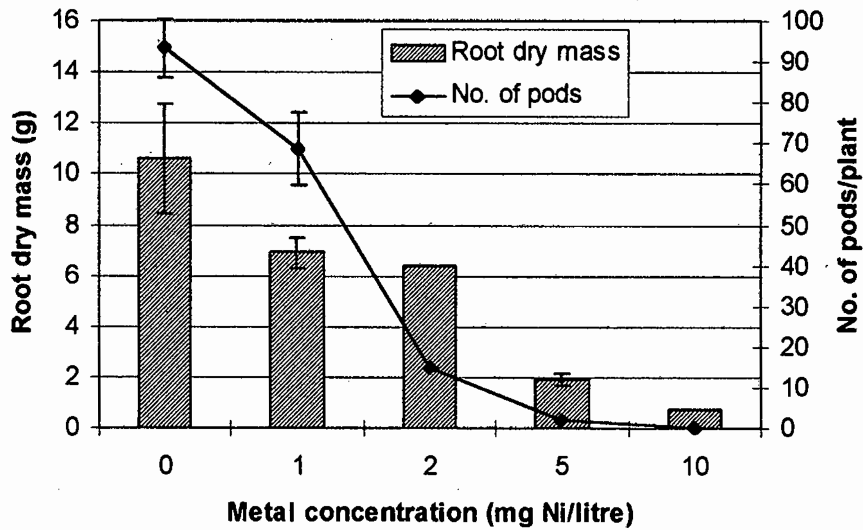
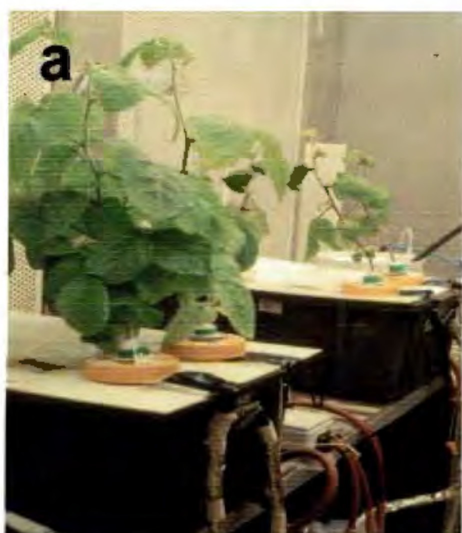


***Fig. 2.2** The effect of metal pollutant concentration on plant growth. Plants grown in a circulating nutrient solution system containing up to 1 mg/litre Cd or 10 mg/litre Ni, as described in section 2.2.1.2. No metal pollutant was added in the case of control plants. n = 3 for control, 0.05 mg/litre Cd, 1 and 5 mg/litre Ni treatments. n=1 for other treatments.*



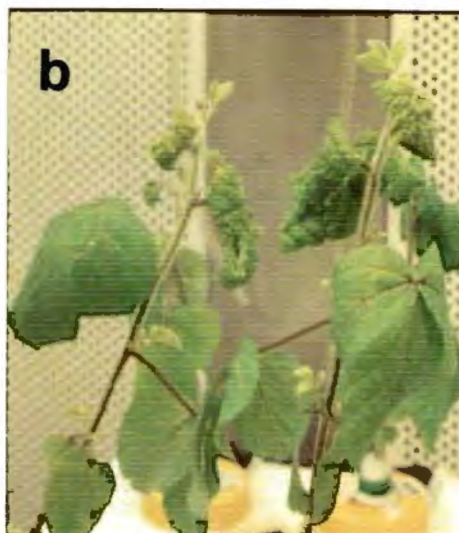


Fig.2.3a *Soybean plants growing in the circulating nutrient solution system. Control plants can be seen in the foreground and plants subjected to a high level of Cd-stress behind.*

Fig.2.3b *Visual toxicity symptoms exhibited by the plants approximately 2 weeks after addition of 0.1mg/litre Cd to the nutrient solution in which the plants were grown. Toxicity symptoms exhibited in response to both Cd and Ni were very similar.*

Fig. 2.3c *Terminal deformed racemes produced by plants grown in nutrient solution amended with 1 mg/litre Ni.*

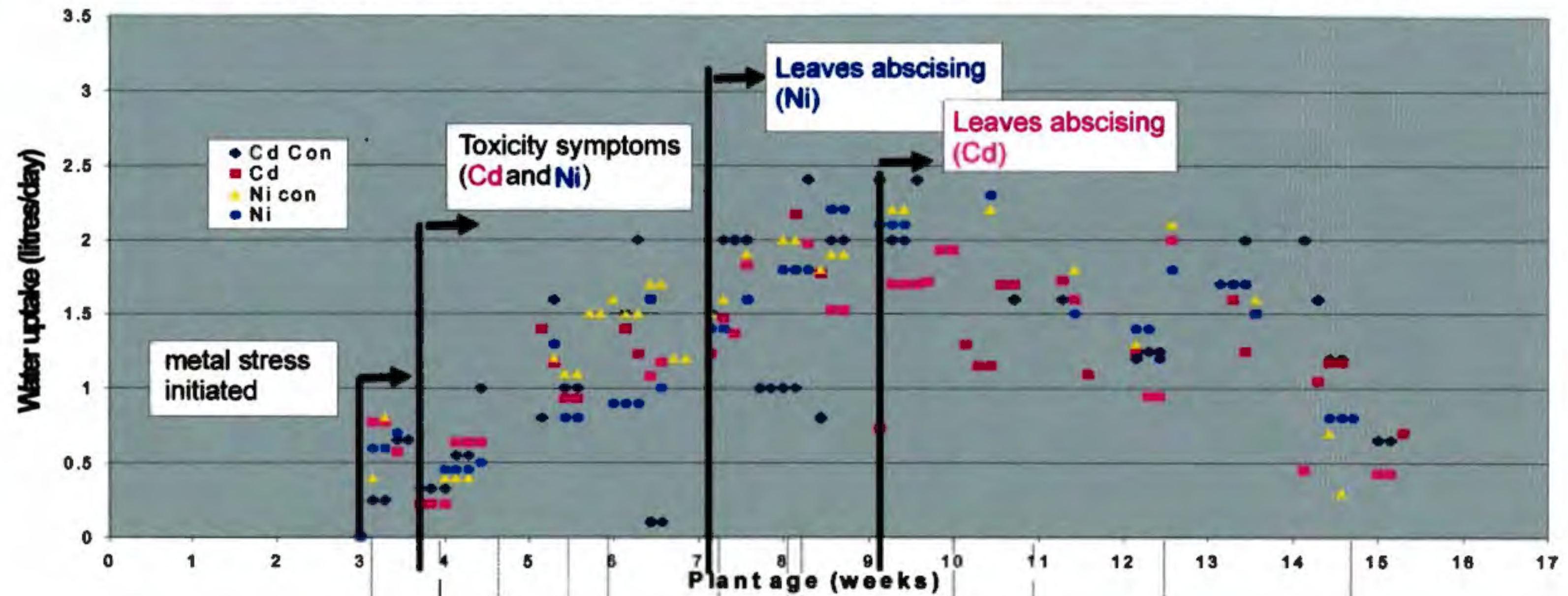
This factor was considered to be insignificant therefore and was not included in the concentration calculations.

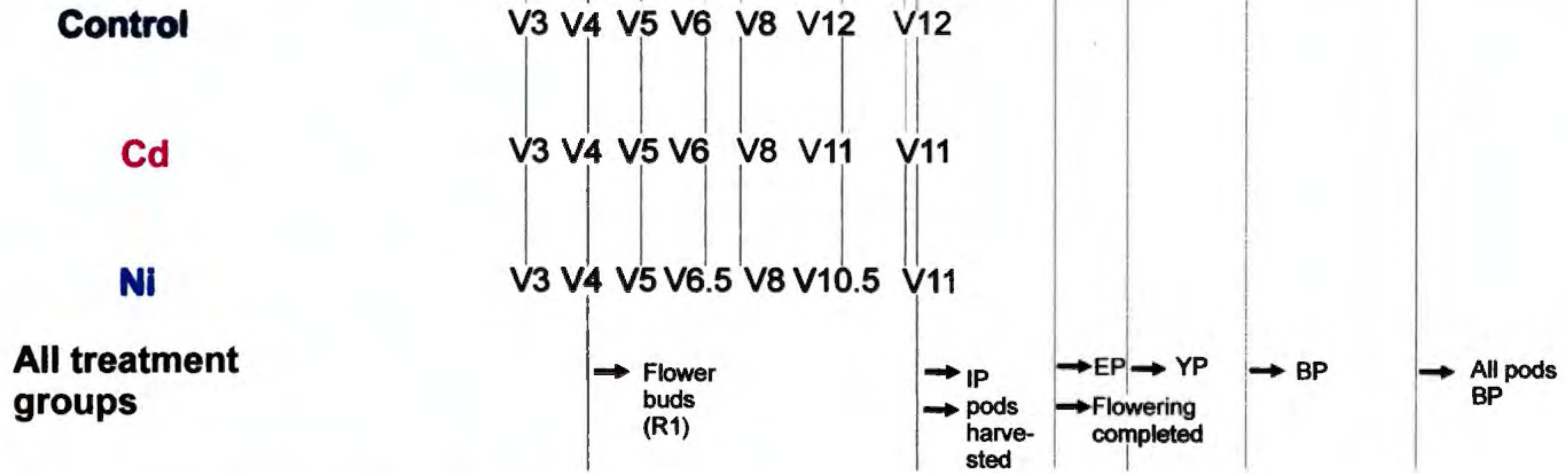
2.3.2 Visual toxicity symptoms

Visual toxicity symptoms exhibited by the plants on exposure to either Cd or Ni were very similar. The earliest symptoms were drooping of the leaves relative to those of control plants and the appearance of a distinctive red colouring of the petioles and leaf veins (Figs. 2.3a and b). These symptoms occurred within 2-4 days of metal application, depending on the concentration of pollutant (higher concentrations induced toxicity symptoms sooner than lower concentrations). After a few weeks, leaves became curled, exhibiting interveinal chlorosis and necrotic lesions. Growth of both shoots and roots was reduced markedly. The roots were stunted, brown in colour and gave rise to a reduced number of lateral roots, compared to the controls. In addition to the above, Ni-treated plants frequently exhibited small red spots in the interveinal regions of mature leaves. The pods produced by metal-treatment plants were for the most part indistinguishable in appearance from those of the controls. Plants treated with Ni however, occasionally produced deformed terminal racemes (Fig 2.3c), composed of pods greatly reduced in size (approximately 10 mm in length compared to 50 mm in the case of the controls). These remained small and green and did not develop further.

Cadmium and Ni both appeared to accelerate senescence. Leaf abscission began earlier in treatment plants compared to the controls and was also more extensive. By the time all pods were brown and ready to harvest, almost all the leaves in treatment plants had abscised whereas approximately 40% still remained on control plants.

Fig .2.4 Major phenological events and changes in water uptake with plant age.





V*=plant growth stage according to Fehr *et al.* 1971

2.3.3 Phenology and changes in nutrient solution parameters

2.3.3.1 Uptake of water

Uptake of water by the plants, as measured by the decrease in volume of the nutrient solution is shown in Fig. 2.4. Although some evaporation may have occurred, this is likely to have been minimal since the growth tanks were covered in plastic. Thus the water deficit is considered to be primarily due to transpiration. Substantial variation is shown by the data. Nevertheless, both controls and metal stress treatments show a similar trend, namely, a steady increase in water uptake, reaching a peak at around 8-12 weeks and then a decrease as the plants approach senescence.

2.3.3.2 Phenological events

Phenological events in the life cycle of the plants are also indicated in the above figure. Plants within the treatment groups were very similar with regard to growth rate, initiation of flowering and the timing of pod formation. For that reason the data for the Cd- and Ni-controls were combined. Shortly after transfer to the circulating nutrient system, plants from all treatment groups reached the V3 stage of growth as described by Fehr *et al.* (1971) i.e. the main stems had 3 nodes with completely unrolled leaves, beginning with the unifoliate node. Twenty eight to thirty days after germination, flower buds appeared on the plants of all treatment groups and opened a few days later (indicated by R1 in the figure). Individuals differed by only 1-2 days in this event. Slightly prior to this, the first toxicity symptoms appeared in treatment plants. Growth on the main stem was complete by 8-9 weeks in both control and metal-treated plants. Control plants were slightly larger in size (12-13 nodes on the main stem) compared to 10-11 for Cd or Ni treated plants. Although not measured, internodes of metal stressed plants appeared slightly shorter than the controls. Control plants also initiated more side branches, so that shoot biomass was greater than in the metal-treatment plants. From 8 weeks onwards, pods at the IP growth stage were harvested. A difference in the timing of leaf abscission between control and metal stressed plants was also noted. Abscission was initiated by week 7 in Ni-stressed plants, week 9 in Cd-stressed plants, but only by week 12 in control plants. Although within a treatment group, plants were relatively uniform with regard to the appearance of the initial toxicity symptoms, thereafter there was

considerable plant to plant variation. Some individuals appeared to be more sensitive than others.

2.3.3.3 Changes in pH

Changes in Δ pH with plant age are given in Fig. 2.5. The data are plotted as the change in pH per day. An alternative and possibly more correct way of plotting the data would have been as total hydroxyl ion production per day. It was felt however that this was not likely to be any more revealing than the current method.

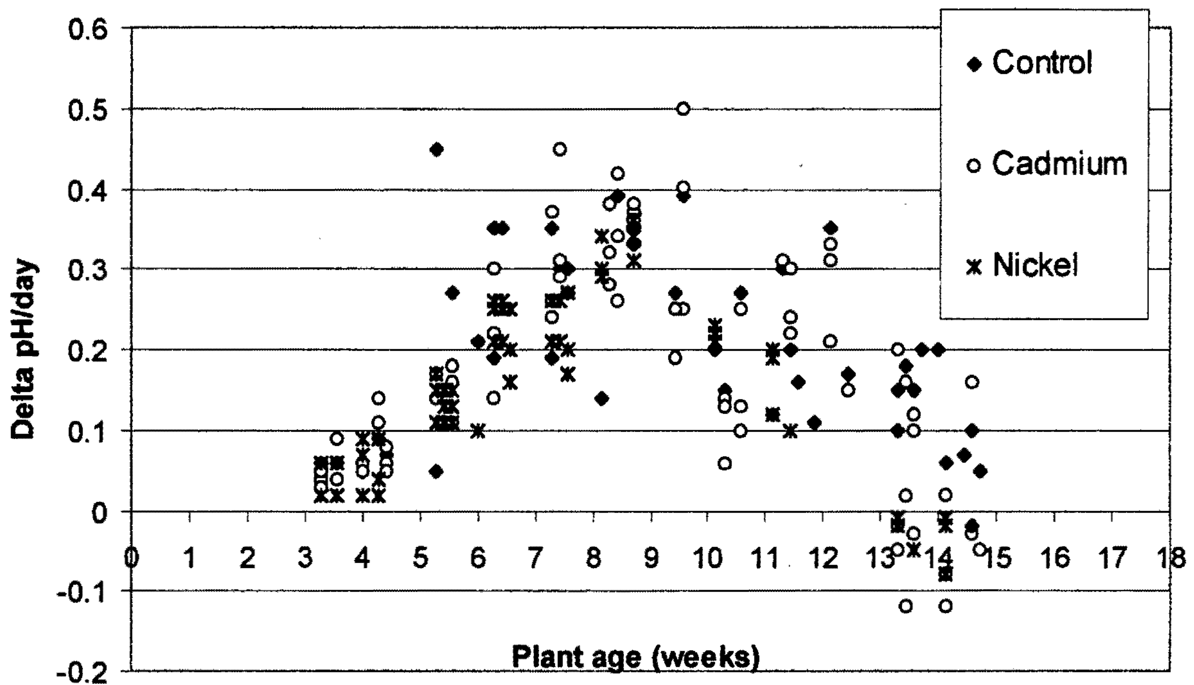


Fig. 2.5 Changes in pH of the nutrient solution with plant age. Control plants grown in standard nutrient solution. Metal pollutant-treatment plants grown in nutrient solution amended with 0.05 mg Cd/litre or 1 mg Ni/litre. Delta pH measured as the difference from the initial pH value of 6.0.

As in the case of water uptake, the daily change in pH of the nutrient solution was small in the beginning of the growth period, rose to a maximum from approximately 7 – 12 weeks and then decreased as the plants approached senescence. On a daily basis, Δ pH was generally positive. At senescence however, in order to bring the pH back to the nominal value of 6.0, it was sometimes necessary to add 0.1 M NaOH to the nutrient solution, since the pH decreased at this stage. This is shown in the figure by data points below the x-axis. Despite the addition of metal pollutants to the nutrient solution, little difference could be discerned between the treatment groups in the efflux of hydroxyl ions from the plants into the surrounding nutrient medium. From these results it was deduced that the rate of plant growth (as suggested by nitrate uptake and hence hydroxyl ion efflux) was maximal in all treatment groups at around 7-10 weeks after germination. Thus it was at this point in the plant life cycle, that nitrate and metal pollutant levels in the nutrient solution were determined.

2.3.3.4 Uptake of nitrate

The results from nitrate assays carried out on the nutrient solution are given in Table 2.2. Freshly prepared standard nutrient solution (prepared in a 1-litre glass volumetric flask) yielded a determined nitrate concentration of 11.6 mM which is close to the actual value of 12 mM (4 mM $Ca(NO_3)_2$ plus 4 mM KNO_3). Nitrate concentration for all treatments when tested on day 0 (i.e. shortly after the growth tanks were filled with fresh nutrient solution) was approximately 12 mM. This indicates that very little nitrate was taken up during the initial exposure of the plants to fresh nutrient solution. The nitrate concentration for the Cd control growth tank at day 0 was lower than expected (10.19mM). This is most likely due to inhomogeneity of the nutrient solution as well as to the element of error involved when preparing a complex solution in bulk (160 litres). All treatments showed a decrease in nitrate content with time, presumably due to plant uptake.

Table 2.2 *Changes in concentration of nitrate (mM) in the nutrient solution (ns) with time. Assays performed on fresh ns as well as at time intervals thereafter. %Plant uptake calculated as the difference in nitrate concentration from day 0. SD in parenthesis. n = 3.*

Treatment	Age of plants (weeks)	Age of ns (days)	Mean absorbance @ 410 nm	Mean concentration (mM)	% Uptake
Standard	-	0	0.565 (±0.06)	11.6 (±1.15)	-
Cd-control	9	0	0.498 (±0.02)	10.19 (±0.35)	0
	"	4	0.481 (±0.02)	9.85 (±0.34)	3.3
	"	7	0.417 (±0.01)	8.54 (±0.06)	16.2
Cd-treatment	9	0	0.592 (±0.01)	12.01 (±0.16)	0
	"	4	0.487 (±0.05)	9.97 (±0.97)	17.0
	"	7	0.419 (±0.00)	8.58 (±0.01)	28.6
Ni-control	7	0	0.683 (±0.01)	12.22 (±0.10)	0
	"	8	0.503 (±0.02)	8.98 (±0.31)	26.5
Ni-treatment	7	0	0.707 (±0.02)	12.63 (±0.31)	0
	"	8	0.501 (±0.01)	8.96 (± 0.11)	29.1

Standard curve for Cd-treatment and standard nutrient solution given by y=0.0977x (r^2=0.9972); for Ni-treatment y=0.112x (r^2=0.9921).

Table 2.3 *Changes in metal pollutant concentration in the nutrient solution (ns) with time. Day 0 represents the concentration in freshly prepared ns. SD in parenthesis. BD = below detection. n = 3.*

Age of ns (days)	Cd concentration (mg/litre)	Ni concentration (mg/litre)
0	0.071 (±0.02)	0.932 (±0.006)
3	0.038 (±0.02)	0.805 (±0.007)
7	0.047 (±0.03)	0.722 (±0.05)
Control	0.008 (±0.006)	BD

2.3.3.5 Uptake of metal pollutants

Table 2.3 shows the removal of metal pollutant from the nutrient solution with time. Nutrient solution samples were taken during the eighth week of plant growth. There is some deviation from the expected value of 0.05 mg/litre Cd and 1 mg/litre Ni on day 0 due to the same reasons outlined for anomalous nitrate values. The concentration of both Cd and Ni in the respective treatment solutions decreased with time and it is considered that this was most likely due to uptake by the plants. The Cd results show a high degree of variability, possibly a consequence of the low concentration of this metal in the nutrient solution.

2.3.4 Effect of metal pollutants on pod development and abscission

Growth and development of pods containing 3 seeds, harvested from control plants is shown in Fig. 2.6. Pods reached the maximum length of 5 – 5.5 cm within 10-14 days and thereafter pod length remained constant. A similar growth curve was shown when pod depth (or extent of lateral growth) was plotted against DAF (days after flowering). By 14 DAF the pods had reached the maximum value for this growth parameter and thereafter did not grow further in this direction. Filling of the pods (i.e. pod "fatness") occurred much more slowly, and at a constant rate until levelling off after 40 DAF. All three growth parameters remained constant after the maximum value had been attained, even after maturation drying had occurred. Preliminary results indicated that pods from Cd- and Ni-treatment plants showed similar growth curves and thus this line of research was not pursued further.

Table 2.4 shows the length of time (given as DAF) for pods to reach each growth stage. Pods were tagged when they were 7 mm long and projected just beyond the senescing corolla. According to Peterson *et al.* (1992), pods reach this size approximately 5 DAF. This value therefore was added to the number of days recorded for each pod to reach a given growth stage. For the two younger growth stages (IP and EP), there was little difference between the DAF values for different treatments. As the pods became older

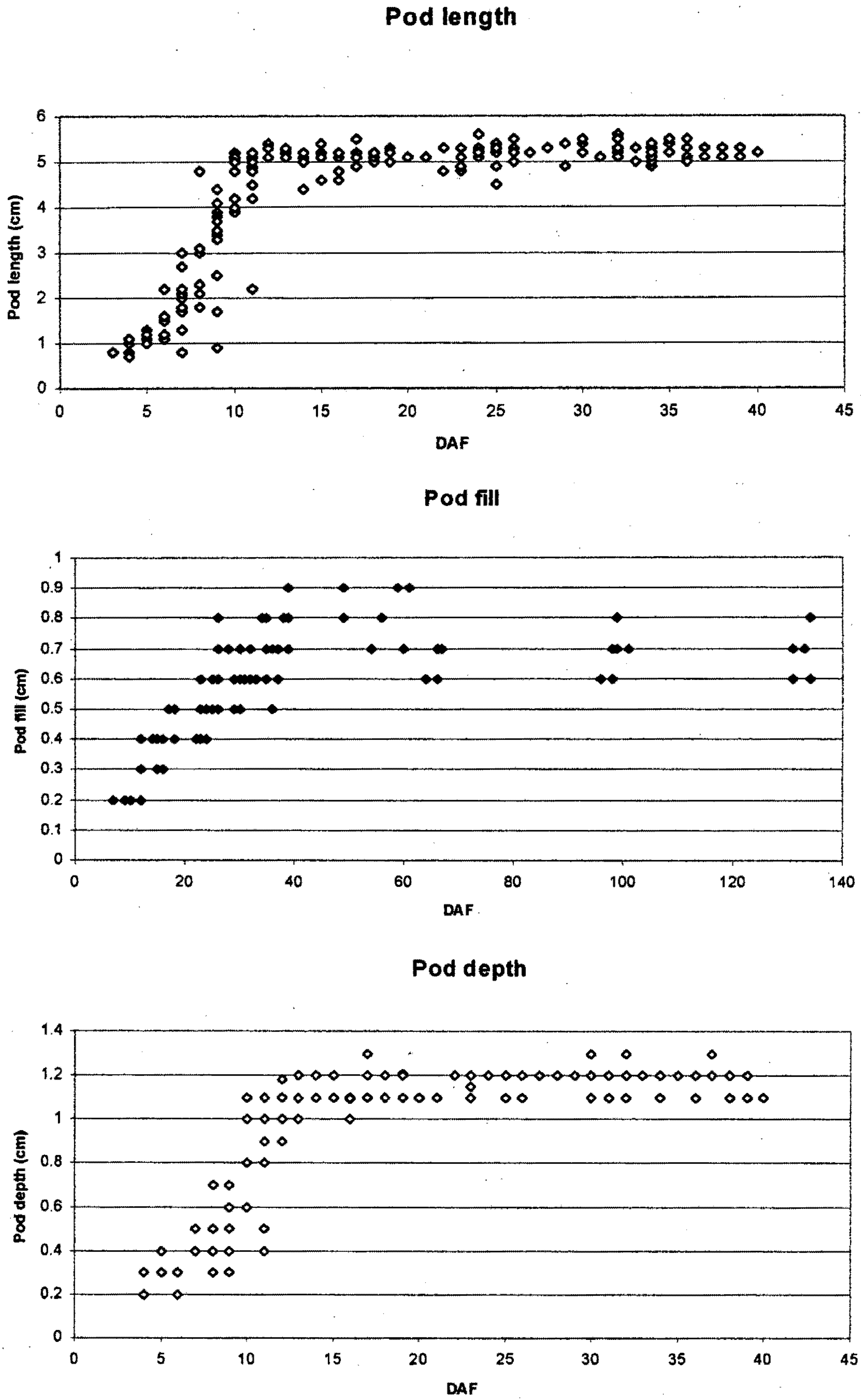


Fig. 2.6 Changes in pod growth parameters with age (DAF).

(YP and BP) however, addition of either metal pollutant to the nutrient solution appeared to decrease the time required for pods to reach each growth stage. Thus at the YP growth stage, the difference in DAF between Ni or Cd treatment pods and the controls was significant. A similar effect was noted at the BP growth stage. Both metals promoted pod senescence to the same extent, since there was no significant difference in DAF between Cd and Ni treatments.

Table 2.5 shows the number of abscised pods expressed as a percentage of the total number of pods tagged per plant. Addition of either Cd or Ni to the nutrient solution appeared to enhance pod abscission slightly relative to the control plants. This effect however this was not statistically significant.

2.3.5 Distribution of metal pollutants within the plant

Table 2.6 shows the Cd and Ni content of roots, leaves, mature (BP) pods and seeds taken from metal-treatment and control plants. Metal content in all parts was higher than in the equivalent organ of control plants. Cadmium enrichment was found almost exclusively in the root. A concentration of 130 μg/g.dm Cd was found in this tissue which represents a considerable accumulation of the metal from the surrounding nutrient solution in which the Cd concentration was only 0.05 mg/litre. Cadmium values for the aerial portions, especially the reproductive parts of the plant, were low in comparison to the roots.

Nickel was also concentrated in the roots with much lower levels in the shoots. The general pattern for Ni accumulation was the same as for Cd i.e. roots >> leaves ≥ seeds > pods. As a general rule, the levels in all plant parts were much higher for Ni than for Cd. The concentration of Ni accumulated in mature seeds was 49 μg/g.dm compared to a concentration of 1 μg/g.dm Cd. In addition, whereas leaf Cd contents were significantly higher than seed levels the difference in Ni levels between seeds and leaves was not significant. Of interest, is the fact that pod metal levels for both

Table 2.4 *Effect of metal pollutants on the length of time (DAF) for pods to reach each growth stage. Plants grown in nutrient solution containing either 0.05 mg/litre Cd or 1 mg/litre Ni. Growth stages as described in section 2.2.3. SD in parenthesis. Sample size (n) indicated.*

Treat-ment	IP		EP		YP		BP	
	DAF	n	DAF	n	DAF	n	DAF	n
Cd-control	16.3 (± 1.31)	20	28.9 (± 2.5)	31	50.4 (± 1.6)	17	54.7 (± 2.9)	27
Cd-treat-ment	16.1 (± 1.72)	24	30.8 (± 2.2)	30	46.2 (± 4.31) *	28	52.1 (± 4.2) *	22
Ni-control	16.5 (± 1.1)	32	28.5 (± 2.5)	15	50.3 (± 2.29)	24	53.7 (± 3.02)	30
Ni-treat-ment	16.9 (±1.15)	37	28.4 (±1.6)	18	47.2 (± 2.94) *	40	49.7 (± 3.62) *	61

* denotes significant difference from the control at $p < 0.05$.

Table 2.5 *Effect of metal pollutants on pod abscission. SD given in parenthesis.*

Treatment	Mean no. pods tagged/plant	Mean no. pods abscissed/ plant	Mean % Abscissed
Cd-control	41.5 (±0.96)	5 (±0.5)	12.05 (±0.21)
Cd-treatment	28.7 (±1.7)	4 (± 0.71)	14.28 (±3.3)
Ni-control	28.5 (±1.3)	3 (±0.1)	10.6 (±0.78)
Ni-treatment	31.8 (±0.87)	3.2 (±0.98)	11.5 (±1.7)

treatments were lower than for seeds, although the result for Cd is not statistically significant.

This chapter is largely concerned with plant development and growth. The effect of metal pollutants on seeds is outlined in the following chapter. Metal levels in the seeds have been presented in this section however, in order to put accumulation in these organs in context with uptake into the rest of the plant. A full discussion of these results is given in Chapter 3.

Table 2.6 *Distribution and concentration of metal pollutants (μg/g.dm) in various parts of plants grown in nutrient solution containing 0.05 mg Cd/litre or 1 mg Ni/litre. SD in parenthesis. BD = below detection. 3<n ≥6.*

Plant part	Cadmium (μg/g.dm)		Nickel (μg/g.dm)	
	Treatment	Control	Treatment	Control
Roots	130.09 (±34.2)	1.31 (±0.19)	1100 (±40.0)	3.1 (±0.1)
Leaves	3.80 (±0.08)	0.43 (±0.17)	48.1 (±1.5)	BD
Pods	0.78 (±0.24)	0.48 (±0.01)	12.5 (±0.69)	BD
Mature seeds	0.96 (±0.15)	0.12 (±0.04)	49.1 (±5.75)	0.2 (±0.01)

2.4 DISCUSSION

Soybeans have been grown successfully in nutrient solution varying in pH from 5.5 (Grusak and Pezeshgi 1994) to 6.4 (Haghiri 1973). Thus maintenance of a strict value between 5.8 and 6.2 was not critical for the growth of the plant. As mentioned in the literature review on metals in the environment however (section 1.3.6.1), pH can exert a profound effect on chemical speciation and hence bioavailability of nutrient and metal pollutant ions. In order to validate speciation modeling of the nutrient solution (Chapter 7) as well as to ensure uniform growth parameters for all plant treatments therefore, this variable was strictly controlled. Imsande and Ralston (1981) and Imsande (1986) cultivated soybeans in a nutrient solution amended with the buffer MES (2-[morpholino] ethanesulphonate). Preliminary trials in this project (data not reported) showed that this was not an effective means of ensuring pH homeostasis, since pH in the growth tanks still escalated. Further more, MES may inhibit nitrate uptake (Dr Cramer, *pers. comm.* 1995) and its addition to the nutrient solution would have complicated modeling of the system. Thus MES was omitted from the nutrient solution and pH was stabilized by adjusting this parameter daily as well as employing a large volume of nutrient solution per plant and frequent nutrient solution renewal. In addition, it was felt that the second and third precautions mentioned would help to minimise accumulation of root exudates and thus potential changes in chemical speciation.

The plant growth system worked efficiently and throughout the project, control plants grown in the circulating nutrient solution were bigger and the seed yield higher than control plants grown in soil (data not shown). A major concern was that due to extensive root growth, zones of nutrient or oxygen depletion might occur. Continuous aeration and circulation of the nutrient solution however helped to keep the system well mixed. This was supported by the fact that the pH difference between the growth tank and reserve tank was small (0.02 $\pm$0.03 n=114). The use of a reserve tank as well as a growth tank resulted in a large volume of nutrient solution per plant and hence minimal nutrient depletion. In addition, due to the design of the system, exposure of roots due to excessive water uptake and transpiration was avoided. A limitation of the system

however, was that once in the large growth tank movement of the plants (so as to minimise microhabitat effects) was possible only during the first month. Thereafter, the roots and shoots became intertwined, and could not be separated without damaging the plants. Another deficiency in experimental design was the small number of plant replicates. For each planting there were 4 control plants and 12 treatment plants and thus seed yield was relatively small. Due to limited space and the labour-intensive nature of the culture technique, growth of a larger number of plants was not feasible.

The general pattern of water uptake with growth is similar to that reported by Grusak and Pezeshgi (1994) for soybeans grown in a nutrient solution system. Water uptake was low whilst the plants were small, rising with increasing plant mass and then again decreasing as leaf abscission began and the plants entered the senescent phase. Both Cd and Ni, in common with many other metal pollutants, disrupt plant water relations (Marschner 1983; Marchiol *et al.* 1996; Pandolfini *et al.* 1996). Cadmium treatment is usually associated with a decrease in plant water content (Hernandez *et al.* 1996). Despite this, no difference in water uptake could be detected in this work between the control and treatment plants. This could possibly have been because the technique used to measure water loss was rather crude and small differences between treatment groups would not have been detected.

The change in Δ pH of the nutrient solution during the plant life cycle generally mirrored the trend in water uptake. As noted in the introduction, this pH change is due mainly to NO_3^- uptake, quantitatively the most important macronutrient for plant growth. From this it follows that the most rapid phase of plant growth was roughly from week 7 to week 11. Grusak and Pezeshgi (1994) also recorded changes in NO_3^- influx and pH in the hydroponic solution used to support soybean growth. They found that uptake of NO_3^- and protons reached a peak at 6 -7 weeks and was diminished by around 10 weeks. Nitrate uptake was thus determined during weeks 9 (Cd-stressed plants) and 7 (Ni-stressed plants) in the present study. Since uptake was never greater than 30% of the total nitrate content, this nutrient is not likely to be limiting under the experimental conditions used in the study. A similar conclusion can be drawn in the case of metal

pollutant influx, which was also determined during the period of rapid growth. Vogeli-Lange and Wagner (1996) found that actively expanding tobacco leaves were a stronger sink for Cd than ready-expanded leaves. It is therefore likely that maximum uptake of metal pollutant ions occurred during the phase of rapid plant growth. The concentration of both Cd and Ni in the nutrient solution remained above 40% of the original starting values. As in the case of nitrate therefore, it is not considered likely that the level of either metal pollutant would become insignificant during any stage of the plant growth cycle. Plant biomass was reduced in the metal-stressed plants of this project and in addition, reduced nitrate uptake in Cd-treated plants has been cited as a reason for poor growth (Ouariti *et al.* 1997). Such an effect however, was not reflected in the Δ pH values for the nutrient solution compared to the controls, nor in the nitrate levels in the nutrient solution. Again it is considered that the method of measurement was possibly too crude to detect slight differences between treatment groups.

The major effect exerted by Cd and Ni in this study, was a general reduction in plant biomass. This was observed in the form of decreased root mass as well as a decline in pod yield and was elicited by both metals. Reduction in plant biomass as a result of heavy metal stress appears to be an almost universal finding (MacNicol and Beckett 1985; Dudka *et al.* 1996; Leita *et al.* 1993; Ouzounidou, Moustakas and Eleftheriou 1997). A few cases of biomass enhancement due to metal pollutants have been reported in the literature, but these were from experiments utilising extremely low concentrations (Mishra and Kar 1974; Breckle 1991; Yang *et al.* 1996a). Biomass reduction has been reported for both subterranean and aerial plant parts, including pods and seeds. Results from the pod abscission experiment indicated that decreased pod yield was not a result of enhanced pod abortion. This will be discussed in conjunction with seed yield in the following chapter. Inhibition of shoot growth was not studied, since extensive leaf abscission occurred, space was limited and this aspect was not considered to be of major relevance to the project. Root growth on the other hand, was measured, since these organs appear to be especially susceptible to metal toxins and reduction in root mass has been used as a convenient criterion of metal tolerance (Ouzounidou *et al.* 1997). Depressed growth in these organs is one of the most

common effects reported for both Cd and Ni (Mishra and Kar 1974; Shah and Dubey 1995; Mattioni *et al.* 1997). Chen and Kao 1995b found that Cd-induced stress inhibited root elongation with a concomitant increase in ionically bound peroxidase (POX) in the root cell walls of these organs. According to these authors, growth inhibition may result from a cell wall tightening process related to formation of cross-linkages among cell wall polymers which is thought to be catalysed by POX. They also found that the levels of proline increased as a response to increased cellular Cd levels. Treatment of the roots with proline in the absence of Cd also resulted in increased POX activity as well as inhibiting root elongation. Proline induction has been found to occur in plant cells as a result of many different types of stress (drought, cold) as well as from Cd excess and is thought to act as an osmolyte (Costa and Morel 1994). An increase in proline has been linked to disruption of plant water relations, which as mentioned above is another well-documented effect of Cd (Marchiol *et al.* 1996; Carlson *et al.* 1975). In addition to inhibition of cell expansion, heavy metal-induced reduction of root growth may be a consequence of decreased photosynthate transport to the roots (Breckle 1991). L'Huillier *et al.* (1996) also found that Ni inhibited root growth by a direct effect on cell division in the meristem.

The toxicity responses of soybean plants to Cd and Ni and to stress from other metals have been well documented in the literature (Boggess *et al.* 1978; Rauser 1978; Smith *et al.* 1985). The fact that both Ni and Cd give rise to similar toxicity symptoms make it tempting to postulate a similar toxicity mechanism for the two metals. As mentioned earlier in Chapter 1 however, these symptoms are likely to be the secondary results from a long chain of metabolic malfunctioning resulting from metal toxicity. The primary toxicity effect may be very different. Rauser (1973 and 1978) also found similarities between visual symptoms elicited by both Cd, Ni and Zn poisoning, although other aspects such as starch accumulation in leaves differed. Appearance of red colouration in soybean petioles seems to be a response to several environmental stresses, not only metal pollutants (Scott and Aldrich 1970), including infestation by pathogens (Brisson, Peterson, Robb *et al.* 1977) and high light intensity (our own observations). According to Brisson *et al.* (1977), discolouration is caused by deposition of phenolic substances

in, or near, the xylem vessels of the vascular bundle. Other members of the *Fabaceae* also give rise to red colouration in the petioles as a response to metal stress (Rauser 1978; Vazquez, Poschenrieder and Barcelo 1989) as does *Triticum* (Ouzounidou *et al.* 1997). No reports could be found in the literature regarding visual toxicity symptoms exhibited in response to metal pollutants by the pods of leguminous plants, or fruits in general. A point to note is that although plants were grown under the same environmental conditions not all plants produced deformed terminal racemes and that plant to plant variation did occur. Soybeans appear to be moderately susceptible to heavy metal damage, although variation in metal tolerance amongst cultivars of soybean has been reported (Boggess *et al.* 1978), which may be related to the efficiency of iron utilisation (Smith *et al.* 1985). Nothing could be found in the literature relating "Crawford", the cultivar used in these experiments, to metal tolerance. Cadmium toxicity symptoms in soybean are considered by some authors to resemble Fe chlorosis (Haghiri 1973) and will be discussed further in the next chapter.

Addition of either metal pollutant to the nutrient solution had very little effect on the phenology of the plants, especially in the early growth stages. The dates of flowering and appearance of the lower nodes were virtually identical between treatment groups. Both pollutants appeared to promote senescence in the latter half of the life cycle however, as shown by the premature leaf abscission exhibited by metal-stressed plants. Nickel may be more effective in promoting this effect compared to Cd since this was first noticed in the Ni-treated plants at 7 weeks and in the latter treatment only 2 weeks later. However, since the two metal-treatment experiments were conducted consecutively rather than simultaneously and since Cd-treatment plants were grown first, it might well be that the leaf abscission effect was not recognised earlier. Vazquez *et al.* (1989) reported that Cd caused premature leaf abscission in beans and that this occurred at the secondary pulvinus, the petiole remaining attached to the stem. A similar pattern of leaf abscission was noted in this study. Pollutant-enhanced senescence was also evident in the reduced length of time taken for pods from metal-stressed plants to reach the later growth stages (YP and BP). Poschenrieder, Cabot and Barcelo (1983) reported that Cd toxicity induced premature senescence in beans.

The growth parameters obtained for control pods agree with findings in the literature (Bewley and Black 1994), namely that pod growth is more or less complete (i.e. pod length and depth reached a maximum) before ovule expansion (increase in pod width). Pods were measured in order to elucidate the seed developmental process and determine where each growth stage was placed on the developmental curve. This aspect will be discussed in conjunction with seed development in the next chapter.

The high levels of metal pollutant found in the roots was anticipated, since it was noted in Chapter 1 (section 1.3.6.2), that as a general rule, metal content is normally higher in roots than shoots. Although the roots were washed with water to remove surface contamination, no attempt was made to distinguish between apoplastic and symplastic uptake. The high metal enrichment values obtained for the roots thus represent metal taken up into the cells as well as metal in the cell walls. Amongst the various organs of the aerial portions of the shoot, levels of metal pollutants are generally lowest in seeds compared to other plant parts (Marschner 1983; Ernst 1996). This was substantiated by the results obtained with Cd in this study and will be discussed further in the next chapter. The relatively low foliar concentrations of Cd and Ni encountered in this study may possibly be due to the fact that the plants were entering senescence when leaves were removed for analysis. In a study conducted by Cataldo *et al.* (1978a) on Ni uptake in soybean plants, at senescence, more than 70% of Ni present in the shoot was remobilized to the seeds. A more accurate estimation of leaf metal concentration would have resulted had the leaves been harvested during the period of active growth.

A perusal of the literature concerning elemental analysis of plant tissue, shows that whilst the various forms of atomic absorption spectroscopy (AAS) are most commonly employed for this purpose (Valdares *et al.* 1983; Duke *et al.* 1985; Lopez, Williams and Cooler 1986; Allen, Parkinson and Rowland 1989; Eklund 1995), ICP or inductively coupled plasma spectroscopy is becoming increasingly popular (Zarcinas, Cartwright and Spouncer 1987). ICP is frequently more sensitive than many AAS techniques (Broekaert 1990) and indeed preliminary experiments showed that AAS was not able to

detect the low levels of Cd found within the seeds (Chapter 3). For both AAS and ICP, mineralization of samples prior to analysis is a mandatory step in order to prevent non-specific absorption due to the presence of organic components (Kucera and Soukal 1995). To achieve this end, variations on wet and dry ashing procedures have been reported in the literature (Azcue and Mudroch 1994). In this project a combination of several methods was employed, taking cognisance of the fact that dry ashing at temperatures greater than 500° C and digestion in H_2SO_4 can lead to Cd loss, as a result of volatilization and $CdSO_4$ precipitation respectively (Kucera and Soukal 1995). It was found that Ni levels in control tissue were sometimes too low for detection (Tables 2.3 and 2.6). A wide range in detection limits was found in the literature for the two elements using ICP-AES. These varied from 0.21 ppm for Cd and 0.38 ppm for Ni (Kos, Budic, Hudnik *et al.* 1996) to 0.002 ppm for Cd and 0.01 ppm for Ni (Broekaert 1990). Although Broekaert (1990) does state that for multi-element determinations (as was usually performed in these experiments) the detection limits may be increased up to a factor of 5 relative to that for single-element determinations. In addition higher SD (standard deviations) are to be expected at low concentrations of Cd and Ni (Kos *et al.* 1996).

It is pertinent to note at this point that the levels of metal xenobiotics used in this study were very low (0.05 mg/litre, or 0.45 μM Cd; 1 mg/litre or 17 μM Ni). This is because seed production was found to be extremely sensitive to the two metal pollutants (Fig. 2.2). Higher metal concentrations may have elicited a more pronounced response, but the reduced number of seeds produced per plant would have made experimentation difficult. Extrapolation of results from plants grown in a nutrient solution system to those in the field is contentious, as is comparison of metal concentrations in the two types of growth medium. The two techniques represent complementary approaches to the problem of ecotoxicology. On one hand, field or pot trials, often yield variable results, but are more representative of the natural state. On the other hand, nutrient solution experiments offer an artificial system, but one in which variables can be carefully controlled. As explained in Chapter 1, due to binding of metal cations by soil particles, not all of the metal is bioavailable. In nutrient solution systems, matrix binding is largely

absent, and if the metal is present in a bioavailable form, it can generally be said that plant uptake will be greater from nutrient solution than from a soil containing the equivalent concentration of a given ion (Cataldo, Garland and Wildung 1983). According to the above authors, many researchers use unrealistically high Cd levels in nutrient solutions. Cataldo *et al.* (1983) used concentrations of from 0 to approximately 0.04 mg Cd/litre in experiments investigating uptake by soybean and claim these values are representative of those found in soil solutions. Similar concentrations were used by McKenna *et al.* (1993) in Hoagland's nutrient solution to correspond to the soil solution levels in polluted soil. Soil solution values of Ni are extremely variable and values of around 40 μg/g Ni have been reported for unpolluted soils (McGrath and Smith 1990; Reeves *et al.* 1996). McIlveen and Negusanti (1994) found that soil levels near a Ni smelter were up to 332 μg/g. As mentioned in the introduction to this study, elevated levels of this metal also occur naturally in some soils. Thus it is felt that the levels of metal pollutants used in this study are comparable to those likely to be encountered in moderately polluted soils.

CHAPTER 3

SEED DEVELOPMENT AND PHYSIOLOGY

3.1 INTRODUCTION

As mentioned in the introduction to this thesis, research on metal pollutants in seeds has largely been directed towards quantifying accumulation within seeds of economic importance and estimating the resultant threat to the food chain. In addition, the effect of such accumulations on seed yield has been studied. Remarkably little attention has been paid to the effects metal pollutants may have on seed functioning. Chaer Borges and Wollum (1980) found that whilst high levels of Cd applied to the soil significantly reduced soybean seed yield (by 24% relative to the control), the seeds produced showed no significant reduction in germination potential as indicated by the tetrazolium dye test. Neither was the nitrogen content of such seeds affected. Investigations into the effect of Pb exposure on the membranes of *Capsicum annuum* (pepper) seeds revealed changes in the fatty acid composition and also in the anti-oxidative capacity of these seeds (Stefanov *et al.* 1995). Singal, Gupta, Joshi *et al.* (1995) found that treatment with Cd altered the phosphorus (P) content of fenugreek seeds harvested from plants grown in soil amended with differing levels of $CdCl_2$, causing complex shifts between lipid, nucleic acid and protein P. Other investigations have been limited to the effect of metal pollutants on the mineral composition of seeds (Bjerre and Schierup 1985; Moraghan 1993).

Precise staging of developing seeds was an important part of this project. In order to construct meaningful profiles of various aspects of seed development (e.g. changes in seed mass, storage reserves) and to examine the effect of metal pollutants thereon, it was essential to ensure that seeds harvested at any one stage had the same developmental status. Several different criteria have been utilised in the literature to assess the extent of soybean maturation. These include chronological age, usually expressed as DAF (Guldan and Brun 1985), seed moisture content

relative to seed mass (Fraser *et al.* 1982), seed size (Meinke *et al.* 1981) as well as seed/pod morphology or colour (Miles *et al.* 1988). Although DAF and seed size or mass are commonly used to describe developmental stages, growth rate can vary depending on the position of the seed within a pod as well as the location of the pod on the plant (Spaeth and Sinclair 1984a and b). In addition, seeds produced at the end of the season tend to be smaller than those produced early in the season (Egli *et al.* 1978). According to Miles *et al.* (1988), seed moisture may be a more consistent quantitative indicator of developmental status. The shortage of seeds in the present study however, necessitated the use of a non-destructive method. Thus seed size was used during the early stages, whilst the seeds were gaining rapidly in mass. Thereafter, pod turgor and colour were used as indices of development.

Several workers have postulated that the toxic effect of metal pollutants is not due solely to the presence of that element in plant tissues, but may also be a consequence of the resulting imbalance in essential minerals (Wallace, Romney, Alexander *et al.* 1977; Ouzounidou *et al.* 1997). Consequently, in addition to metal pollutants, the concentrations of other nutritionally important metals were also determined in control as well as in treatment seeds. Iron was chosen because of its nutritional importance with regard to humans and livestock and because it is frequently limiting in soils (Chaney 1991). The element Zn has frequently been linked to Cd toxicity in plants (Smith *et al.* 1985) and exhibits similar chemistry to Cd (McKenna *et al.* 1993). It was thus considered important to investigate how levels of this element changed in response to amendment of the nutrient solution with Cd. In addition, shifts in the Mn and Mg levels of seeds were examined, because of their important role in plant nutrition (Sandman and Boger 1983).

If orthodox seeds are left in water for a period of time, chemical constituents including ions, are leached out into the surrounding liquid. The extent of this leaching may be estimated by measuring the electrical conductivity (EC) of the soaking water (Deswal and Sheoran 1993). It has been shown that seedling vigour and emergence in the field can be predicted from such experiments and that high leakage rates are

inversely related to seed quality (Hepburn, Powell and Mathews 1984). The practice of using EC measurements of leachates for seed quality assessments is now well established (Mathews and Powell 1987) and has been carried out on a wide range of seeds including soybeans (Hepburn *et al.* 1984; Hampton, Lungwangwa and Hill 1994). Release of high levels of leachate into solution is thought to result from loss of membrane integrity (Siddique and Goodwin 1985). As seeds age, or if they are damaged in some way, degradative changes in cellular membranes cause an increase in leakiness (Stewart and Bewley 1980 cited by Tyagi 1992). It has been postulated that loss of membrane integrity is likely to be the result of free radical reactions and lipid peroxidation (Hendry, Thorpe and Merzlyak 1993). In Chapter 1 it was mentioned that the principal toxicity mechanism of Cu is at the cell membrane level. Although the primary deleterious activities of Cd and Ni are not known, it is very likely that membrane damage is involved to at least some extent. According to Das, Samantaray and Rout (1997), variation between plant species in the degree of tolerance to Cd toxicity almost certainly involves differences in the structure and function of cell membranes. Thus the leakage of seeds harvested from plants grown in metal pollutant-amended nutrient solution will be compared with control seeds in order to assess whether membrane integrity and functioning is compromised by the presence of Cd or Ni within the seed tissues.

For the purpose of this thesis, seeds harvested from plants grown in Cd-amended nutrient solution will be referred to as Cd-*treatment* seeds. Similarly Ni-treatment seeds and control seeds are those harvested from plants grown in Ni-amended nutrient solution or unadulterated nutrient solution respectively. This is to distinguish seeds germinated in a metal contaminated solution as in Chapter 5. Such seeds will be referred to as metal-*germinated* (either Cd or Ni) seeds. In the previous chapter it was shown that one of the major effects caused by addition of either metal pollutant to the growth medium was a marked reduction in pod, and hence seed yield. In this chapter the development and functioning of those seeds is examined.

3.2 MATERIALS AND METHODS

3.2.1 Seed harvesting, processing and storage

Plants were grown as recorded in the previous chapter and pods harvested at the various growth stages. The appearance of the seeds at each development stage is described below. For the sake of completeness, the age (DAF) for each growth stage, as determined for control pods, is also given.

Very Immature Pod (VIP) – ovules 3-4 mm in depth, filling one quarter to one third the depth of the locule. Dark green in colour. DAF = not determined, but less than 15 DAF.

Immature Pod (IP) – ovules approximately 5 mm in depth, filling half the depth of the locule. Dark green in colour. DAF = 16 ± 1.2

Expanded Pod (EP) – Ovules minimum of 6 mm in width (fill). Light green in colour, with no yellowing over the radicle. Ovules covered by white membranous layer formed from the innermost layer of the pod. DAF = 28 ± 2.5

Yellow Pod (YP) - seeds light yellow in colour, bean-shaped and soft. Hilum, variable in colour from light yellow to dark brown/black. Seeds detached from pods. DAF = 50 ± 1.9

Brown Pod (BP) – seeds dry, hard and round in shape. Light cream in colour. Hilum, dark brown/black. DAF = 54 ± 3.01

Whilst still on the plant, the youngest (VIP) seeds were assessed according to a non-destructive method similar to that of (Spaeth and Sinclair 1984a). A tube of black card was placed around each pod and illumination supplied from behind. The silhouette of the ovules within the pods could then be easily seen and measured. IP

seeds were staged by feeling the ovule within the pod and estimating the proportion of the locule depth that was filled. The other three growth stages were staged according to the colour of the pod and by measuring the width ("fatness") of the pods with calipers. Seeds used for the experimental purposes described below, were harvested and sorted into the different growth stages. Those that did not meet the criteria for a particular growth stage were discarded. The rest were frozen in liquid nitrogen and freeze-dried for 48 hr in a Savant RVT 4104 bench-top Vapour-trap freeze drier. After grinding in a coffee grinder, the tissue was stored at -80°C until further processing. Material for each growth stage was bulked in order to minimise seed to seed variation. Mature (BP) seeds destined for biochemical or elemental analyses were processed as above. The rest of the BP seeds were stored according to the following protocol:

The moisture content of the seeds was first determined gravimetrically (see below). If this was found to be more than 12% when expressed on a dry mass basis, the seeds were placed on plastic mesh grids suspended over, but not touching, silica gel. The seeds were maintained at 10°C in sealed containers for approximately 1 week, or until the moisture content had dropped to 9-11%. Seeds were then stored in the dark, at 10°C. Sterilised glass jars were employed containing a little fungicide, in the form of dry Benylate powder (active ingredient, benzimidazole). This storage protocol was used only in the case of mature seeds. Seeds at the younger growth stages were freeze-dried as outlined above.

3.2.2 Elemental analysis

Seeds were analysed for elemental content using ICP-AES as described previously for other plant tissues (section 2.2.1.5). In the case of seeds however, the plant material was freeze-dried rather than oven -dried and a mass of 2 g dried tissue rather than 0.5 g was used. Seeds and pods were analysed for Cd and Ni content as well as for Fe, Mn, Mg and Zn.

3.2.3 Determination of seed moisture content

Seed moisture content was determined according to one of two methods, immediately after excision from the pod. In the earlier experiments, twenty seeds per treatment were chosen at random. Seeds belonging to the VIP, IP and EP growth stages were wrapped in cling film immediately after removal from the pods in order to prevent moisture loss until further treatment. Individual seeds were placed in pre-weighed metal containers and the combined mass of seed and container recorded. After drying at 103°C for 24 hours the seeds were cooled in a desiccator before re-weighing. Subsequent experiments showed that freeze-drying and oven drying gave comparable results. From that point onwards therefore, in order to maximise the harvested material, on removal from the pod, seeds were bulked in groups of roughly 12-25. The bulk fresh mass was recorded and after freeze-drying for 24 hours (VIP, IP seeds) or 48 hours (EP, YP, BP) the seeds were re-weighed. Moisture content was calculated as the difference between fresh and dry mass and expressed as % moisture content per gram dry mass of seed. The freeze-dried tissue was stored and used for biochemical analysis according to the standard protocol.

3.2.4 Standard germination procedure

Twenty-five seeds of a given growth stage or treatment were surface sterilized by immersing in a 1% solution of sodium hypochlorite for 10 min. On a few occasions, immersing seeds directly into liquid caused imbibitional damage. In the case of these seed lots, it was found to be beneficial to first wrap the seeds in damp paper towelling for 4 hours, before initiating the sterilisation procedure. After treatment with sodium hypochlorite, seeds were rinsed three times with ultrapure (Millipore) water and allowed to drain for a few minutes. Two pieces of paper towelling were folded double and placed in the bottom of a plastic container (approximately 80 x 150 x 210 mm in size) which had been surface sterilized with concentrated sodium hypochlorite. The seeds were spread evenly over the surface of the towelling and covered with two more pieces of the same. The towelling was then moistened with 20-50 ml of 0.1% benylate solution. The volume of solution added was dependent on the moisture

content of the seeds (50 ml in the case of YP and BP seeds, 35-40 ml for EP, and 20 ml for IP or VIP). The plastic germination containers were sealed with air-tight lids, wrapped in black plastic to exclude light and placed in a controlled environment chamber set at 25°C day and 20°C night. Seeds were checked daily and the number of seeds germinated (i.e. the number of seeds with radicle protrusion greater than 5 mm) was recorded. Assessment of germination was terminated after 7 days and the final radicle lengths recorded. Results were expressed in terms of mean daily germination (MDG), peak value (PV) and germination value (GV) according to the method of Czabator (1962).

Where MDG represents the completeness of germination and is given by:

$$MDG = \frac{\%\ \text{Final germination}}{\text{Total no. of test days}}$$

Where PV represents the speed of germination and is given by:

$$PV = \text{maximum } T \text{ value}$$

$$\text{and } T = \frac{\%\ \text{Cumulative germination on a given day}}{\text{No. of days since beginning of test}}$$

Where GV represents the composite germination value and is given by:

$$GV = MDG \times PV$$

In addition, in later work, the germination index (GI) was calculated according to the method of Vertucci, Roos and Crane (1994).

$$GI = \%\ \text{final germination} \times \text{mean radicle length}$$

3.2.5 Quantification of storage reserves

3.2.5.1 Lipids

The method used for quantification of lipids was adapted from that of Christie (1973) and Privett, Dougherty, Erdahl *et al.* (1973). Fresh seeds were used, since freeze-dried tissue reportedly gives lower lipid yields (Christie 1973). It was considered unnecessary to perform the extraction under a nitrogen atmosphere, since only the quantity of lipid was determined and not the fatty acid profile. After preliminary experiments, it was found that inclusion of an iso-propanol extraction step as used by Christie (1973), did not increase lipid yields and this was also omitted from the final protocol.

Approximately 1 g of finely ground BP or YP seeds (2.5 g in the case of EP or IP seeds) were weighed out. After adding a volume of 25-40 ml extraction medium (chloroform:methanol 2:1 v/v), samples were liquidised in an Ultra-Turox homogeniser. Samples were filtered through Whatman No. 1 filter paper into measuring cylinders and the total volume made up to 60 ml with the extraction medium. A Folch wash was then carried out on the extracts to remove polar contaminants. The extracts were transferred to a separating funnel and 15 ml of 0.88% KCl solution added. The contents of the separating funnel were shaken together and left until a clear separation between the two phases was visible. The lower, organic phase containing the lipid, was removed by running it into a pre-weighed glass vessel. The lipid sample was concentrated under vacuum in a Savant SpeedVac SC110 rotary concentrator. Samples were stored in a desiccator and weighed daily until constant mass was attained. Results were expressed as mass of lipid per gram of tissue.

3.2.5.2 Carbohydrate

Starch and sugar contents were determined according to the method of Adams *et al.* (1980). Approximately 0.2 g of finely ground, freeze-dried seeds were weighed out and each sample extracted in 5 ml of water added to 25 ml of hot (approximately 70° C) 80% ethanol. The extracts were allowed to stand for 10 min and were then

centrifuged at 1 700g in a Beckman Ultracentrifuge for the same length of time. The supernatant was retained and the pellet re-suspended in a further 30 ml of hot ethanol, incubated once again for 10 min and centrifuged as above. The supernatant was once again retained and the extraction process repeated for a third time. Supernatants for each sample (90 ml in total) were combined and concentrated under vacuum in a Savant SpeedVac SC110 rotary concentrator to a volume of approximately 5 ml. Each sample was then made up to exactly 100 ml with distilled water.

Soluble carbohydrates (sugars) present in the supernatant fraction were determined directly using the phenol-sulphuric acid method of Dubois, Gilles, Hamilton *et al.* (1956) detailed further on. Insoluble carbohydrate (starch) in the pellet was first converted to soluble glucose and then assayed using the method of Dubois *et al.* (1956).

Insoluble carbohydrate (starch) was converted to glucose by adding 15 ml of distilled water to the pellet and autoclaving at 121°C for 60 min. This extract was then cooled and 5 ml of 0.1 M citrate buffer (pH 5.0) containing 5 mg amyloglucosidase and 1 mg alpha-amylase added to each sample. The extract was made up to a total volume of 50 ml using the citrate buffer and incubated at 30°C overnight. Following this, an aliquot of the starch extract (approximately 5 ml) was centrifuged at 25 000g for 5 min. The glucose content of the supernatant was determined using the Dubois phenol-sulphuric acid method as for soluble carbohydrates.

The Dubois phenol-sulphuric acid assay for glucose was carried out as follows. A standard curve was prepared in triplicate with glucose concentrations ranging from 10 μg to 100 μg in the total sample volume of 2 ml. One hundred microlitres of starch extract, or 250 μl of soluble carbohydrate extract were pipetted into a test tube. To each sample or sugar standard, 0.05 ml of 80% phenol followed by 5 ml concentrated sulphuric acid and an appropriate volume of distilled water were added

(to bring the total volume to 2 ml). After standing for 30 min at room temperature, absorbance at 485nm was read on a Hitachi U1100 Spectrophotometer.

3.2.5.3 Protein

Roughly 0.1 g of dry, lyophilised tissue was weighed and ground with a pestle and mortar in buffer. A volume of 10 ml was used for young seeds (i.e. VIP or IP growth stages) and 20 ml for all other growth stages. Two different buffers, 1 and 2 were employed. For the initial extractions, Buffer 1 (50 mM Tris-HCl, pH 7.5, containing 500 mM NaCl and 5 mM $MgCl_2$), modified from Blackman, Wettlaufer, Obendorf *et al.* (1991) was employed. For subsequent work, Buffer 2 (0.2 M Tris-HCl buffer, pH 8.5, containing 1M sucrose and 56 mM 2-mercaptoethanol), modified from Shatters, Abdelghany, Elbagoury *et al.* (1994) was used. The extract was spun at 23 700g for 10 min in a Beckman J2-21 centrifuge. The supernatant was removed and the pellet ground again in a pestle and mortar and re-suspended in a further 20ml of the same buffer. This second extract was centrifuged for 10 min as above, the supernatant removed and combined with that from the first extraction. The pellet was then ground and extracted for a third time in 10ml of buffer and centrifuged at the same speed for 20 min.

A 0.1 ml aliquot of the extract (diluted if necessary) was then mixed with 5 ml of Coomassie Blue G-250 reagent. The reagent was prepared according to the method of Bradford (1976) in 96% ethanol and 85% phosphoric acid. Absorbance was read at 595nm in a Hitachi U1100 Spectrophotometer, exactly 15 min after addition of the Coomassie Blue reagent. The concentration of protein in the sample was determined by comparison with a standard curve prepared from known masses of bovine serum albumin (BSA) diluted in Tris-HCl buffer.

3.2.5.4 Total nitrogen

Total nitrogen (N) was determined using the micro-Kjeldahl method outlined in Stock and Lewis (1986). Finely ground aliquots (0.05 g) of seeds were weighed accurately into thick-walled boiling tubes and 1 ml of distilled water added. Standards were also

prepared by adding appropriate volumes of a 1 mg N/ml ammonium sulphate stock solution to give standards in the range of 0 – 3.0 mg N per tube. Blanks, which contained 1 ml of distilled water but no N were also included. To each boiling tube, 3 ml of acidified salicylic acid (0.25 M) was added followed by a spatula tip of sodium thiosulphate crystals and one selenium Kjeldahl tablet. All tubes were placed in a cold digestion block and heated at 150°C overnight to drive off excess water. The samples were digested further according to the programme given below:

220°C for 1 hour

250°C for 1 hour

280°C for 1 hour

300°C for 1 hour

350°C for 2-4 hours.

The last section of the digestion protocol was terminated when the digest appeared clear. Any of the samples that contained dark material at this stage were cooled to 150°C and reheated in the above sequence using 30 minute intervals for the lower temperatures and 1-2 hours at 350°C. The tubes were then cooled to 150°C and 5-10 ml of distilled water was added. Each digest was made up to a total volume of 50 ml with distilled water, and mixed well.

Total nitrogen content of the digests was determined colorimetrically using the method of Smith (1980). A 0.2 ml aliquot of digest solution was pipetted into a thin-walled boiling tube and 25 ml of (0.12% m/v) disodium-EDTA added. To this was added, 2 ml of Reagent A (0.5% m/v fresh nitroprusside) followed by 2.5 ml of Reagent B. Reagent B was prepared by mixing one part 1.5% sodium hypochlorite with four parts alkaline phosphate buffer (0.7% m/v Na_2HPO_4 solution prepared in 1 litre of 2.1% m/v NaOH). The samples were made up to a final volume of 50 ml with distilled water, mixed until homogeneous and left for 1 hour. Absorbance at 635 nm was read on a Varian Cary 1E Spectrophotometer.

3.2.6 Construction of the seed development profile

Seeds were harvested from metal-stressed, as well as control plants and the following aspects of seed development examined at each growth stage:
Size (length, depth, fill)
Mass (fresh, dry)
Moisture content
Metal pollutant content
Storage reserve content
Content of nutritionally important metals (Fe, Zn, Mg, Mn)
Germination
Other parameters recorded included, pod yield, the number of deformed seeds and the mean number of seeds per pod.

3.2.7 Leakage experiments

All seed leakage experiments were carried out by measuring the change in EC of the soaking solution with a CM100 (Reid and Associates cc) conductivity meter. Prior to experimentation, the perspex wells (cells) were washed in a non-ionic detergent and rinsed in ultra-pure (Millipore water) to remove possible contamination. Each well was filled with 3 ml of Millipore water and the conductivity measured for each cell. Any cells that gave a reading higher than 1.0 μS were rinsed again until the conductivity levels fell below this threshold value. The background conductivity levels were then recorded for each cell. Water used to fill the wells was first stored for at least 24 hr in order to minimize CO_2 and NH_3 absorption effects (CM100 User manual 1996). All measurements were conducted 25° C.

Mature seeds of uniform size were weighed, rinsed briefly in ultra-pure water, blotted dry and then placed individually in each well. Any seeds with visible areas of mechanical damage were discarded. Care was taken to ensure that the moisture

content of control and treatment seeds was approximately the same. To this end, seeds were equilibrated in plastic mesh bags at 10°C, within the same storage jar for approximately 4 weeks prior to experimentation. A sub-sample of each was tested for moisture content in advance of the conductivity experiments.

Conductivity readings were taken immediately after placing seeds in the wells and thereafter every 3 min for 2 hrs. Each seed was then removed from its respective cell, placed in a numbered foil tray and oven dried at 104°C for 24hr. Once cool, seeds were reweighed and placed back in the appropriate well, which had been rinsed and refilled with 3 ml of Millipore water. Conductivity was again measured every 3 min for 2 hr as described above.

Results were calculated by first subtracting the appropriate background conductivity reading for each cell. The rate of increase in conductivity with time, expressed per gram dry mass of tissue, was calculated. This was first performed for each fresh seed followed by calculating leakage rate for the same seed after oven drying. The rate of leakage in oven dried seeds was considered to represent the maximum possible rate for each seed. Percentage relative leakage was expressed by the following:

$$\text{\% Relative leakage} = \frac{\text{Rate of leakage by fresh seed}}{\text{Rate of leakage of dried seed}} \times 100$$

This value was determined for each seed and the mean per treatment group calculated.

3.2.8 Seedling establishment trials

In order to examine the effect of metal-stress on seedling establishment, seeds harvested from metal-stressed or control plants were grown in soil. The

environmental conditions were as described for nutrient solution-grown plants (section 2.2.1). Seeds were first germinated according to the standard procedure and then planted in plastic bags (approximately 230 x 250 x 250 mm in size). The soil used was a mixture of commercial potting soil and acid-washed sand in the ratio of 3:1. Three seeds were planted per pot and the pots watered every second day. The length of time after germination for each plant to reach the growth stages V3, V5 and R1 (section 2.2.3) was recorded, as well as the mean number of pods produced per plant.

3.3 RESULTS

3.3.1 Changes in general seed morphology

In general, reproductive parts (pods and seeds) of metal-stressed plants did not differ in appearance from their control counterparts. Apart from the production of terminal deformed racemes (Fig. 2.3b) in Ni-stressed plants, seeds and pods from metal-treatment plants showed few external toxicity symptoms. A small proportion of the pods however, even those harvested from control plants did exhibit some deformities and this was found to increase on addition of Ni to the nutrient solution. If the deformed terminal racemes mentioned above are discounted, the percentage abnormality in harvested pods rose from 1.9% in the case of the controls, to 11.7% in the case of Ni-pods. Abnormalities usually involved failure of one or more ovules to develop. Alternatively, malformation of pods occurred so that they appeared misshapen or twisted. Very rarely however did the seeds appear to be damaged. The effect of Cd on the percentage of abnormally formed pods was not recorded but was not noticeably higher than for the Ni-treatment.

3.3.2 Effect of metal pollutants on seed growth parameters

Table 3.1 summarises the effect of Cd and Ni on seed mass and moisture content. Percentage moisture content decreased steadily with increasing seed age and was very similar between treatments. The presence of Cd or Ni in the growth medium did not appear to exert any significant effect on the water status of the seeds. For this reason percentage moisture contents given in the table represent the mean of control and treatment values.

As expected, control seeds generally showed a steady accumulation of dry mass with age. An exception to this pattern however was exhibited by BP seeds, which were slightly reduced in mass compared to YP seeds. This was noted in both Cd-control and Ni-control seeds. Seeds harvested from Cd-treatment plants showed a similar increase in dry mass with age until the BP stage. It was found that Cd caused a reduction in seed mass relative to the controls. This effect however was significant ($p \geq 0.001$) only in mature seeds.

In the case of Ni-treatment plants only seed mass for the two oldest growth stages was determined. It was found that a large sample size was necessary to yield accurate results, insufficient numbers of seeds precluded this being done for other developmental stages. The mean dry mass for BP seeds was also slightly less than that of YP seeds. Amendment of the nutrient medium with Ni, did not appear to reduce seed mass and no significant difference between treatment and control seeds was recorded.

The average number of seeds per pod was also examined for mature (BP) pods from Cd- and Ni-treatment plants and is presented in Table 3.2. This was recorded after each harvest and the sample size (n) therefore refers to the number of harvests. Nickel-treatment significantly decreased the mean number of seeds per pod relative to control pods ($P > 0.001$). Cadmium however, appeared to have little effect on this parameter.

***Table 3.1** Percentage moisture content (MC) and the effect of Cd and Ni on dry mass of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. SD and sample size given in parenthesis. *indicates significance at P≤0.001. ND= not determined. Descriptions of development stages given section 3.2.1.*

Seed Develop -ment Stage	Mean % MC	Seed mass (g)			
		Cd-treatment plants		Ni-treatment plants	
		Cd	Control	Ni	Control
VIP	85.5 (±0.01, n=30)	ND	0.0027 (±0.001,n=30)	ND	ND
IP	81.9 (±0.66, n=60)	0.012 (±0.02, n=30)	0.013 (±0.02, n=30)	ND	ND
EP	65.3 (±0.78, n=60)	0.146 (±0.06, n=30)	0.142 (±0.05, n=30)	ND	ND
YP	53.0 (±0.72, n=147)	0.228 (±0.1, n=61)	0.232 (±0.07, n=36)	0.192 (±0.1, n=29)	0.229 (±0.07, n =21)
BP	10.1 (±0.62, n=30)	0.192* (±0.04, n=84)	0.229* (±0.02, n=53)	0.198 (±0.04, n=37)	0.193 (±0.05, n=31)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod

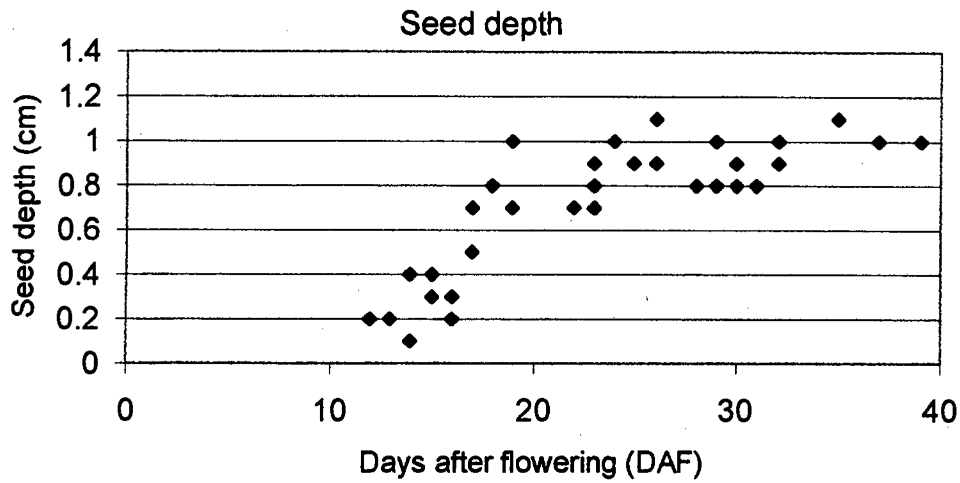
***Table 3.2.** Effect of Cd and Ni on the mean number of seeds per pod for mature (BP) pods harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. SD and sample size given in parenthesis. * indicates significance at P≤0.001.*

Number of seeds/pod			
Cd-treatment plants		Ni-treatment plants	
Treatment	Control	Treatment	Control
1.94 (±0.4, n = 14)	2.12 (±0.19, n = 11)	1.86* (±0.4, n = 24)	2.27 (±0.2, n = 20)

Figure 3.1 shows the change in size of control seeds with development. All measurements were made on pods containing 3 seeds. Preliminary measurements on Cd-treatment seeds did not reveal any marked difference in seed size and so this line of research was not pursued further. It was shown in Chapter 2 that pod expansion in length and depth was completed by approximately 10-12 DAF, whereas pod fill (expansion in width) was complete only by 40 DAF. Using the silhouette method, ovules were visible inside the pod from approximately 11 DAF. Ovule expansion with regards to length and depth was complete by 25 DAF. It was not possible to measure ovule fill from this method due to the orientation of the ovule within the pod. An estimate could be obtained however, by following the changes in pod fill (section 2.3.4). Since the pod walls were slightly less than 1 mm thick and the ovules were oppressed to the pod wall, ovule width was estimated to be approximately pod width, minus 1-2 mm. Developing seeds, as in the case of pods therefore, first completed growth in longitudinal and vertical planes (i.e. length and depth) before filling and increasing in width.

3.3.3 Effect of seed growth stage on metal accumulation

The concentration of metal pollutants in seeds at each developmental stage was determined. Table 3.3 shows these results expressed on a dry mass, fresh mass and per seed basis. The levels of both metal pollutants were higher in seeds from treatment plants compared to those harvested from the controls. Treatment with Cd raised the concentration of this element in mature seeds approximately four times that of control seeds. Nickel accumulation within seeds was higher than Cd accumulation at all growth stages and was approximately 50 times the level of Cd in mature seeds. Nickel levels in control seeds at all growth stages except IP were below detection using the method employed in this study.



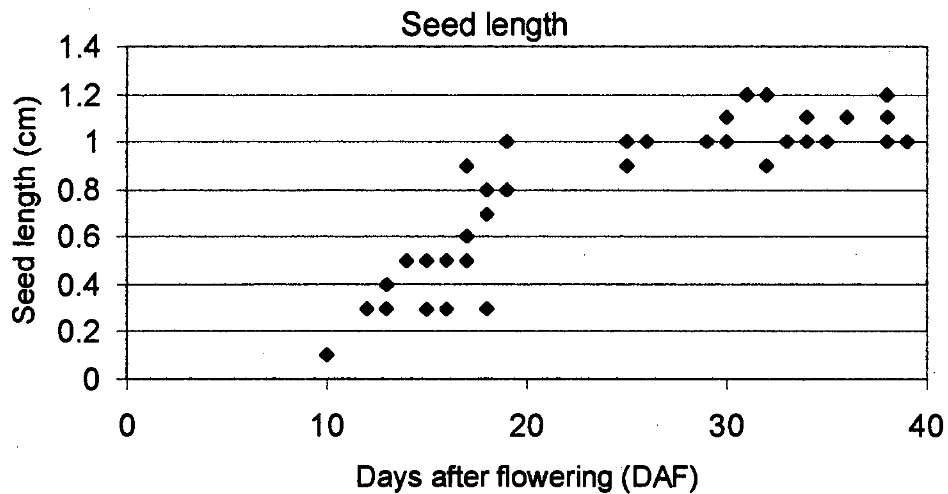


Fig. 3.1 *Changes in growth parameters of control seeds with age (DAF). Growth in the vertical and longitudinal planes depicted as changes in seed depth (cm) and seed length (cm) respectively.*

Table 3.3. *Effect of seed development stage on metal concentration in seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Concentrations in metal-treated seeds given on a dry mass, fresh mass and per seed basis. SD in parenthesis. Sample size n=3-4. BD= below detection. Full description of growth stages given section 3.2.1.*

Seed development stage	Cd-treatment plants			Cd control	Ni-treatment plants			Ni control
	µg/g.dry mass	µg/g.fresh mass	µg/seed	µg/g.dry mass	µg/g.dry mass	µg/g.fresh mass	µg/seed	µg/g.dry mass
IP	1.25 (±0.12)	0.24 (±0.03)	0.015 (±0.001)	0.37 (±0.03)	71.2 (±0.5)	13.5 (±0.1)	0.926 (±0.01)	1.2 (±0.16)
EP	0.84 (±0.09)	0.29 (±0.02)	0.123 (±0.014)	0.34 (±0.06)	49.8 (±5.2)	17.5 (±1.82)	7.07 (±0.74)	BD
YP	0.91 (±0.07)	0.43 (±0.03)	0.208 (±0.015)	0.33 (±0.04)	40.8 (±2.3)	19.2 (±1.1)	7.83 (±0.44)	BD
BP	0.87 (±0.15)	0.79 (±0.14)	0.167 (±0.029)	0.15 (±0.01)	49.1 (±5.8)	44.6 (±5.2)	9.72 (±1.14)	BD

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Both Cd and Ni levels in seeds tended to decrease after the IP growth stage when results were expressed on a dry mass basis. When expressed on a fresh mass basis both treatments showed an increase in metal accumulation relative to the control seeds as the seeds progressed from IP (immature pod) to BP (mature pod) growth stages. On expressing metal pollutant levels per seed, there was a steady increase in accumulation of both metals. An exception to this was the level in Cd-treatment BP seeds which showed a slight decline, a reflection of decreased seed mass (Table 3.1).

Table 3.4 shows the concentrations of metal pollutants (on a dry mass basis) in the pods harvested at different developmental stages. Levels of metal pollutants in pods harvested from treatment plants were always higher than in controls. The concentration of Ni in control pods was below detection for all growth stages. A clear trend in Cd levels was not shown in the case of pods from Cd-treatment plants. Nickel levels in pods tended to decrease with pod development, although, again the trend was not clear. Of interest is the distribution of metal pollutant between pods and seeds. For comparative purposes, seed metal levels (μg/g.dm) have also been included in the table. At all developmental stages, Ni levels were considerably higher in seeds than in pods. There appeared to be little difference between pods and seeds of Cd-treatment plants however, whatever the stage of development.

***Table 3.4** Effect of development stage on metal concentration (μg/g.dm) in pods harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Seed metal levels (μg/g.dm) also shown. SD given in parenthesis. Minimum n=3. BD= below detection. Full description of pod developmental stages given in section 2.2.3.*

Pod development stage	Cd-treatment plants			Ni-treatment plants		
	Cd seed	Cd pod	Control pod	Ni seed	Ni pod	Control pod
IP	1.25	0.95 (±0.02)	0.46 (±0.01)	71.2	31.03 (±5.8)	BD
EP	0.84	1.41 (±0.29)	0.47 (±0.01)	49.8	17.3 (±1.2)	BD
YP	0.91	0.96 (±0.15)	0.52 (±0.02)	40.8	8.49 (±1.8)	BD
BP	0.87	0.78 (±0.24)	0.48 (±0.01)	49.1	12.5 (±0.69)	BD

IP=immature pod, EP=expanded pod, YP=yellow, BP=brown pod

3.3.4 Effect of metal pollutants on storage reserves

3.3.4.1 Lipid

Total lipid content of seeds, expressed on a fresh mass, dry mass and per seed basis is presented in Table 3.5. In general the mass of lipid storage reserves and seed age were positively correlated. When expressed as mg per gram dry mass of tissue, the lipid content increased up until the YP stage and then decreased slightly in all experimental groups except the Ni-control plants. In contrast, when expressed on a fresh mass basis, all treatment groups showed a steady increase in the seed levels of this storage reserve. Both Ni-control and Ni-treatment seeds exhibited increasing lipid content with age when calculated per seed. In seeds from Cd-treatment plants however, lipid was reduced in the oldest growth stage when expressed on a per seed basis.

***Table 3.5** The effect of Cd and Ni on the **lipid content of seeds** harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Lipid expressed as mg/g.dry mass (dm), mg/g.fresh mass (fm) and on a per seed basis. SD given in parenthesis. *Indicates significance at p≤ 0.05. Sample size n= 3-4. ND= not determined. Full description of growth stages given in section 3.2.1.*

Seed develop -ment stage	Cd-treatment plants			Cd-control plants			Ni-treatment plants			Ni-control plants		
	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed
IP	114 (±0.59)	19 (±0.1)	1.368 (±0.07)	141 (±12.2)	24 (±2.08)	1.82 (±0.16)	ND	ND	ND	ND	ND	ND
EP	177 (±11.8)	60 (±4.0)	25.84 (±1.75)	221 (±8.3)	75 (±2.81)	31.38 (±1.14)	173 (±6.6)	62 (±2.36)	25.57 (±0.85)	175 (±10.61)	61 (±3.7)	24.85 (±1.52)
YP	202 (±5.0)	95 (±2.36)	46.06 (±1.14)	212 (±9.2)	100 (±4.34)	49.82 (±2.12)	ND	ND	ND	ND	ND	ND
BP	192 (±10.5)	167 (±9.14)	36.86* (±2.0)	191 (±8.5)	159 (±7.08)	43.74* (±1.8)	168 (±13.5)	153 (±12.27)	33.26 (±2.77)	209 (±15.6)	186 (±13.93)	40.33 (±2.90)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Figure 3.2 shows lipid content (on a per seed basis) reproduced in a graphical form to facilitate interpretation of the effect of treatment with metal pollutant. The presence of Cd in the nutrient solution lowered the lipid content of these seeds at all growth stages although this was significant only for mature (BP) seeds. This was a reflection of the reduced mass of mature Cd-seeds compared to that of the mature Cd-controls (Table 3.1), since no difference was apparent between the two treatments when expressed as mg/g.dm. Although the presence of Ni in the growth medium depressed lipid content, this was not significant when expressed on a per seed basis. From Table 3.5 it can be seen that if the results are expressed on a dry mass basis, although lipid levels in mature Ni-treatment seeds were substantially lower than their control counterparts, because of the high standard deviation, this effect was not significant.

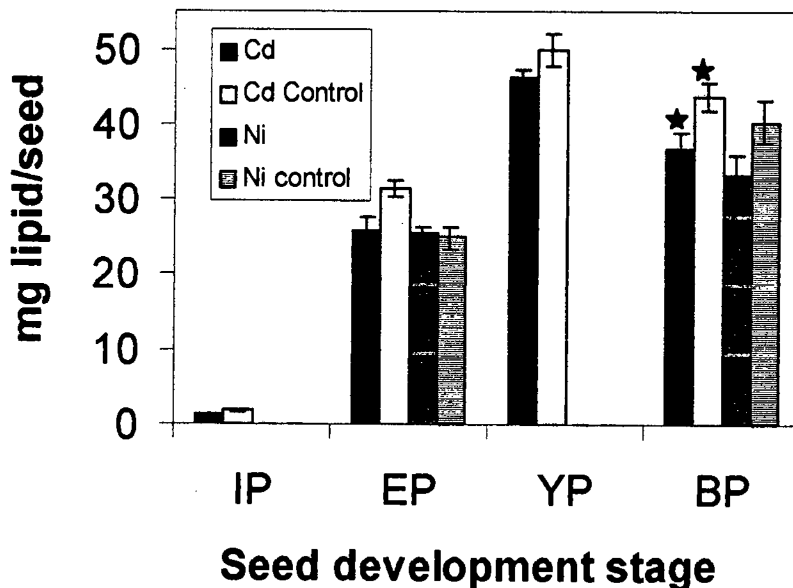


***Fig. 3.2** Effect of Cd and Ni on the lipid content of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Lipid expressed as mg per seed. *denotes significance at $p \leq 0.05$.*

3.3.4.2 Carbohydrates

Tables 3.6 and 3.7 report the starch and sugar content respectively of seeds harvested at the different growth stages. As in the case of lipids, results are reported in three different ways. In addition, the results are given on a per seed basis in Figures 3.3 and 3.4. From Table 3.6 it can be seen that when expressed on a dry mass basis, starch levels generally increased until the EP stage where they were maximal. They then decreased and for Cd-treatment seeds and Cd-control seeds little difference was apparent between the YP and BP growth stages. Starch levels at the IP and YP stages were not determined for the Ni-treatment, but these seeds also showed the highest starch content at EP growth stage and lowest (of those determined) at maturity. Results expressed on a per seed basis showed a similar trend to those expressed as per gram dry mass, namely a peak value at EP and then similar YP and BP levels.

Table 3.7 shows the soluble sugar levels in the seeds. On a dry mass basis, soluble sugar levels generally were high in IP seeds, decreased and then increased at the YP and BP levels. When expressed as per gram fresh mass, or per seed, the levels of this storage reserve were positively correlated with seed age.

Starch content expressed on a per seed basis is shown in Figure 3.3. It can be seen that treatment with Cd significantly decreased the starch content of mature seeds relative to that of the controls. There was no other statistically significant effect however. Figure 3.4 shows that in the younger growth stage, treatment with Cd or Ni had little effect on the soluble carbohydrate level. In mature seeds the metal pollutants appeared to decrease the levels of this storage reserve. This effect was not statistically significant however, most likely because sample size was small in the case of the mature metal-treatment seeds.

The regression equation for the glucose standard curve is given by:

$$\text{Glucose concentration} = 0.0077 \text{ X Abs} \quad (r^2 = 0.997)$$

Table 3.6 *The effect of Cd and Ni on the* ***starch content of seeds*** *harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Starch (represented by glucose) expressed as mg/g.dry mass (dm), mg/g.fresh mass (fm) and on a per seed basis. SD in parenthesis. Sample size n= 2-5. *Indicates significance at P≤ 0.05. ND= not determined. Full description of growth stages given in section 3.2.1.*

Seed develop -ment stage	**Cd-treatment plants**			**Cd-control plants**			**Ni-treatment plants**			**Ni-control plants**		
	mg/g.dm	**mg/g.fm**	**mg/seed**	**mg/g.dm**	**mg/g.fm**	**mg/seed**	**mg/g.dm**	**mg/g.fm**	**mg/seed**	**mg/g.dm**	**mg/g.fm**	**mg/seed**
IP	53.5 (±5.7)	9.1 (±1.0)	0.64 (±0.07)	68.4 (±0.0)	11.6 (±0.0)	0.89 (±0.0)	ND	ND	ND	ND	ND	ND
EP	193.7 (±8.2)	65.9 (±2.8)	28.28 (±1.2)	191.8 (±10.8)	65.2 (±3.7)	27.24 (±1.53)	149.9 (±0.2)	51.0 (±0.07)	21.29 (±0.03)	168.6 (±0.0)	57.35 (±0.0)	23.94 (±0.0)
YP	84.1 (±6.6)	39.5 (±3.1)	19.18 (±1.5)	67.3 (±8.3)	31.6 (±3.9)	15.82 (±1.95)	ND	ND	ND	ND	ND	ND
BP	77.8 (±1.1)	70.7 (±1.0)	14.94* (±0.21)	80.5 (±6.4)	73.2 (±5.8)	18.44* (±1.47)	32.4 (±5.3)	29.46 (±4.8)	6.4 (±1.05)	54.9 (±33.2)	49.91 (±30.2)	10.60 (±6.4)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Table 3.7 *The effect of Cd and Ni on the* ***soluble sugar content of seeds*** *harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Sugar (represented by glucose) expressed as mg/g.dry mass (dm), mg/g.fresh mass (fm) and on a per seed basis. SD in parenthesis. Sample size n= 3-4. *Indicates significance at P≤0.05. ND= not determined. Full description of growth stages given in section 3.2.1.*

Seed develop -ment stage	Cd-treatment plants			Cd-control plants			Ni-treatment plants			Ni-control plants		
	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed
IP	136.2 (±2.4)	23.2 (±0.4)	1.63 (±0.03)	138.2 (±9.8)	23.5 (±1.7)	1.80 (±0.13)	ND	ND	ND	ND	ND	ND
EP	85.9 (±7.5)	29.2 (±2.6)	12.54 (±1.1)	105.4 (±0.9)	35.9 (±0.3)	15.0 (±0.13)	77.3 (±5.0)	26.29 (±1.7)	10.98 (±0.71)	72.7 (±0.0)	24.7 (±0.0)	10.32 (±0.0)
YP	114.3 (±7.4)	53.7 (±3.5)	26.06 (±1.69)	108.9 (±5.4)	51.1 (±2.5)	25.60 (±1.27)	ND	ND	ND	ND	ND	ND
BP	137.8 (±4.1)	125.3 (±3.7)	26.46 (±0.79)	132.1 (±5.4)	120.0 (±4.9)	30.23 (±1.24)	69.6 (±15.3)	63.27 (±14.0)	13.78 (±3.03)	113.5 (±1.3)	103.2 (±1.2)	21.91 (±0.25)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

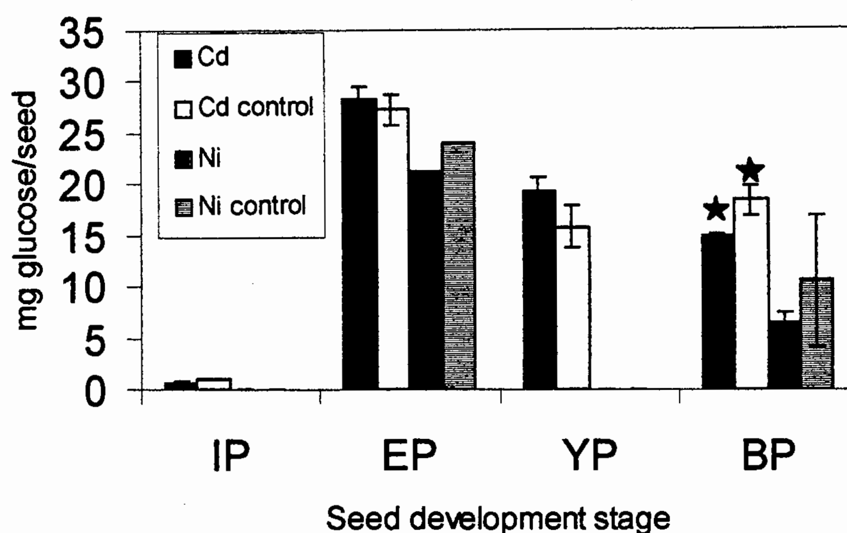


Fig. 3.3 Effect of metal pollutants on the starch content of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Starch expressed as mg glucose/seed. * *indicates significance at* $p \leq 0.05$. $7 \leq n \geq 2$.

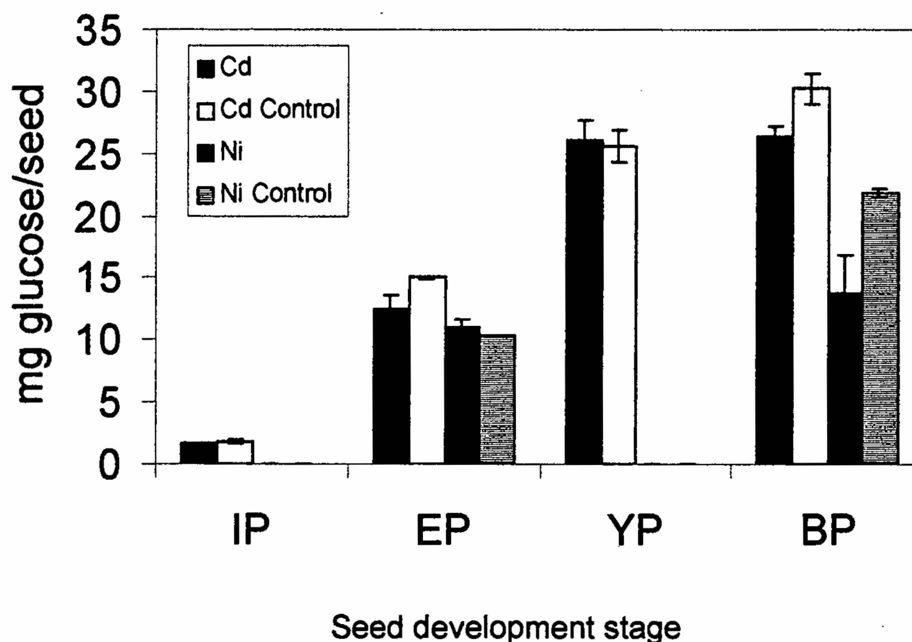


Fig. 3.4 Effect of metal pollutants on the soluble sugar content of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Sugar expressed as mg glucose/seed. $7 \leq n \geq 2$.

3.3.4.3 Protein

The results presented in Table 3.8 show that, when expressed on a fresh mass or per seed basis, protein content was positively correlated with seed age. The protein content of mature seeds was lower than reported in the literature however (Kondo *et al.* 1986). Therefore the protocol was assessed critically and a second extraction buffer employed. Of the two buffers employed, Buffer 2, containing sucrose and 2-mercaptoethanol (Shatters *et al.* 1994), consistently resulted in higher yields for seeds of all growth stages (Table 3.8) and was therefore used for subsequent experiments. Despite this, not all protein was removed into the supernatant, since performing further extractions on the pellet removed still more. The extraction process appeared to be more efficient when used on older seeds compared to VIP and IP seeds and thus in further work the younger growth stages were not assayed. In addition, the effect of de-fatting the seeds in cold acetone (Adams, Norby and Rinne 1982), prior to the protein extraction was examined. This was not found to enhance yields (data not shown) and was not employed in subsequent work.

Attention was also given to the assay method itself. The Folin-Lowry protein determination method (Lowry 1951) gave similar results to the Bradford method (data not shown) and thus this avenue of research was pursued no further. Reproducibility of results was difficult to attain using the Bradford method however, even when the same tissue sample was assayed. Absorbance of the Coomassie Blue-protein complex at 595 nm remained constant for approximately 20 min and then decreased with time. Agitation of the sample in the cuvette also decreased absorbance. Finally it was decided to use the Bradford assay, with the following precautions:

i) Glassware was washed and rinsed especially thoroughly since contamination by detergent has been reported to affect the assay (Bradford 1976).
ii) Addition of the Coomassie Blue G reagent to each test tube was staggered so that the absorbance of each sample was read after precisely 15 min.

Table 3.8 Effect of two buffers on the amount of protein extracted from control seeds at different growth stages. Protein content expressed on a dry mass (mg/g.dm), fresh mass (mg/g.fm) and per seed basis. Seeds harvested from plants grown in standard nutrient solution. SD and sample size in parenthesis. Full description of seed development stages given in section 3.2.1. Chemical composition of buffers given below table. P = amount of protein remaining behind in the pellet.

Seed develop -ment stage	Buffer 1			Buffer 2		
	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed
VIP	138.31 (± 7.9, n=4)	19.37 (± 1.1)	0.373 (± 0.14)	148.0 (±7.3, n=2) P=28.7 (± 5.1)	20.73 (± 1.02)	0.401 (± 0.02)
IP	158.11 (±2.96, n=4)	26.89 (± 0.5)	2.055 (± 0.06)	186.9 (±18.6, n=4) P = 34.95 (± 4.8)	31.79 (± 3.16)	2.431 (± 0.24)
EP	173.71 (±20.26, n=4)	59.09 (± 6.9)	24.667 (± 1.01)	324.1 (±14.7, n=4) P = 19.4 (± 1.9)	110.24 (± 5.0)	46.021 (± 2.09)
YP	200.5 (± 6.3, n=2)	94.13 (± 2.96)	46.52 (± 1.46)	288.2 (±13.0, n=4) P = 12.3 (± 2.8)	135.31 (± 6.1)	66.862 (± 3.02)
BP	203.5 (±7.8, n = 4)	185.0 (± 7.1)	46.60 (± 1.79)	259.1 (±30.0, n=4) P = 11.93 (± 3.9)	235.6 (± 27.3)	59.334 (± 6.87)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Buffer 1: 50 mM Tris-HCl (pH 7.5) containing 500 mM NaCl and 5 mM $MgCl_2$ (Blackman *et al.* 1991)

Buffer 2: 0.2 M Tris-HCl (pH 8.5) containing 1M sucrose and 56 mM 2-mercaptoethanol (Shatters *et al.* 1994)

iii) After addition of the dye, each test-tube was inverted exactly 3 times to mix the reagents. This was repeated after pouring the sample into the cuvette, immediately prior to reading absorbance.

iv) The protein content of control and treatment samples was determined during the same assay.

v) A new standard curve was drawn up every time and the samples were diluted so that the protein concentrations in the test vials were below 100 µg/ml. This latter precaution was deemed to be necessary since the standard curve for the Bradford protein assay is non-linear at higher concentrations (Pierce 1996).

The regression equation for a typical standard curve is given by:

$$\text{Protein concentration} = 106.8 \times \text{Abs} + 0.001 \ (r^2 = 0.987)$$

The effect of metal pollutants on the protein content of soybean seeds using buffer 2 and the standard extraction protocol is also shown in Table 3.9. Neither the presence of Cd nor Ni in the nutrient solution appeared to have a significant effect on the protein content of the seeds harvested from these plants. Protein levels were slightly lower in the Cd treatment for all growth stages but this was not statistically significant. In addition, there was considerable variation between the values obtained for the two control treatments (Cd-control and Ni-control) at a given growth stage.

3.3.4.4 <u>Total nitrogen</u>

Total N values were converted to mg protein by multiplying by a factor of 5.49, which is a conversion factor specific for soybean seeds (Mosse′ and Pernollet 1983). For all treatments, protein content and seed age were positively correlated (Table 3.10). Cadmium decreased nitrogen levels relative to the controls, although this was only significant for the mature seed values, expressed on a per seed basis and is a reflection of reduced seed size. Nickel-treatment, on the other hand, appeared to enhance seed protein levels, the Ni-treatment seeds at EP stage being significantly different from the control.

Table 3.9 Effect of Cd and Ni on the **protein content of seeds** at different growth stages. Protein content expressed on a dry mass (mg/g.dm), fresh mass (mg/g.fm) and per seed basis. Seeds harvested from plants grown in nutrient solution amended with 0.05 mg/litre Cd or 1 mg/litre Ni. ND = not determined. SD in parenthesis. Full description of seed development stages given in section 3.2.1

Seed Devel-opment stage	Cd-treatment plants			Cd-control plants			Ni-treatment plants			Ni-control plants		
	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/see d
EP	267.8 (± 15.9)	91.09 (± 5.41)	38.03 (± 2.26)	290.6 (± 9.5)	98.8 (± 3.23)	41.27 (± 1.35)	221.1 (± 7.0)	75.20 (± 2.38)	42.45 (± 1.3)	200.9 (± 30.2)	68.33 (± 10.27)	28.53 (± 4.29)
YP	262.9 (± 24.21)	123.43 (± 11.37)	60.99 (± 5.6)	268.0 (± 0.02)	125.8 (± 0.0)	62.18 (± 0.0)	ND	ND	ND	ND	ND	ND
BP	206.1 (± 6.6)	187.4 (± 6.0)	47.20 (± 1.5)	212.5 (± 3.8)	193.18 (± 3.46)	48.66 (± 0.87)	225.3 (± 13.2)	204.82 (± 12.0)	44.61 (± 2.61)	230.8 (± 17.4)	209.82 (± 15.8)	52.85 (± 3.99)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Table 3.10 *The effect of Cd and Ni on the* ***protein content (derived from total N determination) of seeds*** *harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Protein expressed as mg/g.dry mass (dm), mg/g.fresh mass (fm) and on a per seed basis. SD in parenthesis. Sample size n= 3. *Indicates significance at P≤ 0.05. Full description of growth stages given in section 3.2.1.*

Seed development stage	Cd-treatment plants			Cd-control plants			Ni-treatment plants			Ni-control plants		
	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed	mg/g.dm	mg/g.fm	mg/seed
EP	349.4 (±8.1)	118.8 (±2.76)	49.6 (±1.18)	372.2 (±20.7)	126.6 (±7.04)	52.9 (±2.94)	391.9* (±12.3)	133.3 (±4.18)	55.7 (±1.75)	367.5* (±8.2)	125.1 (±2.79)	52.2 (±1.16)
BP	377.3 (±15.7)	343.0 (±14.27)	72.4* (±3.01)	397.8 (±3.2)	361.6 (±2.91)	91.1* (±0.73)	407.0 (±26.3)	370.0 (±23.9)	80.6 (±5.2)	379.6 (±14.7)	345.1 (±13.36)	73.3 (±2.84)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

The regression equation for the total N standard curve is given by:

$$\text{Total N concentration} = 11.497 \times \text{Abs} - 0.226 \quad (r^2 = 0.973)$$

3.3.4.5 Seed mineral content

Table 3.11 shows the effect of Cd on seed mineral content. The concentration of the nutritionally important metals (Fe, Mn, Zn and Mg) is expressed as μg metal/g.dry mass of seed tissue for each development stage. The Fe content of seeds from Cd-treatment plants was decreased relative to that in control seeds in all age groups, although this effect was statistically significant for YP and BP seeds only. Cadmium treatment also appeared to decrease the Mn content of YP and BP seeds. Marked effects were noted on the Zn levels in seeds of all ages. Addition of Cd to the nutrient solution in which the plants were grown appeared to have an enhancing effect on the Zn levels in seeds. Similarly, Mg enrichment also occurred in mature Cd-treatment seeds.

Table 3.12 shows the effect of Ni on the mineral content of seeds harvested from plants grown in Ni-amended nutrient solution. As in the case of Cd-treatment seeds, the Fe content of seeds from Ni-treatment plants was significantly decreased in all age groups. Unlike Cd-treatment seeds however, Mg levels were also lowered relative to the controls. The effect of Ni-treatment on the Mn and Zn seed levels was variable and depended on the growth stage. Overall, the most pronounced effect of Ni addition to the nutrient solution, was the reduction of seed Fe content. The level of Fe in Ni-treatment BP seeds was 39% less than in mature control seeds. The reduction was even more pronounced in Ni-treatment IP seeds, which contained 43% less Fe than their untreated counterparts. No significant enhancement of mineral content resulting from Ni treatment was detected.

The effect of the metal pollutants, Cd and Ni, on the mineral content of pods at different development stages, was also studied. Once again (Table 3.13), Cd resulted in significantly lowered Fe levels in tissues harvested from treatment plants.

***Table 3.11** Effect of **Cd treatment on the mineral content (µg/g.dm) of seeds** at different growth stages. Seeds harvested from plants grown in nutrient solution amended with 0.05 mg/litre Cd. SD given in parenthesis, n = 3. Figure given in bold indicates the percent decrease (-) or increase (+) relative to the control value. Asterisks denote significance p ≤ 0.05. Description of seed development stages given in section 3.2.1*

Seed Development stage	Fe			Mn			Zn			Mg		
	Treatment		Control	Treatment		Control	Treatment		Control	Treatment		Control
IP	69.6 (±3.1)	**- 9%**	76.5 (±4.7)	27.8 (±2.6)	**- 8%**	30.4 (±8.8)	63.0* (±2.2)	**+ 16%**	54.5* (±3.2)	1760 (±20.3)	**+1%**	1750 (±43.6)
EP	65.5 (±15.5)	**- 9%**	71.8 (±3.2)	24.2 (±3.3)	**+ 18%**	20.6 (±5.8)	53.3* (±4.2)	**+52%**	35.0* (±4.8)	2227* (±30.6)	**+ 6%**	2107* (±25.2)
YP	52.5* (±4.8)	**- 22%**	67.6* (±10.1)	16.6* (±0.9)	**- 32%**	24.6* (±3.9)	59.5* (±2.7)	**+58%**	37.7* (±2.4)	2723 (±75.7)	**- 6%**	2903 (±513.9)
BP	56.3* (±1.7)	**- 24%**	73.7* (±2.5)	16.6* (±6.3)	**- 45%**	30.2* (±2.2)	60.3* (±0.9)	**+ 93%**	31.2* (±1.0)	2980* (±19.9)	**+10%**	2720* (±132.3)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

Table 3.12 *Effect of* ***Ni treatment on the mineral content (μg/g.d.m) of seeds*** *at different growth stages. Seeds harvested from plants grown in nutrient solution amended with 1 mg/litre Ni. SD given in parenthesis, n = 3. Figure given in bold, indicates the percent decrease (-) or increase (+) relative to the control value. Asterisks denote significance at p ≤ 0.05. Description of seed development stages given in section 3.2.1*

Seed Development stage	Fe			Mn			Zn			Mg		
	Treatment		Control	Treatment		Control	Treatment		Control	Treatment		Control
IP	43.3* (±1.4)	**- 43%**	76.5* (±4.7)	15.4* (±1.2)	**- 49%**	30.4* (±8.8)	48.4* (±1.0)	**-11%**	54.5* (±3.2)	1623* (±50.3)	**- 7%**	1750* (±43.6)
EP	51.1* (±6.3)	**- 29%**	71.8* (±3.2)	26.5 (±7.2)	**+ 29%**	20.6 (±5.8)	40.4 (±4.5)	**+15%**	35.0 (±4.8)	1867* (±159.5)	**- 11%**	2107* (±25.2)
YP	56.2* (±1.5)	**- 17%**	67.6* (±10.1)	25.3 (±3.0)	**+ 3%**	24.6 (±3.9)	34.2* (±0.2)	**- 9%**	37.7* (±2.4)	2417 (±83.3)	**- 17%**	2903 (±513.9)
BP	45.1* (±2.1)	**- 39%**	73.7* (±2.5)	22.7* (±2.2)	**- 25%**	30.2* (±2.2)	35.4 (±2.9)	**+ 14%**	31.2 (±1.0)	2570* (±26.5)	**- 5%**	2720* (±132.3)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

This effect was apparent in pods from all growth stages. The high Fe concentration obtained for the BP control-treatment was unexpected and may possibly be an artefact due to contamination. Despite this, it is still considered that the depression of Fe in mature pods harvested from plants grown in Cd-amended growth medium, was a true effect. Addition of Cd to the nutrient solution appeared to have a variable effect on the Mn and Zn content in pods however and was dependent on growth stage. Magnesium concentration was increased significantly in YP and BP pods by Cd treatment.

A comparison of Tables 3.11 and 3.13 reveals that Fe levels for both treatment and controls were higher for all development stages in the seeds compared to the pods. That was also generally the case for Zn. In the case of Mn and Mg however, pod levels of these metals in both treatment and controls were higher than those of seeds.

Treatment with Ni generally reduced the Fe content of pods (Table 3.14) as well as in seeds, one exception being EP treatment pods. As in the case of the seeds, pod Mn content was depressed relative to that of control pods. The effect of Ni on the Zn and Mg content of these tissues did not show a clear pattern. Seed Fe levels in both treatment and controls were marginally higher than in pods at the same growth stage (Tables 3.12 and 3.14), as were Zn concentrations. Pods tended to be enriched in Mn and Mg relative to the seeds however. This is similar to the distribution pattern of mineral nutrients between pod and seeds brought about by Cd treatment.

Table 3.13 *Effect of* ***Cd-treatment on the mineral content (μg/g.dm) of pods*** *at different growth stages. Pods harvested from plants grown in nutrient solution amended with 0.05 mg/litre Cd. SD in parenthesis, n = 3. Figure given in bold, indicates the percent decrease (-) or increase (+) relative to the control value. Asterisks denote significance *p≤ 0.10 and **p≤ 0.05. Description of pod development stages given in section 2.2.3.*

Seed Development stage	Fe			Mn			Zn			Mg		
	Treatment		Control	Treatment		Control	Treatment		Control	Treatment		Control
IP	31.0* (±7.9)	**- 31%**	45.0* (±6.9)	36.7 (±12.3)	**- 10%**	41.0 (±3.3)	32.7 (±6.0)	**- 30%**	47.0 (±13.3)	3457 (±338.6)	**+14%**	3040 (±396)
EP	14.7* (±3.7)	**- 41%**	25.1* (±6.5)	45.5 (±15.3)	**- 33%**	67.8 (±24.5)	26.5 (±1.1)	**- 12%**	30.3 (±4.3)	4533.3 (±268.6)	**- 4%**	4720 (±198)
YP	10.9* (±2.8)	**- 53%**	23.1* (±4.0)	77.0 (±13.7)	**+36%**	56.8 (±12.9)	31.1 (±19.0)	**+ 48%**	21 (±10.3)	6950* (±98.5)	**+21%**	5735* (±318.2)
BP	14.7** (±3.5)	**- 94%**	231.3** (±81)	80.0 (±22.1)	**+17%**	68.2 (±1.8)	34.4 (±18.1)	**- 63%**	92.2 (±103.)	6757** (±1183.1)	**+33%**	5067** (±135.8)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

***Table 3.14** Effect of **Ni treatment on the mineral content (μg/g.dm) of pods** at different growth stages. Pods harvested from plants grown in nutrient solution amended with 1 mg/litre Ni. SD in parenthesis, n = 3. Figure given in bold, indicates the percent decrease (-) or increase (+) relative to the control value. Asterisks denote significance *p≤ 0.10 and **p≤ 0.05. Description of pod development stages given in section 2.2.3.*

Seed Develop-ment stage	Fe			Mn			Zn			Mg		
	Treatment		Control	Treatment		Control	Treatment		Control	Treatment		Control
IP	30.1* (±4.4)	**- 33%**	45.0* (±6.9)	32.3 (±9.1)	**- 21%**	41.0 (±3.3)	31.2 (±9.1)	**- 34%**	47.0 (±13.3)	2820 (±466.1)	**- 7%**	3040 (±396)
EP	76.4* (±19.1)	**+ 204%**	25.1* (±6.5)	53.7 (±6.9)	**- 21%**	67.8 (±24.5)	45 (±12.3)	**+ 49%**	30.3 (±4.3)	3975 (±35.4)	**- 16%**	4720 (±198)
YP	13.2* (±3.2)	**- 43%**	23.1* (±4.0)	34.4 (±13.7)	**- 39%**	56.8 (±12.9)	33.2 (±16.5)	**+ 58%**	21 (±10.3)	4553 (±687.7)	**- 21%**	5735 (±318.2)
BP	12.6** (±1.9)	**- 95%**	231.3** (±81)	32.1** (±18.8)	**- 53%**	68.2** (±1.8)	18.8 (±10.5)	**- 80%**	92.2 (±103.)	5180 (±81.9)	**+ 2%**	5067 (±135.8)

IP=immature pod, EP=expanded pod, YP=yellow pod, BP=brown pod.

3.3.5 Effect of metal pollutants on membrane integrity

As expected, the rate of leakage in oven-dried seeds was higher than in fresh seeds, although considerable seed to seed variation was noted. For some seeds, leakage was not linear and results from any seed that yielded r^2 values less than 0.9 in the linear regression calculations were discarded. A summary of the percentage relative leakage rates for the different seed treatments is shown in Table 3.15. Because ageing can also be detrimental to membrane functioning (Sung 1996) and because Cd-seeds were 8 months older than Ni-seeds, results from the control treatments were not combined. Neither Cd nor Ni appeared to have a significant effect on membrane functioning as indicated by the very similar relative leakage values of treatment and control seeds. Percentage relative leakage in Ni seeds (both treatment and control) was high compared to that in Cd seeds.

Table 3.15 *Effect of metal pollutants on membrane integrity as indicated by the rate of electrolyte leakage from seeds of the different treatment groups. Results expressed as the percentage leakage rate in fresh the seed, relative to that after oven-drying. Moisture content and sample size (n) are indicated.*

Treatment	% Moisture content	No. of seeds (n)	% Relative leakage
Cd-treatment	11.48 (± 0.42)	30	29.41 (± 15.45)
Cd-control	11.33 (± 0.57)	30	30.75 (± 17.3)
Ni-treatment	12.01 (± 0.46)	20	61.45 (± 23.36)
Ni-control	11.47 (± 0.33)	15	61.8 (± 17.6)

3.3.6 Effect of metal pollutants on seed germination

Seeds were harvested from metal pollutant-treatment plants at different growth stages and the germination capacity determined (Table 3.16). The germination value (GV) generally increased for each treatment with development stage. This was a result of the fact that both the rate of germination (as given by the peak value, PV) and the extent of germination (as given by mean daily germination, MDG) also increased with development stage. Only control VIP seeds were tested, but none of these seeds germinated. Even at the IP stage, only 11% of the control seeds germinated (thus yielding an MDG value of 1.5) and no seeds from Cd-treatment plants. For all growth stages examined, germination of Cd-seeds was always slightly less than that of control seeds. Due to a paucity of seeds, the effect of Ni treatment was only examined for mature (BP) seeds. It can be seen that treatment with either metal pollutant depressed the GV in mature seeds relative to controls. A comparison of PV and MDG values however, shows that for all three treatments the extent of germination (given by MDG) was identical and that 100% germination was achieved for all treatments by the end of the test period.

The rate of germination was significantly reduced in Cd-seeds however (PV = 31), as well as in Ni-seeds (PV = 30) compared to that of the controls (PV = 38). Despite this, the slight depression in seed vigour exhibited by metal-treatment seeds was not supported by the germination index (GI) results, which is calculated from % germination and mean radicle length (section 3.2.4). Slightly longer radicle lengths were exhibited by treatment seeds compared to the controls. These results were not statistically significant however, and although large differences were recorded for the GI values (mean radicle length X 100% germination) these are deceptive. More definitive results may have been obtained if a larger number of replicates had been used; however a lack of sufficient number of seeds prevented this.

***Table 3.16** Effect of Cd and Ni on germination (given by germination value and germination index) of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Description of development stages given in section 3.2.1. Definition of germination terms given in section 3.2.4. SD in parenthesis. ND = not determined.*

Treatment / develop-ment Stage	PV	MDG	GV	Mean radicle Length (cm)	GI	No. of replicates
VIP control	0	0	0	0	0	1
IP control	1.75 (± 1.03)	1.5 (± 1.18)	2.63	ND	ND	3
IP cadmium	0	0	0	ND	ND	1
EP control	14.3 (± 0.8)	13.3 (± 0.6)	190.2	ND	ND	3
EP cadmium	14.5	12.6	182.7	ND	ND	1
YP control	23.0	13.14	302.3	ND	ND	1
YP cadmium	22.3	13.14	293	ND	ND	1
BP control	39.5 (± 5.0)	14.3 (± 0)	539.1	11.26 (± 0.6)	1126	5
BP cadmium	31.4 (± 0.75)	14.3 (± 0)	449.0	13.2 (± 1.2)	1320	4
BP nickel	30.0 (± 0.03)	14.3 (± 0)	429.0	12.7 (± 1.9)	1270	2

IP=immature pod, EP=expanded pod, YP=yellow, BP=brown pod.
PV=peak value, MDG=mean daily germination, GV=germination value (=PV x MDG);
GI=germination index (= % germination x mean radicle length).

3.3.7 Effect of metal pollutants on seedling establishment

Seedlings from all treatment groups appeared to be very similar in their growth rates (Table 3.17). This was demonstrated by the fact that similar lengths of time were taken by seedlings grown from metal pollutant-treatment seeds and control seeds to reach given growth stages. In addition, no significant differences in final seed yields were detected between treatments and all seedlings appeared healthy (data not shown). The results did however emphasise the high productivity of plants grown in the nutrient solution system, since the mean number of days to flowering was 28 in nutrient-grown plants compared to approximately 40 when grown in soil. Furthermore, the mean number of pods produced per plant was severely curtailed in this experiment, compared to the mean value of 95 pods/plant obtained from control plants cultivated in the nutrient solution system.

3.4 DISCUSSION

Seeds of all treatment groups progressed through the expected developmental phases as discussed in the literature review of seed development (section 1.4.1). Figure 3.5 shows the generalised seed development curve given in Chapter 1. The different seed growth stages used in this study, have been superimposed on the developmental curve. Positioning of the growth stages is approximate however, since the development curve is generalised and a detailed developmental profile was not carried out. Values for DAF are taken from Table 2.4 and represent the mean of the two control treatments.

Dissection of seeds at the VIP stage showed that the embryo occupied approximately half of the central space and was still surrounded by liquid endosperm. The VIP stage was thus during the period of histodifferentiation. Unfortunately it was not possible to include this growth stage in the majority of the work due to a lack of material.

Table 3.17 *Effect of metal pollutants on seedling establishment in soil. Mature seeds harvested from plants grown in nutrient solution containing either 0.05 mg/litre Cd or 1 mg/litre Ni, germinated and grown in soil to maturity. Number of plants = 18 per treatment. SD in parenthesis. "Crawford" = seeds from the original, non-polluted seed batch. Description of plant growth stages given in section 2.2.3.*

Treatment	Mean no. days to reach V1	Mean no. days to reach V5	Mean no. days to flowering (R1)	Mean no. of pods/plant
Cadmium	31.8 (± 5.8)	37.6 (± 2.4)	38.4 (± 3.5)	8.6 (± 4.6)
Cadmium control	35.9 (± 2.8)	43.4 (± 4.3)	42.3 (± 3.5)	8.5 (± 3.5)
Nickel	32.0 (± 4.8)	40.8 (± 3.5)	40.3 (± 2.3)	10.6 (± 4.5)
Nickel control	33.4 (± 4.6)	40.5 (± 1.2)	40.7 (± 1.4)	8.5 (± 5.2)
"Crawford"	33.0 (± 4.4)	38 (± 3.4)	40.8 (± 2.8)	9.0 (± 3.4)

V3 = 3 nodes on main stem, terminal leaf completely unrolled.
V5 = 5 nodes on main stem, terminal leaf completely unrolled.
R1 = flowers present at any node.

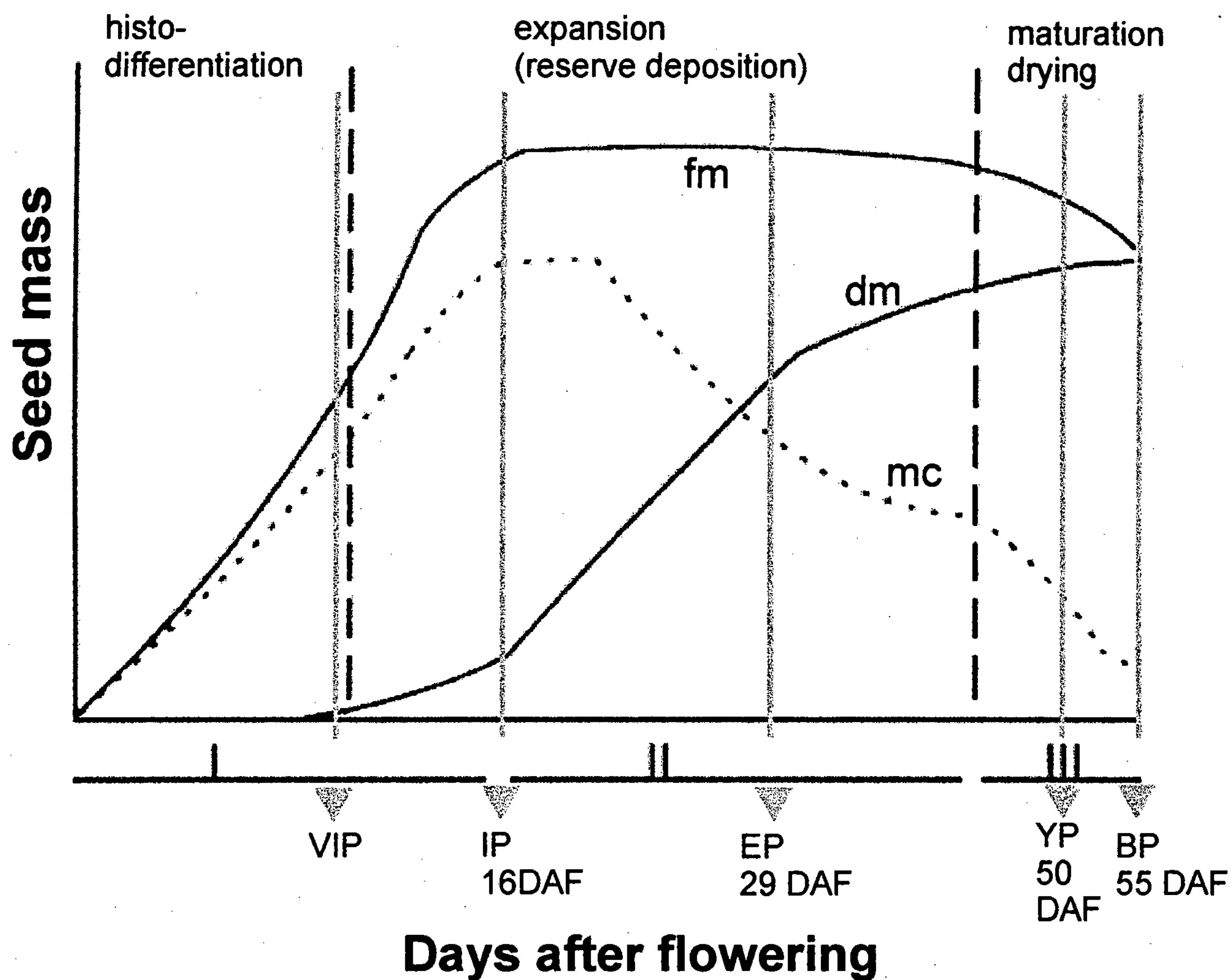


Fig. 3.5 Generalised seed development curve. Changes in fresh mass (fm), dry mass (dm) and moisture content (mc) with seed development are shown. Description of development phases (I, II, III) given in Chapter 1. Approximate DAF of development stages used in the study also given. Adapted from Bewley and Black (1994).

Although the precise DAF of the VIP growth stage was not determined it was approximately 12. These seeds contained 86% moisture and had accumulated only 1% of final dry mass.

By the IP development stage, the central cavity was completely filled by the embryo. Control pods reached this point at 16 DAF. From Figure 2.6 it can be seen that pod growth with respect to length and depth had reached completion by this growth stage. Seed growth with regard to length and depth was only 50% of maximum however (Figure 3.1). Pod (and therefore seed) fill, were approximately one third of the final maximum size. At this growth stage, mean seed moisture content was 81% and dry mass approximately 6% of the final mass. It is considered that IP seeds had just completed phase I and were at early seed fill (phase II). According to Bils and Howell (1963), cell division is completed by approximately 14 DAF, however the length of this period may vary with cultivar. It is considered therefore that very little, if any cell division would be taking place in IP seeds.

The next growth stage, EP, was reached by control seeds on average 29 DAF. Seed growth in length and depth was completed by this stage, but not seed fill (Figures 2.6 and 3.1). Seeds had amassed 62% of final dry weight, contained 65% moisture and were considered to be at mid seed-fill (phase II).

The Yellow pod (YP) development stage was reached 50 DAF, when the seeds contained 53% moisture and had attained 101% of final mass. Seeds were detached from the parent plant at this point and the chlorophyll content had undergone degradation. Seeds were considered to be in phase III of the generalised development curve i.e. they were undergoing maturation drying. Loss of chlorophyll from the pods has been cited by many workers to be a reliable indication of physiological maturity or PM (Crookston and Hill 1978; TeKrony *et al.* 1979). Seed moisture content at PM appears to be relatively constant with cultivar and growing conditions. Values of 54-62% moisture content at PM have been reported in the

literature (TeKrony *et al.* 1979), which compare favourably with the results from the present study.

By the BP growth stage, seeds contained approximately 10% moisture, 100% dry mass and had completed maturation drying. Several researchers (Bils and Howell 1963; Privett *et al.* 1973) have reported a decrease in mass of soybean seeds between PM (i.e. YP seeds) and at harvest (BP seeds). Similar results were found in this study for Cd-treatment and control seeds. Cadmium and Ni-treatment seeds differed from this scheme only in that, as discussed in Chapter 2, the YP and BP growth stages were reached earlier than the controls. In addition, Cd-treatment seeds were slightly smaller in size.

Amongst the various organs of the aerial portions of the shoot, levels of metal pollutants are generally lowest in seeds compared to other plant parts (Marschner 1983; Ernst 1990) and this was substantiated by the results obtained with Cd in this study. It is fortunate that seeds tend to be protected from accumulation of this toxic metal. Itai-itai disease (section 1.3.4.2) however, is caused by high Cd levels in rice (Marschner 1983). Furthermore, high levels of Cd in flaxseed (Grant and Bailey 1997) and wheat grain (Gavi *et al.* 1997; Hart *et al.* 1998) have also given cause for concern. Although the value of 1 mg/g.dm Cd obtained in this project is relatively low, it is ten times the recommended level for Cd in legume crops as prescribed by the World Health Organisation in 1992 (Petterson and Harris 1995). Seed levels of Cd that have been reported for legume species in the literature are presented in Table 3.18. Also shown are the concentrations of Cd in the growth medium used to cultivate the plants. From the table it can be seen that the seed Cd levels obtained in this study correspond roughly to those that would be expected from plants grown in a moderately polluted soil.

The relatively high seed concentrations of Ni obtained in this study were similar to those found by Halstead, Finn and MacLean (1969) as well as by Cataldo *et al.* (1978a). Nickel appears to be an exception to the rule that metal accumulation is

Table 3.18 *Summary of seed Cd and Ni concentrations (µg/g.dm) reported in the literature for soybeans (Glycine max) and other plant species. The growth medium used to cultivate the plants is indicated as well as the Cd concentration supplied.*

Plant species	Growth medium	Growth medium Cd (mg/kg)	Seed Cd (µg/g.dm)	Reference
CADMIUM				
Pisum sativum	Soil from polluted area	280	1.02	Stefanov *et al.* (1995)
Glycine max	Cd amended soil	2.5 40	3.9 29.0	Bingham *et al.* (1975) " "
Phaseolus vulgaris	" "	2.5 40	0.2 1.7	" " " "
Glycine max	Unpolluted soil	Not given = background	0.06	Wolnik *et al.* (1983)
Glycine max	Soil from polluted area	Not given	1.4	Yoshida (1986)
Glycine max	Cd amended soil	Not given	1.8	Jones *et al.* (1973)
Glycine max	Cd amended soil	0 2.5 5.0	0.29 1.88 2.51	Chaney and Hornick (1978) " " " "
Glycine max	Cd amended soil	12.1	2.62	Chaer Boges and Wollum (1980)
Glycine max	Cd amended soil	12	0.18	Ham and Dowdy (1978)
Pisum vulgare Faba vulgaris	Cd amended soil " "	10 10	2.64 1.18	Cimino and Toscano (1993) " "
NICKEL				
Phaseolus vulgaris	Ni amended soil	Not given	32.1	Lange-Hesse *et al.* (1994)
Glycine max	Ni amended soil	440	16.3	Ham and Dowdy (1978)
Avena sativa	Ni amended soil	56 84	55.01 37.8	Poulik (1997) " "
Avena sativa	Ni amended soil	500	68	Halstead (1969)

lowest in seeds in comparison to other plant parts (Sajwan *et al.* 1996) and accumulation of Ni in oat grain has been reported to be an increasingly serious problem (Poulik 1997). Seed levels reported in the literature are summarized in Table 3.18. Few references to legumes were available and thus other plant groups have been included. Nickel is more mobile within plants than Cd, as shown by the elevated concentrations of this element in all plant parts, examined in this study. The concentration of Ni used in the nutrient solution was twenty times higher than that of Cd. Calculation of the concentration factor (i.e. the ratio of the concentration of metal accumulated in seeds to that in the growth medium) yields values of 20 for Cd and 50 for Ni. Thus the magnitude of accumulation in soybean seeds was greater for Ni than for Cd, and was not simply a result of a higher supplied concentration.

Accumulation patterns of the two metal pollutants in pods relative to seeds suggests that pods pose very little barrier, or exert little screening effect on metal pollutants, since higher levels relative to the seeds would then have been expected. In the case of Cd, little difference was found between the two plant parts, low concentrations of this element being found in both. Similar results were obtained by Ham and Dowdy (1978), when investigating uptake of metals into soybean from soil amended with sewage sludge. Although other experiments with radioactive Cd in soybean plants showed that Cd levels were higher in pods compared to seeds (Haghiri 1973). Cimino and Toscano (1993) examined uptake of Cd from sludge- or metal-amended soils into pea and bean seeds. They also found that Cd levels in the pods were significantly higher than in the seeds for both species. The results reported in the present study with regard to Ni, show that seed levels of this element were consistently higher than those of pods for all growth stages. Similar results were reported by Cataldo *et al.* (1978a) who examined distribution of $^{63}Ni^{+2}$ in *Glycine max.*

It was shown in Chapter 2 that both Cd and Ni markedly decreased the number of pods produced per plant and that this effect was not due to abortion of pods at the IP growth stage or later. Thus these metals exerted an effect on early events, such as flower production or fruit set. Metal pollutants, including Cd and Ni commonly inhibit

cell division (Breckle 1991; L'Huillier *et al.* 1996). Furthermore, the highest metal levels (when expressed as μg.metal/g.dm of seed tissue) were found during the earliest stage of seed development and gradually decreased, presumably due to accumulation of storage reserves. These two factors may explain why the major toxicity effects of the metal pollutants were exerted very early during the reproductive phase. According to Peterson *et al.* (1992), pod abortion in soybean (in the absence of metal pollutants) usually occurs early in embryogenesis, from 3 to 9 DAF and even during normal seed development, soybeans are susceptible to abortion of flowers and immature pods. Nothing could be found in the literature concerning the stage at which metal pollutants might be expected to exert an effect on seed development. In this study, the very early stages were not examined because of experimental difficulties (the flowers are very small in size and a large number are produced per plant). Hence, further work is required to determine if lowered seed yields resulting from metal-pollutant stress are due to fewer flowers being produced, increased pod abscission during early embryogenesis, or both. It should be noted however, that over and above any specific toxicity effects on embryogenesis, the fact that leaf abscission in metal-treatment plants was increased relative to control plants, would have meant that reduced photosynthate was available. This in turn would be likely to reduce seed production.

Once committed to pod formation however, the two metal pollutants exerted differing effects on seed development. Nickel treatment resulted in reduced numbers of seeds per pod, but seed mass was equivalent to control seeds. Cadmium treatment resulted in the same number of seeds per pod as the control, but individual seeds were smaller. This suggests a very different role for each element. Since Ni decreased seed number, seed or flower production had been reduced. Cadmium treatment on the other hand resulted in decreased seed mass. Thus Ni stress occurs early during seed development, whilst Cd stress occurs after seeds are established. The resulting difference in seed mass may be explained in terms of the amount of photosynthate available from the parent for reserve accumulation. Because Ni-treatment reduced the number of seeds during early development, there was more

photosynthate available per seed for reserve accumulation. With greater numbers of seeds reaching the stage of nutrient deposition, Cd treatment resulted in reduced storage reserve accumulation and this affected seed mass. Cieslinski *et al.* (1996a) concluded that yield reduction in strawberry fruit when grown in Cd-amended soil resulted mainly from decreases in fruit number rather than average weight per berry. Moraghan (1993) when investigating the effect of the same metal pollutant found similar effects on yield parameters of flaxseed. On the other hand, Singal *et al.* (1995), examined the effect of Cd on seed mass of fenugreek, and found, that at all developmental stages there was a general decrease in seed size as the concentration of the metal pollutant increased. Data presented in Chapter 2, indicated that both Cd and Ni significantly decreased the length of time taken for pods to reach the YP and BP growth stages. Final seed mass is determined by the duration as well as the rate of seed fill or storage reserve deposition (Egli 1975; Spaeth and Sinclair 1984a and b). Thus it might have been expected that both metals would result in smaller seeds relative to the controls. As explained above however, the reduced number of seeds per pod in Ni seeds may have confounded this effect. In addition, many factors can affect final seed mass, including growth conditions (Beaver *et al.* 1985) and presumably the presence of pollutants. Thus it is possible that Cd exerted a stronger inhibitory effect on the processes leading to seed fill, than Ni.

It was mentioned in the literature review that metal pollutants often reduce the moisture content of plant tissues either by affecting water uptake or increasing transpiration. In this study, variation in seed moisture content between treatments at equivalent developmental stages was found to be minimal. The moisture status of the seed is directly related to its stage of development, water loss being a controlled process, independent of the maternal tissues (Fraser *et al.* 1982; Saab and Obendorf 1989). This suggests that the mechanisms that protect and regulate seed water status are not particularly sensitive to metal pollutants.

Carbohydrate storage reserves (both starch and sugar) of the Ni-control and Ni-

treatment seeds were lower than that of Cd-treatment. The reason for that is not clear because the same protocol was used throughout. It is possible that variation between seed batches was responsible, and that a larger sample size would have circumvented this problem. Unfortunately lack of sufficient seeds precluded this solution. For this reason the control values for the two treatments were not combined. Reduction of starch levels in mature seeds by Cd-treatment was a reflection of the reduced seed size of Cd-seeds since this was not shown when expressed on a dry mass basis.

Protein levels obtained using an extraction buffer and the Bradford or Folin-Lowry protein assay were consistently lower than those recorded in the literature (approximately 35-40%; section 1.4.3). This was despite the fact that the same or similar extraction methods have been used in the literature for determination of protein in soybean seeds (Meinke *et al.* 1981; Shatters *et al.* 1994). Several reasons may be responsible for this result. Firstly protein content varies from one cultivar to another, and it is possible that "Crawford" naturally contains low protein levels. Secondly, inaccuracy in the protein estimation protocol, may be a consequence of one or more of the buffer components reacting with the Coomassie Blue dye. It is apparently well known that protein detection methods (including those employing Coomassie Blue) are affected by a wide range of chemicals (Bio-Rad 1995; Pierce 1996). Although neither Tris, sucrose, nor 2-mercaptoethanol, the components of the extraction buffer, have been reported to be incompatible with this method (Bradford 1976; Pierce 1996). Thirdly, considerable variation occurs naturally in the protein content of seeds harvested from different nodes (Escalante and Wilcox 1993), which may explain the high variability obtained in the present study.

Total N estimation using the Kjeldahl method detects not only N present in proteins but also non-protein N such as in free amino acids, nucleic acids, alkaloids, complex lipids etc. (Mosse′ and Pernollet 1983). The above authors are of the opinion that derivation of protein levels from total N content is more accurate than colorimetric techniques. The micro-kjeldahl method has been used by several authors to

determine the protein content of soybean seeds (Yazdi-Samadi *et al.* 1977). Use of the micro-Kjeldahl method in this study yielded apparently higher protein values than by assaying protein *per se* and these were more comparable with those expected from the literature. Total nitrogen content when expressed as per gram dry mass was not affected by the presence of Cd within the seeds. Similar results were found in seeds of this species by Chaer Borges and Wollum (1980). Nickel on the other hand, was found to increase the total N (and thus protein) contents of EP seeds, although this effect was not significant in those at the mature stage. The reason for enhanced N levels in such seeds is not clear. Nickel is essential in plants, such as legumes, in which ureides are an important form of transportable N (Marschner 1986). It is conceivable that increased N in the seeds may possibly be a consequence of disruption in ureide metabolism brought about by an excess of Ni.

Soybean seeds have been shown in the literature to exhibit a steady increase in protein content with seed age (Dornbos and McDonald 1986). This increase is due mainly to accumulation of storage proteins, since the proportion of other proteins (e.g. enzymes, trypsin inhibitor) remains constant throughout seed development (Adams *et al.* 1982). If the results are expressed as mg/g.dm however a steady increase is not always apparent (Hill and Breidenbach 1974b). Dornbos and McDonald (1986) found protein content of the soybean cultivar "Williams 79" (expressed as mg protein/seed) reached a maximum at 40 DAF and then declined slightly before termination of the experiment at 48 DAF. Other authors (Yazdi-Samadi *et al.* 1977) found that when expressed as mg/g.dm the protein level remained more or less constant during seed development, presumably, a consequence of increasing levels of other seed storage reserves.

Very little could be found in the literature concerning the effect of metal pollutants on the mineral content of seeds, although there is considerable literature concerning effects on vegetative plant parts (e.g. Khan and Khan 1983; Hernandez *et al.* 1996; Obata and Umebayashi 1997). This is surprising considering the importance of seeds in human nutrition and yet is consistent with the general paucity of metal

pollution studies with regard to these tissues. Bjerre and Schierup (1985) found that addition of Cd to the soil resulted in a significant decrease in the Mn content of oat grain. Slight, but non-significant decreases in Fe and Zn content were observed, depending on the soil. Seed Cd level in the above experiment however, was very low (0.026 – 0.061 ppm), and thus a more pronounced effect may have been observed if this was higher. The above results agree with those found in the present study, where treatment with both Cd and Ni resulted in reduced Mn levels in seeds and pods. One of the most consistent effects of both metal pollutants on seed metabolism was a marked reduction in Fe content. Such a decrease was recorded in seeds as well as pods and was apparent in all but one of the growth stages examined. Nickel was more effective in promoting this reduction than Cd, possibly because of the higher concentrations of the former in the nutrient solution as well as in the seed tissues. Iron chlorosis has been reported in the literature as a possible toxicity mechanism of Cd (Haghiri 1973; Smith *et al.* 1985) and of metal toxicity in general (Schmidt *et al.* 1997). Trifoliate leaves of treatment plants were pale in colour (section 6.4). Despite this, not all studies reporting Cd-induced chlorosis have demonstrated depressed Fe levels. It would appear that in some cases, disruption of Fe metabolism rather than reduced uptake or translocation of this metal *per se* might result in the same symptoms (Williams 1967). Smith *et al.* (1985) suggest that vascular blockage, possibly caused by the red pigmentation at the pulvini, may result in nutrient balances and deficiencies in the leaves and thus lead to foliar chlorosis in soybeans. These authors compared the effect of Cd on foliar iron contents in three different soybean cultivars varying in iron efficiency. They concluded that the response of the three cultivars to cadmium was dependent directly, or indirectly, on their relative ability to utilise iron efficiently. The effects of metal pollutants on the levels of all mineral ions in plant tissue appear to be complex and depend on the plant species, tissue as well as the relative concentrations of ions in the growth medium (McKenna *et al.* 1993). Much of the confusion is likely to be due to chemical interactions and speciation effects in the growth medium. Addition of metal pollutant to the growth medium may result in competition with nutritionally important metals for binding sites at the root plasmalemma, thus limiting uptake of some elements. On

the other hand, in the case of experiments employing plants grown in pots, competition for binding sites on soil particles may occur, resulting in enhanced uptake (Bjerre and Schierup 1985). Speciation modelling of the growth medium can be used to explain some of these interactions. The effect of metal pollutants on the balance of mineral ions therefore will be discussed further in Chapter 7.

Results from this study indicate that Cd is more phytotoxic than Ni, since lower levels of the former were required to reduce seed production. Similar findings have been obtained by other researchers, working on a range of plant species (Carlson *et al.* 1975; Nieboer and Richardson 1980; Mattioni *et al.* 1997). MacNicol and Beckett (1985) surveyed the literature and reported the upper critical limit (i.e. the lowest tissue concentration that elicits a toxic effect) for Cd and Ni in a range of plant species and tissues. The upper critical limit for Cd in soybean leaves was 4-6 mg/kg. Whilst, in bush bean leaves (soybeans were not reported) a value of 10-83 mg/kg for the upper critical limit for Ni was reported. Unfortunately in the above work no critical limits for any metals in seeds were given. Since Ni is an essential element, at least in legumes (Brown *et al.* 1987) it is perhaps to be expected that toxicity effects are only apparent at higher levels and that this element should be more mobile in plants.

Orthodox seeds are remarkable in their ability to withstand desiccation for extended periods of time and then imbibe water and resume as fully hydrated organisms. One of the factors that facilitate this characteristic is the plasmalemma, which is able to survive desiccation (Seewaldt, Priestley, Leopold *et al.* 1981). It is thought that as the degree of seed hydration increases, structural and physical alterations of the lipid bilayer occur (Seewaldt *et al.* 1981; Leprince, Hendry and McKersie 1993) and with slow rehydration membrane structure is regained (Simon 1978). According to Simon (1978) leakage of solutes out of the cytoplasm of seeds will occur until membrane integrity is gradually re-established. If seeds are non-viable this leakage continues indefinitely or until the concentration of electrolytes in the surrounding medium is the same as in the cytoplasm. Despite the fact that metal pollutants have been demonstrated to exert a deleterious effect on cell membranes (Das *et al.* 1997), this

was not substantiated by the conductivity results from these studies and no difference was found in relative leakage rates between treatment and control seeds. A possible explanation for these findings is that the presence of metal pollutants in the seeds affected membrane permeability to only a limited extent, if at all and that the conductivity assessment was not sensitive enough to detect this.

The reason for the high percentage relative leakage in Ni seeds (both treatment and control) compared to that of Cd seeds in not clear. These results are contrary to those expected, since membrane integrity is considered to decrease with seed age and Cd seeds were approximately 8 months older than the Ni seeds. Therefore higher leakage rates in Cd seeds would have been expected. Cadmium and Ni seeds originated from different seed lots and it is possible that physical cracks or damage to the Ni seeds may have been present (although none were visible) and that this resulted in enhanced leakage. Smith and Berjak (1994) noted that steeping of dry seeds with large cotyledons directly in water is likely to induce severe imbibitional damage and may not be a true measure of the physiological status of the membranes. These authors stressed the need for caution when drawing conclusions from conductivity tests.

Despite accumulation of both metal pollutants within the seeds and the resultant effects on size and storage reserve deposition, germination was affected to only a limited extent. Tests revealed that in mature seeds, both Cd and Ni reduced the rate of germination as indicated by the lowered PV levels relative to the controls. Nevertheless, the extent of germination in these seeds was not compromised and 100% germination was attained consistently by all treatment groups. In addition, the ability of metal-treatment seeds to establish healthy seedlings did not appear to be impaired. Similar findings were reported for soybean seeds by Chaer Borges and Wollum (1980) where, despite a Cd concentration of up to 2.62 μg/g.dm, the germination potential of such seeds was not significantly reduced.

CHAPTER 4

LOCALISATION OF CADMIUM AND NICKEL WITHIN THE SEEDS

4.1 INTRODUCTION

It was shown in the previous chapter, that seeds harvested from soybean plants grown in nutrient solution amended with either 0.05 mg Cd/litre or 1 mg Ni/litre contained elevated levels of these metals. Approximate mean concentrations of 1 μg Cd/g.dm or 50 μg Ni/g.dm were present in such seeds. In this chapter, the tissue-specific localisation of these metals was examined, in order to obtain information pertaining to potential toxicity effects as well as to indicate possible barriers to uptake.

Several different methods have been used to localise metal pollutants in botanical samples. One of the most popular techniques is energy dispersive x-ray analysis (EDX), coupled to a scanning electron microprobe. This technique has been used to locate Ni (L'Huillier *et al.* 1996) and Cd (Khan, Duckett, Frankland *et al.* 1984) in *Zea mays* roots. Zinc and Cd localisation in the roots of a hyper-accumulator plant (Vazquez *et al.* 1992) and the distribution of Al in *Eucalyptus* seeds have also been examined using this technique (Egerton-Warburton, Griffin and Kuo 1995). Monma, Sugimoto, Hashizume *et al.* (1990) mapped Zn, Mg, P and Ca in phytin globoids of soybean seeds by analysing excised axes using EDX.

Particle induced x-ray emission (PIXE) is another technique, conceptually very similar to EDX, but differing in that protons rather than electrons are usually used to bombard the specimen, resulting in the production of X-rays (Watt and Grime 1987). PIXE is particularly suited to biological tissues, since when used in a largely organic medium, this technique results in reduced background radiation compared to EDX and is consequently more sensitive (Cookson 1987; Przybylowicz, Mesjasz-Przybylowicz, Prozesky *et al.* 1997). Analytical sensitivities exhibited by PIXE can be better than one

part per million for most elements in the periodic table above Na (Watt and Grime 1987). Despite this, compared to EDX, PIXE has been less frequently employed, at least for biological materials. This is most likely due to the fact that a nuclear accelerator is necessary for generation of the high-energy particles required for specimen bombardment (Maenhaut 1990).

Chemical methods have also been reported in the literature for localisation of Cd and Ni in plant tissues. L'Huillier *et al.* (1996) localised Ni in the leaves and roots of *Zea mays* using two different histochemical techniques, namely AgS (silver sulphide) as well as by the dimethylglyoxim method. Cadmium was also localised at the cellular level in bean root by means of the AgS method (Vazquez *et al.* 1992b). Histochemical localisation techniques however, generally suffer from the disadvantage of being qualitative rather than quantitative. The radioisotopes ^{63}Ni, ^{115}Cd and ^{109}Cd have been used to locate these metals within plant tissues. Cataldo *et al.* (1978a) examined the distribution of Ni between the testa, embryo and cotyledons of soybean seeds using ^{63}Ni. Autoradiography has also been used to obtain more detailed information on Cd distribution and sequestration in calcium oxalate crystals (Van Balen, Van de Geijn and Desmet 1980 cited by Costa and Morel 1993).

In the current study, elemental distribution on a coarse scale was first determined by physically separating the seeds into testa, cotyledon and axis. This was followed by metal content determination using ICP-AES for each tissue. The concentration of the required element in the bulk sample must be at least 100ppm for EDX analysis (D. Gerneke *pers. comm.* 1997) and for this reason it was considered that Cd and Ni localisation in the seeds was possibly not amenable to this technique. Consequently, more detailed elemental mapping of the seeds was carried out using particle induced x-ray emission (PIXE).

In general, most work localising Cd and Ni in plant tissues has been conducted on roots (Khan *et al.* 1984; Vazquez *et al.* 1992b; Van Steveninck, Barbare, Fernando *et al.* 1994). Few reports of distribution patterns in seeds could be found, particularly for Cd.

One of the reasons for such a gap in the literature is probably the low levels of Cd that accumulate in seeds compared with other organs, which makes localisation problematical. Reports of Ni distribution patterns in seeds are more frequent, most likely a reflection of the higher levels generally found for this element. The seeds of the Ni hyper-accumulator plant *Senecio coronatus* were investigated with regard to metal distribution patterns by Przybylowicz, Pineda, Prozesky *et al.* (1995) using PIXE and the gross distribution of this element between the major seed parts of soybeans was reported by Cataldo *et al.* (1978a).

4.2 MATERIALS AND METHODS

4.2.1 ICP-AES analysis

Seeds from each treatment group were separated into testa, cotyledon and axis. To minimise the number of seeds required, tissues from both Cd-control and Ni-control seeds were combined in approximately equal proportions to form a pooled sample. Due to the small tissue mass of the embryo axes and the paucity of seeds, only two replicates were analysed for axis metal content. Quantitative determination of metal pollutant concentration was carried out according to the standard protocol outlined in sections 2.2.1.5 and 3.2.1.2, excepting that in the case of Cd-axes and Ni-axes, samples were made up to a final volume of 8 and 20 ml respectively.

4.2.2 PIXE analysis

4.2.2.1 Cadmium

It was considered that the bulk Cd content (namely $\approx$ 1μg/g.dm) of mature seeds harvested from plants grown in the standard 0.05 mg/litre Cd-amended nutrient solution, was possibly too low for mapping using PIXE (Przybylowicz *pers. comm.*

1996). Consequently, localisation of this element was carried out on mature seeds harvested from plants grown in nutrient solution amended with 1 mg/litre Cd. Since not enough seeds were available for ICP analysis, the bulk concentration of metal pollutant in these seeds was not determined. Following the trends discussed in Chapter 3 (Table 3.18), it is considered probable that these seeds contained significantly higher levels of Cd than those from plants grown in nutrient solution amended with the standard pollutant concentration of only 0.05 mg Cd /litre.

Sample preparation was according to a method detailed in Przybylowicz, Mesjasz-Przybylowicz, Pineda *et al.* (1999). Seeds were immersed in isopentane cooled by liquid nitrogen, for 2 min. When frozen, the seeds were broken into pieces using a pestle and mortar and then trimmed with a glass knife into fragments approximately 5 x 5 mm in size. Care was taken to avoid contamination by using non-metallic implements for cutting and handling the samples. Frozen material was separated into testa, cotyledon and axis and then placed in glass flasks attached to a Savant freeze drier. The samples were freeze-dried for 48 hr before mounting on aluminium targets with araldite epoxy cement. Specimens were given a light coating of carbon in a Balzers sputter coater, transferred to a desiccator and kept under vacuum until viewing.

4.2.2.2 Nickel

Nickel-treatment seeds were analysed subsequently to the Cd-treatment seeds and further changes had been made to the preparation procedure. Nickel seeds harvested from plants grown in the standard 1 mg/litre Ni-amended nutrient solution, as well as control seeds, were first partially imbibed before excision. Seeds were placed on a tray suspended above water in a closed container. After 4 hours, the softened seeds were cut longitudinally through the hilum and radicle with either a scalpel blade or with a glass knife prepared on a LKB (type 7801B) Knifemaker. Subsequent sample preparation steps were the same as for Cd-treatment seeds excepting that Formvar coated targets were employed instead of aluminium holders.

Possible contamination due to the use of scalpel blades during specimen preparation

was a concern and therefore samples cut in this manner were compared with those excised with a glass knife. Tissue prepared using the two sectioning methods exhibited similar distribution patterns of key elements and no evidence of metal contamination from the blade was apparent. Since excision with scalpel-blades yielded superior samples compared to those excised with glass-knives, the former were used throughout.

The distribution and concentration of Cd and Ni as well as other elements, was determined using the nuclear microprobe at the National Accelerator Centre, Faure, South Africa. The main features of this microprobe have been described by Prozesky, Przybylowicz, van Achtenberg *et al.* (1995). Particle induced X-ray emission (PIXE) and proton Back-Scattering (BS) were used simultaneously. Measurements were carried out using a proton beam of 3 MeV. Beam current was between 600 nA and 1.5 nA and the beam size was 3 μm^2 and 4 μm^2, respectively. Total accumulated charge was from 200 nC to 3 µC. A LINK Si(Li) detector with 80 mm^2 active area and 8 µm Be window was positioned 37 mm from the target, at an angle of 135°. An additional 40-µm Al filter was used to shield the detector from back-scattered protons and to attenuate X-rays from major, light elements. Back-scattered protons were detected with a 100-µm thick, annular Si surface barrier detector. The sample area scanned varied from 0.5 mm^2 to 5 mm^2.

4.3 RESULTS

4.3.1 Metal distribution as determined by ICP-AES analysis

Distribution of the two metal pollutants in mature seeds as determined by ICP-AES is shown in Table 4.1. When expressed as µg/g.dm, Cd was distributed equally between the testa and cotyledons with very little accumulating in the axis. Nickel concentrations on the other hand were highest in the axis, intermediate in the testa and lowest in the

Table 4.1 Distribution of Cd and Ni within the tissues of mature (BP) soybean seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Analyses performed using ICP-AES. The approximate number of seeds required per sample is given for each treatment. The same mass for treatment and the equivalent control was used. n=2 for axes, n=3 for other tissues. SD in parenthesis. BD = below detection.

Seed tissue	Cadmium (μg/g.dm)			Nickel (μg/g.dm)		
	Treatment	Control	Seeds/ Sample	Treatment	Control	seeds/ sample
Testa	1.52 (±0.51)	0.04(± 0.01)	40	77 (±3.0)	BD	20
Cotyl-edon	1.53 (±0.19)	0.05 (±0.01)	15	55.7 (±1.9)	BD	15
Axis	0.04 (±0.06)	0.01 (±0.00)	80	99.2 (±3.4)	0.98(±1.2)	50

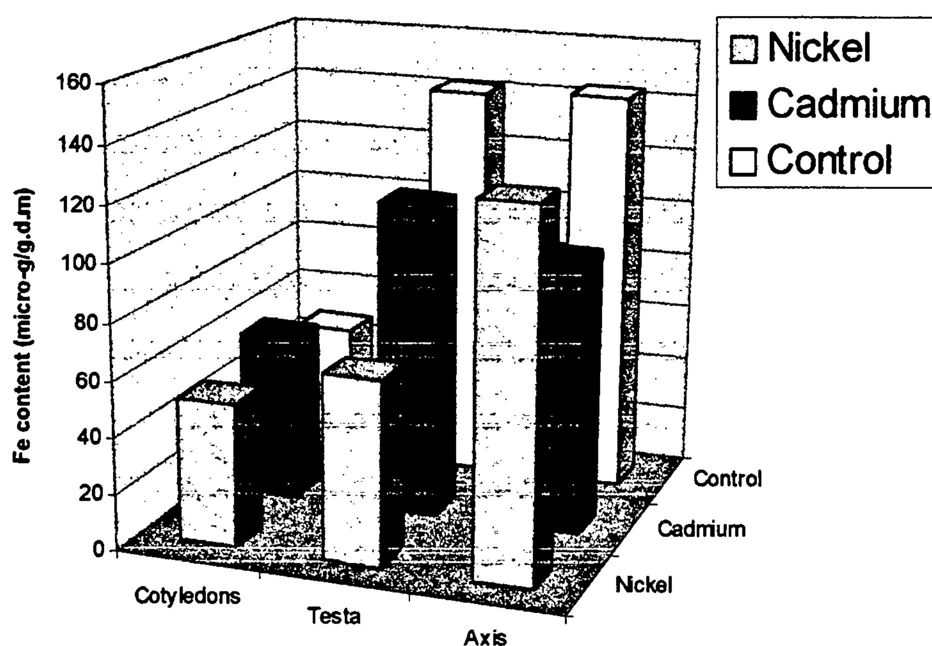


Fig. 4.1 The effect of metal pollutants on the Fe content (μg/g.dm) in different parts of seeds harvested from plants grown in 0.05 mg/litre Cd or 1 mg/litre Ni. Analyses performed using ICP-AES. Reduction of Fe content in testa is statistically significant ($p \leq 0.05$) for both Cd and Ni. n = 3 for cotyledons and testa, n = 2 for axis.

cotyledons. For both treatments, the general trend of pollutant distribution within the tissues of control seeds was similar to that of treatment seeds, excepting that concentrations in the former were either very low, or below the detection level of the technique.

The effect of Cd and Ni on the distribution of the nutritionally important metals Fe, Mg, Mn and Zn was also examined. Distinct patterns were not obtained for the last three metals and are therefore not reported here. The distribution of Fe in seeds did exhibit distinct changes in response to metal pollutants however and is shown in Figure 4.1. Neither metal pollutant exerted a significant effect on the Fe content of cotyledons. Both metal pollutants however, reduced Fe levels in the testa and axis relative to the value in the equivalent tissues of control seeds. Iron depletion in the testa was statistically significant for both Cd- and Ni-treatments. Cadmium appeared to be more effective than Ni in reducing Fe levels in the embryo axis. Due to the small sample size (n=2), the statistical significance of Fe reduction in the axis is difficult to ascertain.

4.3.2 Metal distribution as determined by PIXE analysis

4.3.2.1 Cadmium

The levels of this metal in the various tissues of Cd-treatment seeds were too low for distribution maps to be synthesised using PIXE. Consequently, rather than dividing the counts obtained from a scanned region into individual pixels, the total average Cd concentration for that area was reported. Representative regions were chosen on the sample (e.g. cotyledon, hilum, testa) and the Cd concentration determined (Table 4.2). The plumule could not be distinguished from the surrounding cotyledonary tissue and was therefore not included. Although Cd was detected in the seed coat, this was below the detection limit except in the region of the hilum. Both samples examined showed enrichment in this area (17 and 10 μg/g.dm respectively). Two cotyledonary tissue fragments were examined, each from a different seed. Cadmium was present at a concentration of 10 μg/g.dm in one sample, but not detected in the other, indicating that there may be some variability. No Cd enrichment was found in the seed radicle.

Table 4.2 *Concentrations of Cd (µg/g.dm) in tissues of mature seeds as determined using PIXE. Seeds harvested from plants grown in nutrient solution containing 1 mg/litre Cd.*

Tissue	Sample No.	Conc. (µg/g.dm)	Error (1σ)	MDL	Scan size (mm)
Testa (surface)	1	B.D	2.6 E03	-	0.78 x 2.08
Testa (hilum)	1	16.9	5.5	8.3	0.78 x 2.08
Testa (hilum)	2	10.1	3.5	7.2	1.16 x 1.12
Cotyledon	1	9.8	3.3	7.1	2.03 x 2.36
Cotyledon	2	B.D	-	-	2.03 x 2.36
Radicle tip	1	B.D	-	-	1.55 x 1.87

B.D = below detection. MDL= mean detection level.

4.3.2.2 Nickel

The levels of Ni in control-treatment seeds were too low for mapping, however elemental concentrations at different sites were determined. Nickel concentrations from selected regions varied from 0.6 µg/g.dm in the region of the cotyledons, to 19 µg/g.dm in the radicle. The concentration of Ni in the tissues of Ni-treatment seeds on the other hand, was sufficiently high for mapping this metal pollutant, as well as several other elements (supplied at normal physiological levels in the nutrient solution). Figure 4.2a shows a photograph of a seed used to produce elemental maps. Various parts are indicated, as is the area scanned by the microprobe. The brown discolouration of the tissue within the scanned area is the result of slight beam damage. The image formed by mapping total x-rays (scale in counts) emitted by the sample is shown in Fig. 4.2b. The different seed parts can be readily distinguished.

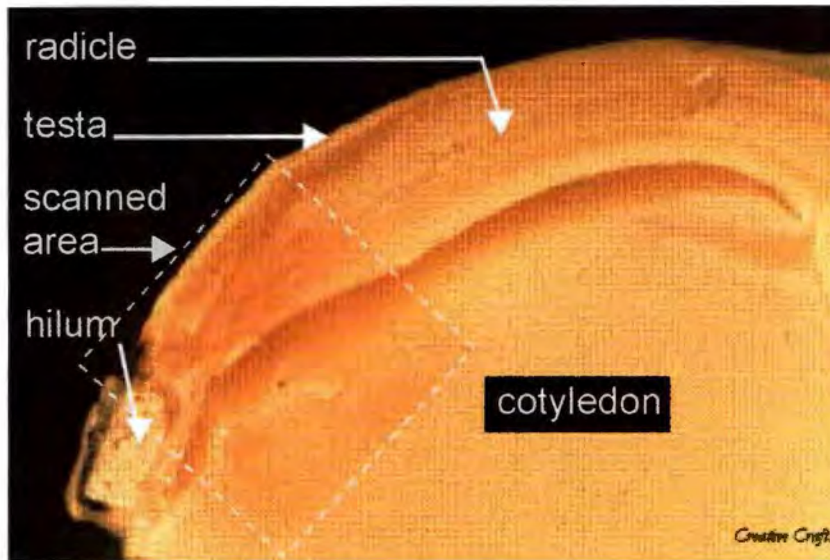


Fig. 4.2a *Photograph of Ni-treatment seed after PIXE analysis. The various seed parts are indicated, as is the area scanned by the nuclear microprobe. Seed harvested from plants grown in nutrient solution containing 1 mg/litre Ni.*

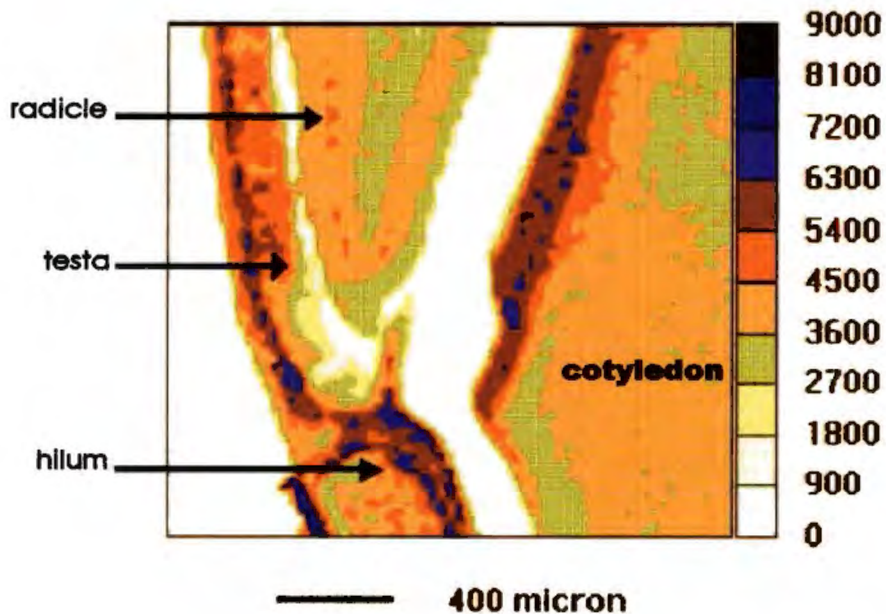


Fig. 4.2b *Map of the total x-rays (in counts) emitted by the seed sample shown in Fig. 4.2a during PIXE analysis.*

Elemental distribution maps from the above sample are shown in Figures 4.3 and 4.4. Mapping of Ca was found to be useful in delineating general seed anatomy, since this element is present in all plant cell walls (Jarvis 1984; Trewavas and Malho 1997). By examining the distribution of this element, the different seed tissues (e.g. embryo root cap, central vascular tissue) could be differentiated. Seed anatomy was also investigated using light microscopy (section 6.3.1.1). A comparison of the Ni distribution pattern with that of Ca, reveals that the metal pollutant was localised primarily in the radicle. The highest levels of Ni (up to 100 µg/g.dm) were found in this tissue. Intermediate levels of the element were found in the testa, especially the outermost layers and in the hilum. The lowest tissue levels (30-40 µg/g.dm) were recorded in the cotyledon, in which Ni appeared to be homogeneously distributed. Figure 4.3 (d) shows the localization pattern of Ni in the radicle tip. On comparison with the Ca map for this area (Fig. 4.3 (c)) it can be seen that the metal pollutant was concentrated specifically in the tissues of the cortex and root meristem, but was largely absent from the root cap and central vascular area.

Elemental maps were also obtained for other elements supplied in the nutrient solution at normal physiological concentrations (Figure 4.4). Manganese, Fe, and Zn showed distribution patterns similar to that of Ni. Namely, enrichment in the meristematic region of the radicle tip but low concentrations in the central vascular tissue. Both Mn and Fe were also present at high concentrations in the procambium as well as in the testa. In contrast to Ni and the elements described above, S (Figure 4.4 (d)), and Cl (not shown) were both evenly distributed throughout the seed tissues. The exception to this homogeneous distribution, was a narrow band of cells at the outer edge of the cotyledon, which indicated localised accumulation of both elements.

4.4 DISCUSSION

Results from ICP analysis of Cd-treatment seeds revealed that although accumulation of this metal did occur in the testa and cotyledons, entry of Cd into the embryo axis appeared to be restricted. These results were generally supported by the results of PIXE, which also indicated that the axis was low in Cd, with much higher levels in testa and cotyledon. Cadmium in the testa was primarily located in the region of the hilum, the point at which the vascular connection from the mother plant enters. This suggests that transport of this element may be limited in the developing seed. The absence of Cd in one of the cotyledon samples is difficult to explain and highlights the main disadvantage of using PIXE. Namely, because analyses are relatively time-consuming and expensive, only a very small number of samples can be scanned. Thus seed-seed variation could cause incorrect conclusions to be drawn and for this reason, point concentration determinations especially, should be considered tentative until confirmed by further analyses. Results from ICP analyses on the other hand represent the bulk metal content for the entire tissue, within more than one seed. Variation in metal content between hilum and the rest of the seed coat would not be reflected in this value therefore and may account for the low Cd concentration in the testa when determined using ICP. Similarly, seed to seed variation, which might have caused the high cotyledon Cd result in Table 4.2 would possibly be attenuated in the bulk Cd concentration, resulting in lower overall values as reported for ICP determinations in Table 4.1.

As mentioned in the introduction, very little has been reported in the literature concerning the distribution of Cd in seeds. Petterson and Harris (1995) reported that the hull to kernel (i.e. testa to embryo) ratio of Cd in *Lupinus angustifolius* ranged from 1.54 to 1.8. These authors postulated that elevated levels in the testa were possibly due to high levels of metal-binding phytochelatins in these tissues. Phytochelatins were not isolated from seeds during the course of this study. Investigation of the distribution and quantification of these plant metabolites within metal-stressed seeds is an avenue of research that should be investigated further, since this may shed light on accumulation

patterns of metals within the various tissues.

Nickel distribution patterns derived from ICP analyses, also supported the results obtained from PIXE. In contrast to Cd, the axis showed considerable Ni enrichment, intermediate levels were found in the testa and lowest levels in the cotyledons. This element did not appear to be especially localised within the hilum relative to the rest of the seed coat, although as explained above, further research is necessary to confirm this. Przybylowicz *et al.* (1995) found that in seeds of the Ni hyper-accumulator *Senecio coronatus,* this element accumulated in the seed coat covering the radicle as well as in the radicle itself. Cataldo *et al.* (1978a) also found similar distribution patterns of Ni between the testa, embryo and cotyledon of *Glycine max*, although they reported that levels in the cotyledons were slightly higher than those of the testa (seed hull). Witkowski, Weiersbye-Witkowski, Przybylowicz *et al.* (1997) found that in the leguminous seed *Burkea africana*, Ni also accumulated within the axis. In contrast to the results found in the present study however, elevated levels in *Burkea* were localised within the vascular tissue of the axis, in addition to the radicle tip.

The obligatory role of Ni in the metallo-enzyme urease has already been mentioned (section 1.3.5.2). Seeds of soybean and other legumes are rich sources of urease (Eskew *et al.* 1984). Two isoenzymes have been isolated from soybean plants, namely: ubiquitous urease, a constitutive enzyme, present in all organs, as well as embryo-specific urease which is synthesised only in developing embryos (Stebbins, Holland, Cianzio *et al.* 1991). In addition, a background urease is usually present, although it is not yet clear if this is of plant origin, or is produced by bacteria infecting the seeds (Stebbins *et al.* 1991). An early hypothesis suggested that urease in the seeds of soybean and other legumes was responsible for degradation of urea, thought to originate from the ureides, allantoin and allantoic acid (Atkins, Pate, Ritchie *et al.* 1982; Jolivet and Mosse' 1983). A similar role has also been assigned to this enzyme in other parts of the plant (Eskew *et al.* 1984). Rainbird, Thorne and Hardy (1984) however, demonstrated that ureides were not taken up directly into developing soybean seeds. A current hypothesis suggests that the bulk of urea in seeds originates from argenine

and to a much lesser extent from ureides. The putative role of urease in germinating seeds is degradation of urea originating from argenine, thus releasing N from storage proteins making it available for incorporation into metabolically active proteins (Stebbins and Polacco 1995). Alternatively, a role in seed chemical defence has also been postulated (Polacco and Holland 1993).

It follows from the above discussion that the localisation pattern of Ni in soybean seeds may well be linked to the distribution of embryo-specific urease. In addition, Mishra and Kar (1974) state that Ni is often (but not always) concentrated in areas of highest metabolic activity. Hence, it is possible that the radicle, being an actively growing region becomes enriched with this metal during seed development. Alternatively, whilst under going the partial imbibition treatment during sample preparation, this element may have become concentrated in the radicle for the same reason. Another possible explanation for the distribution pattern of Ni is due to association with phytate in the inclusions of protein bodies. Although it is well known that different plant species exhibit specific patterns of metal ion sequestration in the globoid inclusions, the reasons and mechanisms responsible for this are unclear (Lott *et al.* 1995). A time-series investigation of Ni accumulation in developing seeds using PIXE and ICP might shed light on this problem. Only mature seeds were used for mapping in this project, partly due to a lack of plant material (in the case of ICP analysis) and limited accessibility to the microbeam (in the case of PIXE). A study of developing seeds may show whether Ni is taken up directly from the endosperm into the radicle, or is redistributed from the rest of the embryo to the axis tip.

The globoid crystals of seeds have been subjected to fairly extensive elemental analysis (Mazzolini, Legge and Pallaghy 1981; Batten, Ockenden and Lott 1994; Egerton-Warburton *et al.* 1995), since these are the major sites of metal deposition in seeds (Lott *et al.* 1995). The elemental composition of globoid crystals in protein bodies of wheat grain, grown on soil treated with sewage sludge, has been examined by Spitzer, Webber and Lott (1981). These authors found that such wheat grain did not show accumulation of Cd or other toxic metals in the globoid crystals. They concluded

that the mineral storage system in wheat is specific in its metal content. Similar results were found with regard to Pb uptake into the globoid crystals of seeds from *Capsella* grown close to a busy highway, as well as those from *Lycopersicon*, grown on Zn/Ni enriched soil (Spitzer, Lott, Vollmer 1980). Lott *et al.* (1995) concluded that toxic metals seem to be excluded from the globoid crystals in several species, and that in general developing seeds seem to obtain the elements they require and exclude elements not needed. In the light of the above statement it is interesting that Egerton-Warburton *et al.* (1995) found that Al did accumulate in the globoid crystals of *Eucalyptus calophylla* seeds. These authors found that the nutrient status of the soil did have a controlling effect on the mineral content of the globoid inclusions. Van Steveninck *et al.* (1994) found that whereas excess Zn was bound by phytate in the roots of soybean and other plant species, Cd was not. However in the tissues of *Lemna minor,* exposure to Cd did induce the formation of Cd-phytate (Van Steveninck, Van Steveninck and Fernando 1992). No attempt was made in the present study to detect the presence of Cd or Ni in the globoid inclusions of the seeds using EDX, since it was considered that the concentrations of these metals were too low (Gerneke *pers. comm* 1996). Further work using seeds containing higher levels of the metal pollutants needs to be carried out in order to enable localisation of Cd and Ni at the ultra-structural level.

It is important to differentiate between metal concentration in seed tissues and the proportion of the total pollutant load. Of the total Cd load in mature seeds approximately 17% was in the testa, 83% in the cotyledons and 0.14% in the axis. In the case of Ni-treatment seeds, 42% was localised in the testa, 43% in the cotyledons and 15% in the axis. Thus, although the lowest concentrations of Ni were demonstrated to be in the cotyledons, because these tissues comprise the bulk of the seeds, most of the metal was present in these parts.

It is possible that because Cd (in contrast to Ni) is a non-essential element, it is excluded from sensitive areas such as the axis. Fifteen percent of the total Ni load was found in the axis whereas very little Cd was present in this tissue. The presence of a toxic metal in the embryo axis could pose a serious threat to radicle extension and thus

future seedling growth. Germination was only slightly depressed in seeds from metal-treatment plants however (section 3.3.6), although this effect may have been exacerbated if higher levels of either Cd or Ni were present. The hypothesis that Cd is more toxic than Ni is substantiated by the fact that even extremely low levels of this metal in the axis were able to depress seed vigour to almost the same extent as the higher concentration of Ni.

The implications of high levels of a toxic metal in the testa are not clear. On one hand, this is not likely to represent a serious risk to developing seedlings, since this is maternal tissue, sloughed off early during the germination process and is not essential for future seedling growth. On the other hand however, this tissue plays a critical role in supplying the developing seed with nutrients (section 1.4.5.3). It is considered that the testa controls the entry of nutrients and other substances into the developing seed (Murray 1987) and hence protection of the embryo may be the reason for high levels of Cd in this tissue. The relatively high levels of Cd in the testa may possibly, in turn, be the reason for depressed seed mass due to impaired functioning of this tissue. From the point of view of seed consumption by animals or humans however, the testa is usually removed during the milling process.

Accumulation of toxic metal pollutants in the cotyledons may have serious implications in regard to storage reserve accumulation and may account (possibly in conjunction with reduced functioning of the testa) for the reduced mass of Cd-treatment seeds compared to their control counterparts. Eighty-three percent of Cd in the seeds was located in the cotyledon, the principal site of storage reserve deposition, whereas only forty-three percent of Ni was located in these organs. This may be one of the reasons why reserves were lowered in seeds exposed to Cd, but not in Ni-treatment seeds.

In Chapter 3 (section 3.3.4.5) it was shown that both Cd and Ni affected the mineral balance of mature seeds and that the Fe content in particular was reduced. Results from ICP analyses in this chapter indicate that significant reduction did occur in the testa. This depletion was brought about by both Ni and Cd, although the effect was

more pronounced in the case of the former metal pollutant. Such a reduction in the Fe contents of the testa may possibly be an indication of impaired functioning of this tissue due to the presence of metal pollutants. Alternatively it may result from lowered concentrations of this element in the phloem, a consequence of altered metabolism in the mother plant. This question will be considered further in Chapter 7. Cadmium appeared to be more effective than Ni in reducing Fe levels in the axis, despite the low levels of Cd in these tissues. It should be remembered however, that due to the small sample size, the axis results are only tentative.

CHAPTER 5

EFFECTS OF EXOGENOUS APPLICATION OF METAL POLLUTANTS ON GERMINATION AND SEEDLING ESTABLISHMENT

5.1 INTRODUCTION

In Chapter 2, the effect of increasing concentration of metal pollutants on plant growth was examined. It was shown that plants exposed to Cd or Ni exhibited reductions in both growth and seed production. In the above-mentioned experiments however, seedlings were exposed to the metal pollutants only three weeks after imbibition. The aim of this chapter is to examine the effect of exogenous applications of metal pollutants on the germination process itself, as well as on the earlier stages of seedling development. Thus seeds were imbibed in water containing differing concentrations of the metal pollutants and various aspects of seed germination and seedling establishment were examined. These aspects included the rate and extent of germination and in the case of seedlings, determination of the efficiency of photosystem II activity, as a measure of impairment of photosynthetic functioning.

Up to this point, this project has primarily been concerned with the process of seed development. As explained in section 1.4 and elsewhere in this work, metabolic and cytological changes during development of orthodox seeds are dominated by synthesis and accumulation of storage reserves, as well as the onset of desiccation tolerance and reduction in moisture content. The ultimate result is the formation of a propagule that, in the quiescent state, is able to withstand unfavourable environmental conditions. Seed germination and seedling establishment, on the other hand, involve axis elongation, utilisation of the storage reserves laid down in the embryo and the formation of a self-sustaining new plant. According to Bewley and Black (1994), seed germination begins with uptake of water by the seed

(imbibition) and ends with the start of elongation by the embryonic axis, usually the radicle. A variety of metabolic processes are involved including, protein hydration, respiration, sub-cellular structural changes, macromolecular synthesis and cell elongation. The above authors point out that strictly speaking, germination does not include seedling growth and mobilization of storage reserves, which are post-germination events and take place during seedling establishment. Germination is considered to have terminated once the radicle has penetrated the testa (Mayer and Shain 1974). The initial phase of radicle elongation during germination is considered to be largely due to water uptake and expansion of the cells of the embryo axis. Only during the second phase of elongation, during seedling establishment, does cell division generally take place (Bewley and Black 1994). In this study, germination was deemed to be complete when the radicle was 5 mm long (section 3.2.4) and had just emerged through the seed coat.

Various parameters have been defined in toxicology to quantify the magnitude of the toxic effect a chemical substance may have on living organisms. Some of the most frequently employed parameters are LC_{50} and EC_{50} (Dallas and Day 1993; Fargasova 1994; Bonifacio and Montano 1998). For acute toxicity testing of the effect of a chemical substance on a given organism, the above parameters can be defined as follows (DWAF 1996):

Lethal concentration – 50% (LC_{50}):- The concentration of a chemical substance that corresponds to a cumulative probability of 50% for death of the test population.

Effective concentration – 50% (EC_{50}):- The concentration of a chemical substance that corresponds to a cumulative probability of 50% for an adverse effect at a specific time of observation.

Estimation of the above parameters is important for comparing the toxicity of different substances (including metal pollutants), for comparison of tolerance ranges of differing organisms, as well as for establishment of environmental criteria or limits (Bonifacio and Montano 1998). The LC_{50} values for the toxicity effect of Cd and Ni

on soybeans will be determined by ascertaining the concentration at which 50% of the test population do not germinate. The EC_{50} values will be defined as that concentration after a test period of 7 days, that elicits a 50% reduction in radicle length compared to that of control seeds.

Measurement of chlorophyll fluorescence has been used extensively for detecting signs of environmental stress in plants, including that caused by metal pollutants (Maksymiec and Baszynski 1996; Moustakas *et al.* 1996; Guidi, Nali, Ciompi *et al.* 1997). According to Bolhar-Nordenkampf, Long, Baker *et al.* (1989), chlorophyll fluorescence is an extremely sensitive tool and can be used to detect signs of stress in plants, long before any visible symptoms are apparent. Measurement of chlorophyll fluorescence usually involves maintaining the photosynthetic tissues in darkness for a period of time (dark-adaptation). This is followed by exposure of the leaves to a light source of various intensities and measurement of the resulting fluorescence emitted by the chlorophyll molecules in these tissues. Several parameters of the fluorescence induction kinetics (the "Kautsky effect") exhibited by such tissues can be examined. Interpretation of such induction kinetics has been reviewed recently by Krause and Weis (1991), Bolhar-Nordenkampf and Oquist (1993) and Govindjee (1995) amongst others. Consequently only a few salient points from this complex field of study will be noted here. The following discussion is taken largely from Bolhar-Nordenkampf *et al.* (1989).

In essence, there are several different processes that are in competition with each other for the light energy that is absorbed by chlorophyll. These include fluorescence, photosynthesis as well as heat dissipation. Any change that affects photosynthetic rate, or dissipative heat emission will cause complementary changes in fluorescence emission. At room temperature, virtually all measured fluorescence originates from photosystem II (Lichtenthaler and Miehe' 1997). A typical chlorophyll fluorescence induction curve as obtained from a dark-adapted leaf is shown in Fig. 5.1. Part A of the curve represents the initial, fast kinetics followed by part B, the slow induction kinetics phase which is closely coupled to CO_2 uptake and O_2 evolution.

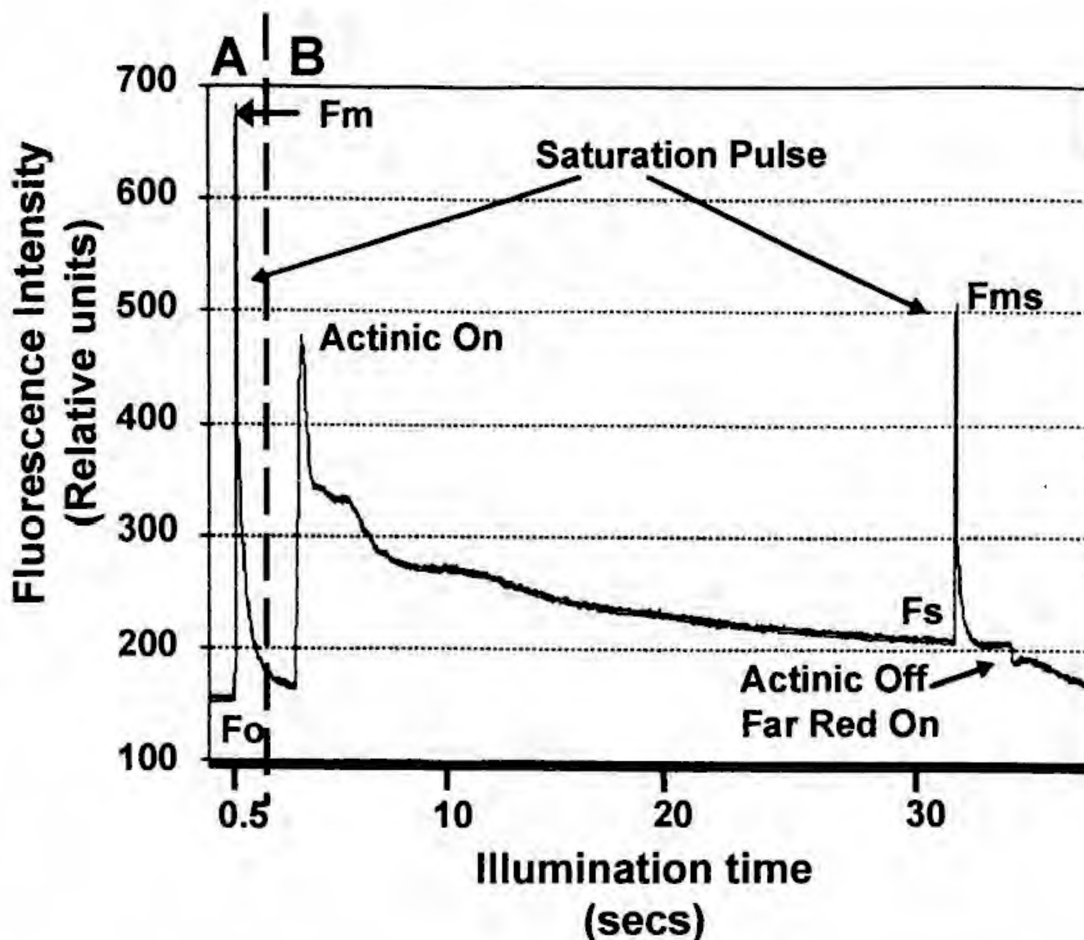


Fig. 5.1 *Characteristic fluorescence induction kinetics ("kautsky curve") of a dark-adapted leaf. Changes in intensity (relative units) of fluorescence emitted by plant tissues, plotted against length of time of illumination with a weak modulated light source. Part **A** represents the initial fast kinetics and part **B** the slow kinetics phase. In addition to continuous illumination with a weak light source, various other light sources supplied as shown; actinic light to induce photosynthesis, pulses of saturating light (saturation pulse) to fully reduce the system, as well as far red light to oxidise the system. Fo = ground fluorescence exhibited when all reaction centres of PS II are open (oxidised). Fm = maximal fluorescence exhibited when all reaction centres closed (reduced). Fs and Fms = steady state and maximal fluorescence respectively under conditions of photosynthesis i.e actinic light supplied. (Adapted from Opti-Sciences OS-500 User's Guide 1993).*

Where:

F_o = minimal or ground fluorescence exhibited when all reaction centres of PS II (photosystem II) are open (i.e. the primary electron acceptor is oxidised).

F_m = maximal fluorescence, exhibited when all reaction centres are closed and thus there is no photochemistry to quench excitation energy.

F_v = variable fluorescence, and $F_v = F_m - F_o$

F_s and F_{ms}= the steady state and maximum fluorescence values respectively of a sample during exposure to light-driving photosynthesis.

From an analysis of the induction kinetics, various deductions can be made regarding the effect of environmental stress on the photosynthetic process, as well as on the mechanism of photosynthesis itself (Schreiber, Schliwa and Bilger 1986; Krause and Weis 1991). There is an inverse relationship between photosynthetic performance and chlorophyll fluorescence emission (Lichtenthaler and Miehe' 1997). Chlorophyll fluorescence analysis however, does not simply provide a means to detect direct effects on the photosynthetic apparatus, but is also sensitive to other physiological processes that feed back into photosynthesis. Hence factors that affect, for example, stomatal opening, carbon assimilation, or ATP utilization can also indirectly affect fluorescence emission (Bolhar-Nordenkampf *et al.* 1989; Sivak and Walker 1985). The empirical parameter F_v/F_m has been found to lie within the range of 0.75-0.85 for a wide range of plant species and can be shown to be proportional to the quantum yield of photochemistry (Bolhar-Nordenkampf and Oquist 1993). A very good correlation has been reported between photoinhibition of photosynthesis as induced by excessive excitation and a decrease in the F_v/F_m ratio. Photoinhibition has be observed as a secondary stress response if the photosynthetic process as a whole, is restricted by some other stress factor (Bolhar-Nordenkampf and Oquist 1993), such as the presence of metal pollutants.

5.2 MATERIALS AND METHODS

5.2.1 The effect of exogenous metal pollutants on seed germination

Seeds were sterilised according to the standard procedure described in section 3.2.1. Ten seeds were placed on filter paper in aseptic petri dishes and either 8 ml of ultra-pure water (control) or the same volume of an appropriate concentration of metal-pollutant solution were added. Each treatment was carried out in triplicate. The range of concentrations employed is shown in Table 5.1 for Cd and in Table 5.2 for Ni. Petri dishes were wrapped in black plastic to exclude light and left for 7 days in a controlled chamber with environmental parameters set as for section 2.2.1. The number of germinated seeds was recorded at the end of the week, as was the mean radicle length. Values of the toxicological parameters LC_{50} and EC_{50} were derived according to the definitions given in section 5.1.

5.2.2 The effect of exogenous metal pollutants on seedling establishment

Based on results from the germination experiments (above), the following metal concentrations were selected for examination of seedling establishment:

Cadmium: 3, 5, 10, 25 and 225 mg/litre.

Nickel: 10, 25, 50, 125 and 700 mg/litre.

Controls: no metal pollutant (0 mg/litre).

During the experiment, tissue samples were removed from the sub-set of treatment groups listed above. Forty-eight hours after the initiation of imbibition, tissue was excised from both radicle tips and cotyledons for examination by transmission electron microscopy (TEM). The procedure for tissue preparation and results from this portion of the study are presented in the next chapter.

In addition to recording the final percentage germination, the germination rate of this sub-set of concentration treatments was also examined by recording the number of

germinated seeds each day. Mean daily germination (MDG), the peak value of germination (PV) and the germination index (GI) were calculated as described in section 3.2.4.

5.2.2.1 Uptake of metal pollutants

Seven days after initiation of the imbibition treatment, nine seedlings from each metal concentration group were reserved for seedling establishment experiments. The remaining seedlings were prepared for elemental analysis. To this end, testas were removed and discarded and the cotyledons separated from the axis. For each concentration treatment, axes and cotyledons were washed separately in 3 X 200 ml of distilled water to remove all extraneous Cd or Ni. Tissue samples were prepared for ICP-AES elemental analysis according to the standard method (2.2.1.5). The final rinse water used to wash the seedlings was also analysed for metal content.

5.2.2.2 Seedling growth

Seedlings selected for the establishment experiments were placed in one-litre hydroponics jars, three seedlings per container. Two of the jars were filled with half-strength Hoagland's nutrient solution amended with the same concentration of metal pollutant as the imbibitional solution. The third container, was filled with standard nutrient solution and was referred to as the "x mg/litre Cd/Ni *recovery* treatment". Seedlings were placed in a controlled growth cabinet with environmental parameters set as in section 2.2.1.

Twenty–one days after commencement of the experiment (and two weeks after placement of the seedlings in nutrient solution), chlorophyll fluorescence of each seedling was measured. Leaf samples were also removed at this point for quantification of photosynthetic pigments. These two procedures are described below. In addition, the remaining unifoliate leaves were harvested for elemental analysis using ICP-AES according to the standard procedure. Roots for each treatment group were separated into main root and lateral roots and the dry mass of each recorded separately.

5.2.2.3 Quantification of photosynthetic pigments

Photosynthetic pigments were extracted from each unifoliate (primary) leaf measured for chlorophyll fluorescence. To prevent chlorophyll degradation, all steps were performed under conditions of low light intensity. A portion, approximately 20 X 20 mm^2 was excised from the middle of each leaf and the remaining tissue reserved for determination of moisture content. Immediately after excision, leaf samples were weighed and then frozen in liquid nitrogen. Each sample was ground in 100% acetone using a pestle and mortar until all colour was removed from the tissue and the volume of acetone noted. Samples were then centrifuged at 4 000 g for 5 min to remove any solid material. Absorbance of the pigment extracts at 470, 644.8 and 661.6 nm was measured in a Cary 1E Varian spectrophotometer. The concentrations of chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (Chl_{a+b}) and of total carotenoids (C_{x+c}) were determined from the equations of Lichtenthaler (1987), as given below:

$$[Chl_a] = 11.24\ A_{661.6} - 2.04\ A_{644.8}$$

$$[Chl_b] = 20.13\ A_{644.8} - 4.19\ A_{661.6}$$

$$[Chl_{a+b}] = 7.05\ A_{661.6} - 18.09\ A_{644.8}$$

$$[C_{x+c}] = (1000\ A_{470} - 1.90\ C_a - 63.14\ C_b) / 214$$

5.2.2.4 Measurement of chlorophyll fluorescence

Differences were examined between metal-treatment, recovery-treatment and control leaves with regard to the induction kinetics of chlorophyll fluorescence. Emission from the adaxial leaf surface of the unifoliate leaves was measured using an Opti-Sciences OS-500 modulated fluorometer after first dark-adapting the leaves for a period of 30 min. All measurements were carried out under the controlled environmental conditions described in section 2.2.1. Minimal fluorescence (F_o) was measured under a weak modulated light of intensity $< 3\ \mu mol/m^2/s$. This level was

set, by incrementally adjusting the intensity until the threshold value was reached at which variable fluorescence was induced. The intensity of the modulated light was then set just lower than this value. Maximal fluorescence (F_m) was induced by illuminating the sample with a saturating light pulse (≅ 8 mmol/m^2/s) for 0.8 sec. Thereafter, the leaf was exposed to continuous actinic light at an intensity of ≅ 600 μmol/m^2/s in order to observe and measure changes in fluorescence emission under conditions of photosynthesis. The value of steady state maximal fluorescence (F_{ms}) was determined by again supplying a saturating light pulse. Finally far red light was supplied to re-oxidise PS II.

The following parameters were recorded:
Fv/Fm (where Fv = Fm –Fo)
and
Photosynthetic Yield = (Fms - Fs)/ Fms

One measurement per leaf was taken. Sample size per treatment varied from n = 2 to n = 6. The value of Fv/Fm and Yield were calculated for each reading and using this data, the mean value per treatment determined.

5.3 RESULTS

5.3.1 Determination of the toxicological parameters for cadmium and nickel

The effects of exogenous applications of Cd and Ni on final percentage germination are shown in Tables 5.1 and 5.2. In the case of both metal pollutants, there was a general decline in both percentage germination and mean radicle length with increasing metal concentration and this was reflected in the germination index (G.I.). Both germination and axis elongation were more sensitive to Cd than to Ni. In the

***Table 5.1** Effect of different concentrations (mg/litre) of exogenous Cd on germination. Percentage germination, radicle length (mm), germination index (GI), mean daily germination (MDG) and the peak value of germination (PV) are given. Standard deviation in parenthesis. LC_{50} = 400 mg Cd/litre; $EC_{50} \cong$ 25-30 mg Cd/litre. n = 3 for metal treatments and n = 5 for control.*

Cd conc. (mg/litre)	% Germination	Mean radicle length (mm)	GI*	MDG*	PV*
0 (Control)	100 (± 2.6)	103 (± 25)	10300	14.3	42 (± 6.7)
3	97 (± 5.6)	76 (± 18)	7372	13.9	42 (± 6.7)
5	93 (± 5.7)	85 (± 8)	7905	13.3	41 (± 2.9)
10	100 (± 0.3)	75 (± 41)	7500	14.3	37 (± 5.8)
25	90 (± 10)	**46** (± 17)	4140	12.9	43.3 (± 7.6)
50	85 (± 2.5)	36 (± 19)	3060	12.1	-
100	85 (± 2)	19 (± 12)	1615	12.1	-
125	93 (± 0)	26 (± 11)	2418	13.3	-
150	90 (± 14)	19 (± 9)	1710	12.9	-
180	90 (± 1.1)	18 (± 8)	1620	12.9	-
200	60 (± 0)	17 (± 6)	1020	8.6	-
225	80 (± 0)	12 (± 5)	960	11.4	38.3 (± 2.9)
300	75 (± 7.1)	11 (± 4)	825	10.7	-
400	**50** (± 0)	12 (± 6)	600	7.1	-
500	40 (± 14)	11 (± 4)	440	5.7	-

* Germination index (GI) = % final germination X radicle length; *MDG = mean daily germination (% germination/7); PV = peak value and indicates the rate of germination (see section 3.2.4).

Table 5.2 *Effect of different concentrations (mg/litre) of exogenous Ni on germination. Percentage final germination, radicle length (mm), germination index (GI), mean daily germination (MDG) and peak value (PV) are shown. Standard deviation in parenthesis. $LC_{50} \cong 2000$ mg Ni/litre; $EC_{50} \cong 125$ mg Ni/litre. n = 3 for metal treatments and n = 5 for control.*

Ni conc. (mg/litre)	**% Germination**	**Mean radicle length (mm)**	**GI***	**MDG***	**PV***
0 (Control)	100 (± 2.6)	103 (± 25)	10300	14.3	42 (± 6.7)
10	100 (± 0)	86 (± 4.5)	8600	14.3	38.3 (± 2.9)
25	93 (± 6)	75 (± 2)	6975	13.3	38.3 (± 7.3)
50	100 (± 0)	93 (± 55)	9300	14.3	43.4 (± 2.9)
100	100 (± 0)	83 (± 21)	8300	14.3	-
125	100 (± 2.6)	**53** (± 2)	5300	14.3	38.5 (± 2.9)
150	95 (± 7.1)	46 (± 14)	4370	13.6	-
200	95 (± 7.1)	36 (± 9.9)	3420	13.6	-
250	95 (± 7.1)	19 (± 2.1)	1805	13.6	-
400	85 (± 7.1)	20 (± 2.8)	1700	12.1	-
500	75 (± 6)	16 (± 3.6)	1200	10.7	-
700	70 (± 0)	15 (± 0.5)	1050	10	33.4 (± 2.9)
800	80 (± 2.5)	18 (± 1.1)	1440	11.4	-
1 000	80 (± 2)	15 (± 0)	1200	11.4	-
1 500	65 (± 1.6)	12 (± 2.8)	780	9.3	-
2000	**47** (± 5.8)	9 (± 0)	423	6.7	-

*Germination index (GI) = % final germination X radicle length; *MDG=mean daily germination; PV = peak value and indicates the rate of germination (see section 3.2.4).

case of Ni, percentage germination was depressed only at concentrations of 150 mg Ni/litre or higher, whereas in the case of Cd, this occurred at concentrations greater than 10 or 25 mg Cd/litre. As a consequence, the LC_{50} and EC_{50} values for Cd are much lower than those found for the other metal. These values are reported below:

Cadmium:

LC_{50} = 400 mg Cd/litre

$EC_{50} \cong$ 25-30 mg Cd/litre

Nickel:

$LC_{50} \cong$ 2000 mg Ni/litre

$EC_{50} \cong$ 125 mg Ni/litre

Results from both tables indicate that radicle extension was more sensitive to metal toxicity than germination. This is shown by the fact that mean radicle length was decreased in all concentration treatment groups relative to that of control seeds, but germination was only affected by the higher metal levels.

The effect of the metal pollutants on the rate of germination is also shown in Tables 5.1 and 5.2. As described in section 3.2.4, the mean daily germination value (MDG) is an indicator of the extent of germination, whereas the peak value (PV) indicates the germination rate. Although differences were recorded in PV for the different treatment concentrations and the rate of germination in the 25 mg Cd/litre treatment group appeared to be slightly higher than that of control seeds, these differences were not statistically significant. A similar finding was recorded for the Ni-treatments (Table 5.2).

5.3.2 Effect of metal pollutants on seedling establishment

5.3.2.1 Toxicity symptoms exhibited at the end of the imbibition period

Seven days after initiation of the imbibition treatment, control seeds consisted of a radicle with several lateral roots. The two cotyledons were still closed around the epicotyl, which was comprised of an apical bud and two rudimentary primary leaves. Toxicity symptoms exhibited by seedlings included the appearance of brown necrotic

lesions on the surface of cotyledons and at the radicle tip. Healthy seedlings were yellow-pale green in colour, whereas those subjected to concentrations equal to or above 25 mg Cd/litre or 125 mg Ni/litre tended to be yellow-beige in colour. There was an increase in the severity of symptoms with increasing metal pollutant concentration. Toxicity symptoms appeared to be generalized and no distinctions could be made between the responses elicited by the two metals.

5.3.2.2 Seedling metal content at the end of the imbibition period

The effects of exogenous applications of Cd or Ni on seedling establishment are summarized in Tables 5.3 and 5.4. For the sake of completeness, percentage germination and mean radicle length for each concentration treatment are included. At the end of the germination/imbibition period, the internal tissue concentrations of metal pollutants were determined and are recorded in column 4 of each table. The rinse water from washing the seedling parts contained negligible levels of the two metals (data not shown). Thus seedling parts were not contaminated by external metal pollutant and the values recorded in the table represent metal concentrations within the tissues.

The Cd content of both cotyledons and axes generally increased as the concentration of this metal in the imibitional solution increased. All treatments showed higher Cd tissue levels than control seeds. In the case of the highest concentration (225 mg Cd/litre), tissue contents were not determined as the seeds were non-viable and already decomposing. The Cd content of the axes was consistently higher than that of the cotyledons and the difference between the two seed parts increased with increasing exogenous metal concentration. Thus in the 3 mg Cd/litre treatment, there was a difference of only 0.007 μg Cd/g.dm between axis and cotyledon metal content. In the 25 mg Cd/litre treatment however, there was a difference of nearly 32 μg Cd/g.dm between the two seed parts. In general, tissue metal concentrations were considerably lower than that of the external solution. An exception to this trend was the 25 mg Cd/litre treatment, in which the axis metal content was higher than the concentration of the ambient imbibitional solution.

Table 5.3 *Summary of the effects of exogenous Cd on germination and seedling establishment. Seeds imbibed in Cd solution for 1 week, transposed to nutrient solution containing either the same concentration of Cd (x treatment) or water (x recovery). Plant growth stage determined as in section 2.2.3. ND = not determined. Standard deviation in parenthesis. n = 3 for metal analyses and germination, n = 6 for root mass determinations.*

Germination/imbibition				**Seedling establishment**				
Conc. (mg/l)	**% germ.**	**Mean radicle length (mm)**	**Tissue Cd content (µg /g.dm)**	**Appearance and growth stage at end of experiment**		**Total root dry mass (g per plant)**	**Lateral root dry mass (% of total)**	**Cd content of primary leaf (µg/g.dm)**
3	97	76	Cots =0.084 (±0.01) Axis =0.091 (±0.03)	3 Treatment	Moderately stressed; V2	0.075 (±0.015)	2.6 (±2.4)	0.091 (±0.003)
				3 Recovery	Healthy; V2.5	0.251 (±0.01)	47.7 (±7.9)	0.079 (±0.007)
5	93	85	Cots =0.093 (±0.02) Axis =0.137 (±0.08)	5 Treatment	Moderately stressed; V1	0.067 (±0.027)	1.7 (±1.1)	0.115 (±0.02)
				5 Recovery	Healthy; V2	0.217 (±0.031)	54.6 (±9.6)	0.073 (±0.004)
10	100	75	Cots =0.218 (±0.01) Axis =0.204 (±0.09)	10 Treatment	Stressed; V0	0.031 (±0.009)	2.9 (±0.91)	0.23 (±0.19)
				10 Recovery	Healthy; V2.5	0.285 (±0.111)	55.4 (±5.8)	0.07 (±0.007)
25	90	46	Cots = 5.7 (±3.0) Axis = 37.4 (±7.1)	25 Treatment	Very stressed; V0	0.036 (±0.002)	0	0.136 (±0.06)
				25 Recovery	Healthy; V2	0.277 (±0.321)	45.5 (±5.3)	0.086 (±0.009)
225	80	12	ND	225 Treatment	All seedlings died	ND	ND	ND
				225 Recovery	All seedlings died	ND	ND	ND
Control	100	103	Cots = 0.02 (±0.02) Axis = 0.05 (±0.07)	Control	Healthy; V3	0.351 (±0.311)	89 (±17.1)	0.04 (±0.012)

Seeds subjected to imbibition in solutions of Ni exhibited a similar pattern of metal uptake. Tissue metal content and imbibitional solution concentration were also found to be positively correlated. Furthermore, Ni concentration in the axes was also higher than in the cotyledons. In contrast to the uptake values obtained for Cd however, the Ni content of all germinated seedling tissues was higher than that of the ambient imbibitional solution. Tissues from the highest concentration treatment (700 mg Ni/litre) were not analyzed since these seeds were non-viable.

5.3.2.3 Effect of metal pollutants on seedling toxicity symptoms

Figure 5.2 shows the appearance of seedlings exposed to different concentrations of Ni on termination of the seedling establishment experiments. The appearance of both Cd- and Ni-germinated seedlings was very similar and therefore the former is not shown. In addition, toxicity symptoms exhibited by the seedlings were similar to those described previously for plants exposed to the metals at later stages in the life cycle (section 2.3.2) and will not be repeated here. The most noticeable effect once again, was the reduction in both root and shoot biomass. The general appearance and average plant growth stage (section 2.2.3) of each treatment group at the end of the experiment is shown in column of five of Tables 5.3 and 5.4. In general, shoot elongation and leaf formation was inhibited with increasing metal content as shown by the plant growth stage. The recovery treatments were healthy and only slightly smaller in size than control plants. Nonetheless, seedlings from the 225 mg Cd/litre recovery group as well as the treatment group died, as did the seedlings from the two highest Ni concentration groups.

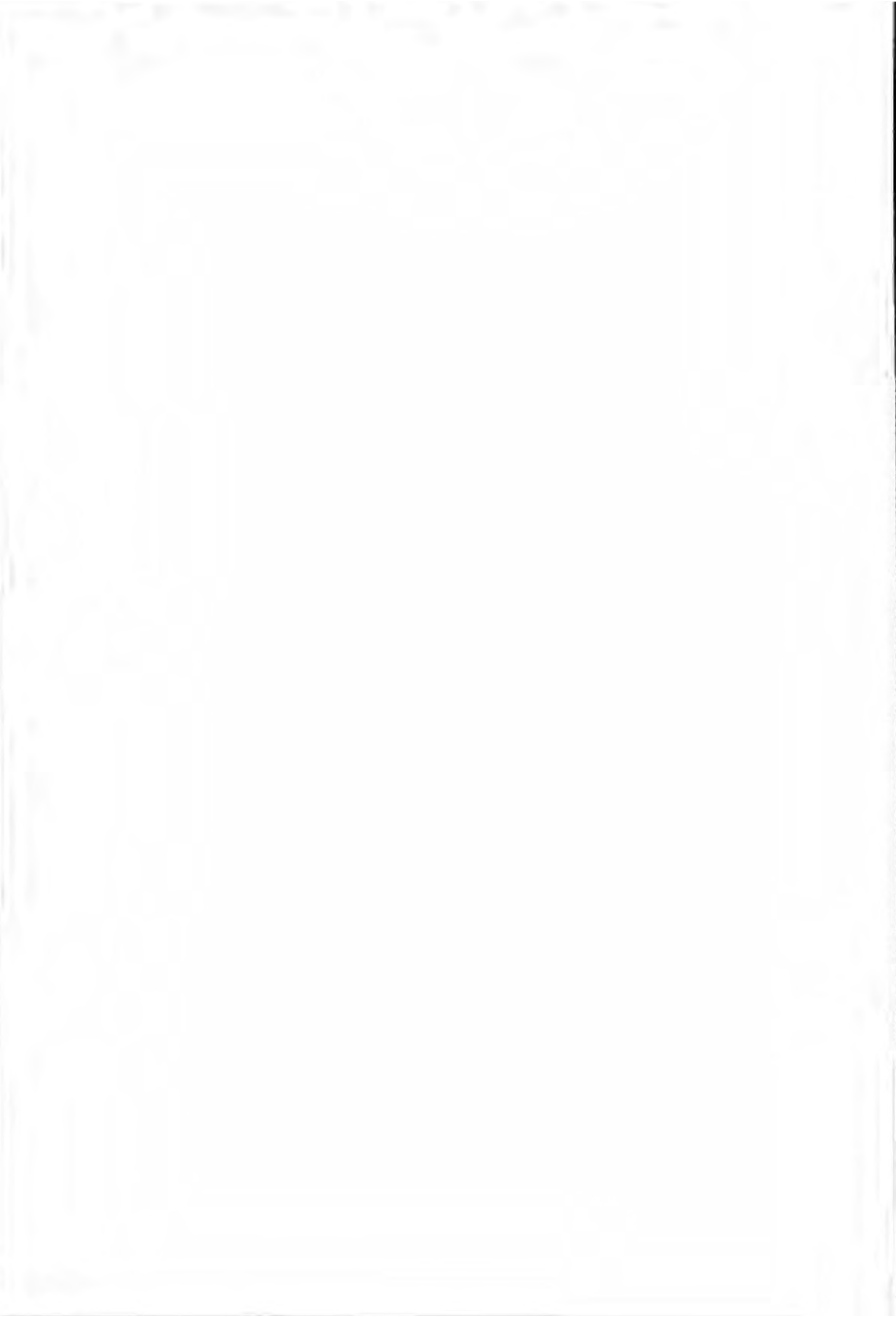
5.3.2.4 Effect of metal pollutants on root biomass

Reduction in root biomass was found to be positively correlated with metal pollutant concentration. Root biomass of the seedlings is shown in Tables 5.3 and 5.4. The root mass of the recovery treatments was higher than that of the equivalent metal-treatment seedlings, but was always lower than that of control seedlings. Lateral root growth appeared to be especially sensitive to the presence of both Cd and Ni. In the case of Cd-treatment groups, lateral roots comprised less than 3% of the total root mass and in the case of Ni treatment groups this was even lower.

Table 5.4 *Summary of the effects of exogenous Ni on germination and seedling establishment. Seeds imbibed in Ni solution for 1 week, then transposed to nutrient solution containing either the same concentration of Ni (x treatment) or water (x recovery). Plant growth stage determined as in section 2.2.3. ND = not determined, BD = below detection. Standard deviation in parenthesis. n = 3 for metal analyses and germination, n = 6 for root mass determinations.*

Germination/imbibition				Seedling establishment				
Conc. (mg/l)	**% germ**	**Mean radicle length (mm)**	**Tissue Ni content (µg/g.dm)**	**Appearance at end of experiment**		**Total root dry mass (g. per plant)**	**Lateral root dry mass (% of total)**	**Ni content of primary leaf (µg/g.dm)**
10	100	86	Cots. = 11.1 (±1.1) Axis = 71.3 (±2.3)	10 Treatment	Moderately stressed; V1	0.049 (±0.03)	0.4 (±0.2)	307 (±34)
				10 Recovery	Healthy; V2.5	0.208 (±0.13)	59 (±15.1)	BD
25	93	75	Cots. = 37.9 (±8.6) Axis = 67.1 (±11.5)	25 Treatment	Very stressed; V0	0.019 (±0.005)	0	ND
				25 Recovery	Healthy; V2	0.141 (±0.1)	45 (±8.9)	BD
50	97	76	Cots. = 80.3 (±4.9) Axis = 207 (±31.3)	50 Treatment	Very stressed; V0	0.018 (±0.002)	0	ND
				50 Recovery	Healthy; V1.5	0.134 (±0.08)	50 (±9.1)	BD
125	100	42	Cots. = 181 (±2.8) Axis = 624 (±23.1)	125 Treatment	All seedlings died	-	-	-
				125. Recovery	All seedlings died	-	-	-
700	70	15	ND	700 Treatment	All seedlings died	-	-	-
				700 Recovery	All seedlings died	-	-	-
Control	100	103	Cots = BD Axis = 0.08 (±0.01)	Con.	Healthy; V3	0.351 (±0.311)	89 (±17.1)	BD

Fig. 5.2 *Toxicity symptoms exhibited by seedlings exposed to differing concentrations of Ni, at the end of the seedling establishment experiment. Note, the symptoms exhibited by Cd-treatment seedlings were very similar and are therefore not shown.*



Transfer of seedlings to the standard nutrient solution resulted in enhanced lateral root production, a response that was demonstrated by both Cd- and Ni-recovery treatments. Lateral root production in the recovery treatments however, was always less than in the control treatment, in which they comprised 89% of the total root biomass.

5.3.2.5 Effect of metal pollutants on leaf metal content

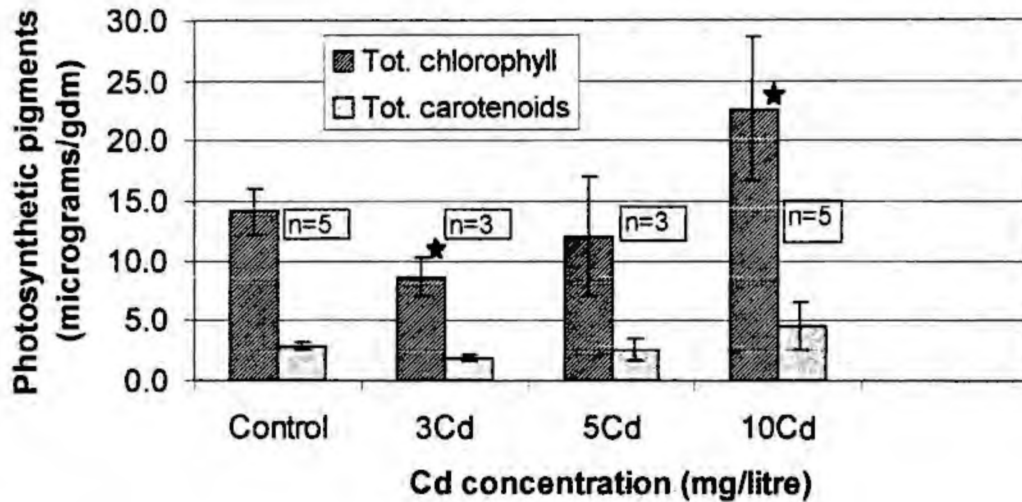
The metal pollutant content of the primary leaves of Cd-treatment seedlings generally increased with increasing concentration of Cd in the nutrient solution. Although in the 25 mg Cd/litre treatment, the metal content in the leaves was slightly lower than expected. The Cd content of the recovery treatment leaves was approximately the same for all concentration groups and was at a value slightly lower than the Cd leaf content of the 3 mg/litre metal treatment. Cadmium concentrations of both metal treatment and recovery treatment groups were higher than that of leaves from control seedlings.

The surface area of leaves from metal-treatment seedlings was reduced compared to control plants (Fig. 5.2). This was especially marked in the treatments containing high concentrations of Ni. Consequently, the Ni concentration in the primary leaves could only be measured for the 10 mg Ni/litre treatment. In the case of the higher concentration groups, not enough leaf material was produced, and therefore metal analyses were not performed on these tissues. The Ni contents of the recovery treatment leaves were all below the detection limit.

5.3.2.6 Effect of metal pollutants on photosynthetic pigments

The effect of increasing Cd concentration on the concentration of photosynthetic pigments in the unifoliate leaves of seedlings is shown in Figure 5.3. Total chlorophyll (Chl a and Chl b) and total carotenoid (carotene and xanthophyll) contents per gram of dry leaf tissue are shown for each treatment group. Cultivation of seedlings in low concentrations of Cd (3 and 5 mg/litre) resulted in a reduction in

A



B

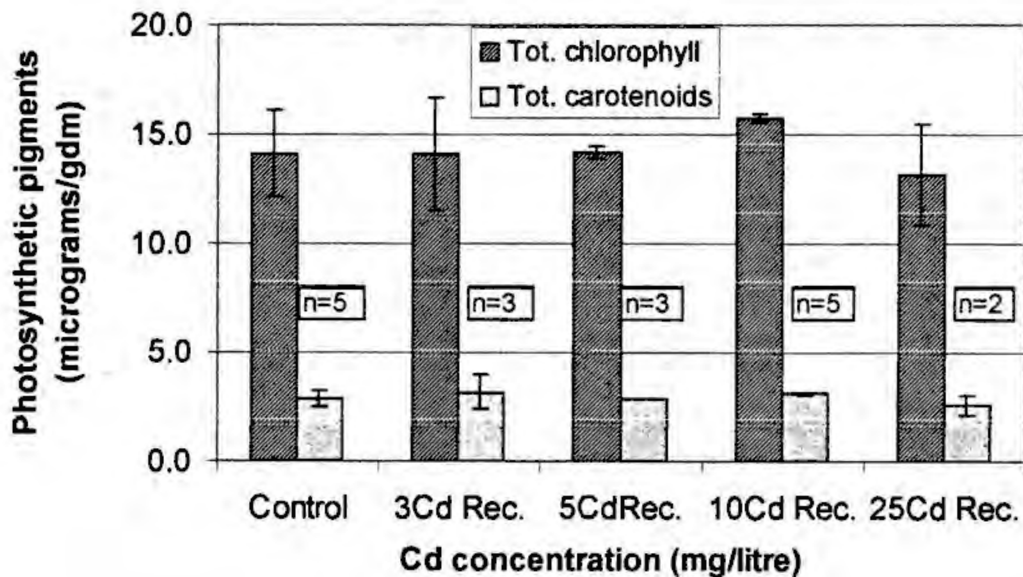


Figure 5.3 *The effect of Cd concentration on the total chlorophyll (Chl a and Chl b) and total carotenoid (carotene and xanthophyll) content of the unifoliate leaves of seedlings. Seedlings imbibed in Cd solution and then cultivated in nutrient solution containing the same concentration of the metal (**A**), or standard nutrient solution (recovery treatments; **B**). Standard deviation and sample size indicated. Asterisk indicates significant deviation from the control at* $p \leq 0.05$.

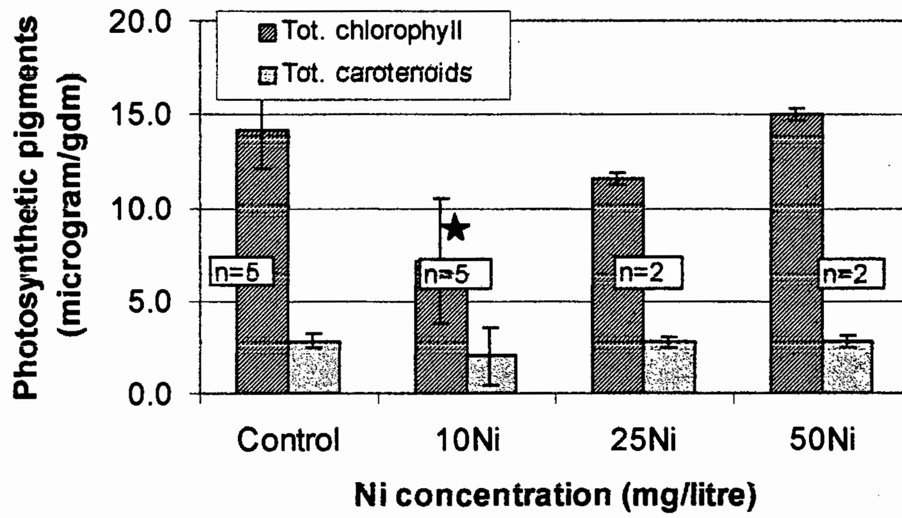
leaf chlorophyll content relative to that of control seedlings (Figure 5.3 A). Higher concentrations of Cd (10 mg Cd/litre) on the other hand, resulted in increased chlorophyll content compared to control leaves. The values obtained for this treatment however, were variable, despite the relatively high sample size (n=5). It was not possible to analyse for photosynthetic pigment content of the 25 mg Cd/litre treatment group due to insufficient leaf material. Although a similar trend to that of chlorophyll was exhibited in total carotenoid content (namely, a reduction at the lower concentration treatments and increased carotenoid content in the higher Cd concentration treatments), the differences were not significant. The overall difference in carotenoid content between the treatment groups was not so marked as in the case of total chlorophyll.

Figure 5.3 B shows the photosynthetic pigment content of the Cd recovery treatments (i.e. seedlings that had been placed in standard nutrient solution after germination in a solution of Cd). No significant difference was found between the total chlorophyll and total carotenoid content of treatment groups relative to the controls.

The effect of increasing Ni concentration on the photosynthetic pigment content of seedlings is shown in Figure 5.4. Low concentrations of Ni (10 mg Ni/litre) significantly reduced the total chlorophyll content relative to leaves from control seedlings. Chlorophyll content in the 50 mg Ni/litre treatment group however was approximately the same as that of control seedlings. There was no significant difference in the carotenoid content of the Ni-treatment seedlings compared to that of the control.

Figure 5.4 B shows the effect of Ni concentration on the photosynthetic pigment content of the Ni recovery treatments. Unfortunately, due to a lack of plant material, few assays could be performed on the higher concentration treatments and it thus not possible to draw definitive conclusions. The data do however, suggest that in contrast to the Cd recovery treatments where no lasting effect on chlorophyll content was observed, the content of this pigment was reduced at higher metal con-

A



B

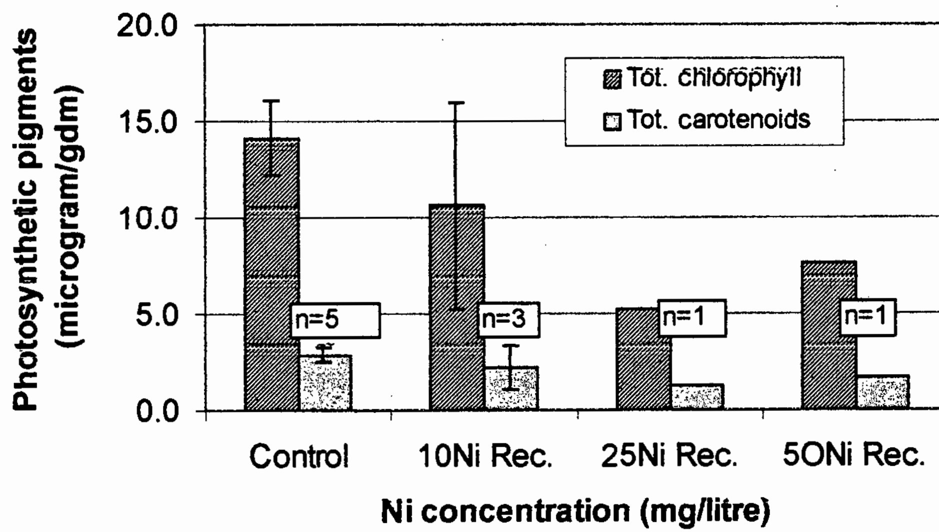


Figure 5.4 *The effect of Ni concentration on the total chlorophyll (Chl a and Chl b) and total carotenoid (carotene and xanthophyll) content of the unifoliate leaves of seedlings. Seedlings imbibed in Ni solution and then cultivated in nutrient solution containing the same concentration of the metal (**A**), or in standard nutrient solution (recovery treatments; **B**). Standard deviation and sample size indicated. Asterisk indicates significant deviation from the control at $p \leq 0.05$.*

centrations. Total carotenoid content was also slightly reduced in the 25 and 50 mg Ni/litre recovery groups, although once again this requires further investigation.

5.3.2.7 Effect of metal pollutants on chlorophyll fluorescence

Figures 5.5 and 5.6 illustrate the effect of metal pollutants on chlorophyll fluorescence in the unifoliate leaves of seedlings. The value of Fv/Fm for control leaves was approximately 0.8 and thus fell within the range of 0.75-0.85 normally exhibited by unstressed leaves (Bolhar-Nordenkampf and Oquist 1993). The value of this ratio for the 5 mg Cd/litre and the lowest Ni concentration treatments was below this limit. There was a tendency for the Fv/Fm ratio to decrease in response to increasing Cd concentrations, although at higher concentrations such an effect was not noticeable. In general, the Fv/Fm ratio was not decreased in the Cd recovery treatments relative to the controls. One exception to this however was the 3 mg Cd/litre recovery treatment.

In contrast to the Fv/Fm ratio, photosynthetic Yield showed a clear trend. Yield tended to decrease in response to increasing Cd concentration in the metal treatment plants, although this was not statistically significant in the higher treatments groups. Only a slight decrease was apparent in the photosynthetic yield of the Cd recovery treatments.

The effect of increasing Ni concentration on the fluorescence parameters of seedlings is shown in Figure 5.6. Nickel decreased the Fv/Fm ratio in the 10 mg Ni/litre treatment group. Because of a lack of leaf material it was not possible to statistically analyse higher concentrations of the metal. In the case of the recovery treatments, even high concentrations Ni did appear to depress the Fv/Fm ratio. As in the case of Cd, Ni also appeared to depress Yield, this parameter being significantly lower in the 10 Ni treatment group than in control leaves. Only in the case of the highest Ni concentration recovery treatment (50 mg Ni/litre recovery treatment) was Yield significantly lower than the controls.

5.4 DISCUSSION

Considering the low concentrations of Cd and Ni that were found to be toxic to plant growth in the circulating nutrient solution system (section 2.3.1), the very high concentrations required to inhibit germination were unexpected. Perusal of the literature however, reveals that the germination process appears to be relatively insensitive to most metal pollutants (Bishnoi *et al.* 1993; Davis *et al.* 1993), including Cd and Ni (Misra, Pandey and Singh 1994; Singh *et al.* 1994). Fargasova (1994) found that the LC_{50} concentration for germination of *Sinapis alba* in the presence of Cd was 692 mg/litre, which is rather higher than the value of 400 mg/litre obtained in this study. This may be due to species-specific differences in the sensitivity of germination to Cd. In comparison to germination, radicle extension in this work was found to be much more sensitive to the presence of metal pollutants, a finding also supported by other studies (Shaw 1995; Xiong 1998). EC_{50} concentration values reported in the literature for the effect of Cd on radicle growth were 48 mg/litre for *Sinapis alba* (Fargasova 1994) and 38 mg/litre in *Enhalus acoroides* (Bonifacio and Montano 1998). Although, in the latter case it is not clear if the authors were referring to radicle or shoot extension. These values are comparable to that of 25-30 mg Cd/litre found in the present work. The enhanced toxicity of Cd compared to Ni was reflected in the higher LC_{50} and EC_{50} values obtained for the latter and is consistent with the findings reported elsewhere in this study. Corradi, Bianchi and Albasini (1993) postulated that germination of *Salvia sclarea* seeds was relatively insensitive to Cr (VI) ions because the seed coat acted as a barrier to uptake. Only once the protruding radicle had ruptured the seed coat, did appreciable uptake of Cr (VI) occur resulting in inhibition of radicle growth. The specialized structure of the testa in soybeans was discussed in section 1.4.5.1. Seed coats of legumes have been reported to be relatively impermeable to water (Esau 1960) and presumably to substances dissolved in water such as metal ions. Hence, although uptake of Cd and Ni in the very early stages of germination was not examined in the present study, it is possible that an impermeable testa prevented uptake until rupture by the emerging radicle. An alternative reason for the relative insensitivity of germination compared to radicle growth may lie in the fact that mitosis and cell division appear to

be sites of toxicity action by many metal pollutants (L'Huillier *et al.* 1996; Das *et al.* 1997). Thus the initial phase of radicle elongation which is primarily due to cell expansion and leads to protrusion through the testa (i.e. germination) would be expected to be relatively insensitive to metal pollutants. Radicle elongation during seedling establishment on the other hand, may be inhibited due to perturbation or reduction in cell division, brought about by the toxic nature of the metal pollutants.

Several researchers have reported enhanced seed germination in the presence of Ni (Welch 1981; Misra *et al.* 1994). Wu and Yun-Hsing 1958 (cited by Mishra and Kar 1974) found that low concentrations of $NiSO_4$ exerted beneficial effects on the germination of soybean seeds. The reason for this stimulation of germination is not known, although it has been postulated that it may be due to the fungicidal properties of Ni (Mishra and Kar 1974). An alternative hypothesis is concerned with the role of Ni as the functional metal in the enzyme urease. The beneficial effects of Ni on seed germination may be related to the activity of urease in germinating seeds and the nitrogen economy of developing seedlings (Welch 1981). An enhanced supply of Ni could conceivably result in increased urease activity and thus more rapid availability of nitrogen to the germinating seed. No stimulation of germination was recorded in this study. Due to the fact that the seeds employed were highly viable and germinated rapidly however, possible stimulation due to the presence of the metal was difficult to detect. Such an effect may be better examined by employing a larger sample size and assessing percentage germination at smaller time increments than 24 hr.

Whatever the reason for the insensitivity of germination to metal pollutants, once the radicle had emerged from the seed coat, due to the role in absorption of nutrients, metal pollutants accumulated in these tissues. Analyses of metal pollutant content in seedlings, seven days after imbibition, revealed a pattern of highest metal pollutant concentrations in the roots and lower levels in the upper shoots or cotyledons. This is similar to the trend exhibited by mature plants (Chapter 2) and was applicable to both Cd and Ni. A point of interest was the accumulation of Ni within the seedlings which was greater than ambient concentration in the imbibitional solution. Similar

high tissue levels of Ni were reported in some organs of mature plants (section 2.3.5). This indicates that accumulation of Ni occurs even during the very early stages of plant growth (before flowering) and is not the result of developing seeds acting as a sink for this element.

Growth of lateral roots appeared to be especially sensitive to the presence of metal pollutants, a finding supported by the work of L'Huillier *et al.* (1996) who studied the effect of Ni on *Zea mays* cultivars. According to Breckle (1991) high concentrations of metal pollutants can also lead to a reduction in the number of root hairs, which in turn can reduce metal uptake. This may be the reason for the lower Cd content in the primary leaves of plants exposed to 25 mg/litre Cd compared to the 10 mg Cd/litre treatment. Whilst 3% of the total root biomass of the 10 mg Cd/litre treatment consisted of lateral roots, there was a complete absence of lateral roots in the higher concentration treatment group. It is thus conceivable that this resulted in reduced Cd uptake and translocation to the aerial shoots. Such and effect was not observable earlier on in development however, since the metal content of the tissues of the 25 mg Cd/litre treatment was higher than the 10 mg Cd/litre treatment when assayed at the end of the imbibition period.

Total chlorophyll content in the primary leaves of seedlings cultivated in nutrient solution amended with metal pollutant, appeared to respond in a similar manner to the presence both Cd and Ni. Low concentrations of the metals resulted in a decrease in the leaf content of this pigment relative to the controls. At higher concentrations of metal pollutant however, the levels of chlorophyll were increased. Total carotenoid contents tended to show very little difference between treatment groups. Several workers have examined the effect of metal pollutants on the chlorophyll and carotenoid levels in leaves. A decrease in pigment content in response to increasing metal concentrations has usually been reported (Angelov, Tsonev, Uzunova *et al.* 1993; Corradi *et al.* 1993). Gallego *et al.* (1996) reported that sunflower leaf discs floating in a Cd solution contained lower chlorophyll concentrations than controls. Krupa, Siedlecka, Maksymiec *et al.* (1993) found that both chlorophyll and carotenoid content, expressed as mg per leaf, and decreased in

the presence of Ni. In contrast, Shaw (1995) examined the effect of exposure to either Hg or Cd on the photosynthetic pigments in the primary leaves of germinating *Phaseolus aureus* seedlings. In response to the presence of either Cd or Hg, total chlorophyll and carotenoid contents were found to increase when expressed as mg pigment per gram fresh mass of leaf tissue. The above author attributed such an effect to either, an increase in the number of chloroplasts per cell, or alternatively a decrease in cellular volume leading to an increase in the number of cells per unit weight, or both. In the present study it is possible that two opposing trends are in operation. Firstly a decrease in pigment content due to the inhibitory effect of metal pollutants on pigment synthesis and/or enhanced degradation. Secondly, a decrease in the volume of the leaf cells accompanied by a decrease in the proportion of sclerophyllous tissue in the cell walls. Such an effect may cause an increase in total chlorophyll content when expressed on a dry mass basis. It is proposed that reduction of cell volume occurred only at higher metal pollutant concentrations, reduced pigment concentration on the other hand is considered to occur even at the lowest levels of metal exposure. The effect of the two opposing trends may be expected to result in reduced pigment contents at low metal exposure levels and increased pigment contents at higher metal exposure levels, which is the pattern exhibited in this present study.

Whilst more detailed experimentation is required, the following points lend support to the above hypothesis. Firstly, several research workers have reported that Cd inhibits biosynthesis of chlorophyll (Somashekaraiah *et al.* 1992) and can result in increased chlorophyll degradation (Shaw 1995). Exposure to Ni has also been reported to lead to reductions in photosynthetic pigments in leaves (Krupa *et al.* 1993). No reports could be found in the literature of enhanced rates of pigment synthesis resulting from treatment with a metal pollutant. It was considered to be unlikely therefore that the increased pigment concentrations in response to metal pollutant treatment were due to stimulation of biosynthesis. Secondly, many studies have reported that metal pollutants result in reduced leaf area (Vazquez, Poschenrieder and Barcelo 1989; Bishnoi *et al.* 1993). Goshroy and Nadakavukaren (1990) demonstrated that high levels of Cd (11 mg Cd/litre) resulted in reduced leaf

area in the primary leaves of soybean, but that lower levels of the metal pollutant, did not elicit a response. In the present study, although not assessed quantitatively, it was clear that the area of the primary leaves in the higher concentration treatments was decreased in metal-stressed seedlings compared to the controls (Fig. 5.2). In addition, such leaves were of a darker green colour. Nothing could be found in the literature concerning changes in cell wall composition or reduction in the sclerophyllous component of unifoliate leaves in response to metal pollutants. Without such changes it is difficult to explain why chlorophyll content per gram dry mass increased in the higher concentration treatments. The hypothesis therefore is speculative at this stage and requires further investigation.

Another point of interest concerning the effect of metal pollutants on photosynthetic pigments was reported by Barcelo, Vazquez and Poschenrieder (1988). These authors found that exposure of *Phaseolus vulgaris* seedlings to Cd resulted in differing responses between unifoliate (primary leaves) and trifoliate leaves. Photosynthetic pigment levels were significantly decreased in trifoliate leaves but to a limited extent in unifoliate leaves. The authors ascribed this difference to the fact that the unifoliate leaves were present prior to exposure of the plants to Cd (Cd was supplied only 6 days after imbibition), thus cell division and chloroplast development had already taken place in the primary leaves. The trifoliate leaves on the other hand, were initiated subsequent to metal pollutant treatment and hence developed during continuous exposure to the metal. They postulated that Cd is an inhibitor of chlorophyll synthesis in new leaves where active chlorophyll production occurs. In the present study, despite the fact that metal pollutants were supplied during the entire duration of the experiment, a similar difference in the effect of Cd and Ni on the two types of leaves was noted. Although the chlorophyll content of trifoliate leaves was not determined, it was reported in section 2.3.2 that one of the symptoms of metal pollutant toxicity was chlorosis of the leaves. On the other hand, as mentioned above, chlorophyll synthesis in unifoliate leaves, although reduced was not severely depressed and these leaves appeared dark green in colour and reduced in size. Ogren and Rinne (1973) (citing McAlister and Krober 1951) reported that removal of soybean cotyledons immediately after emergence resulted

in seedlings that were chlorotic for the following 10 – 15 days. If the cotyledons were removed at a later stage after seedling emergence however, no chlorosis was noted. A possible implication of these experimental observations is that in soybean seedlings, chorophyll precursors are synthesized within the cotyledons prior to translocation to the developing primary leaves. In subsequently formed leaves on the other hand, synthesis occurs *in situ*. Thus the differential effect of metal pollutants within unifoliate and trifoliate leaves may be due to differences in toxicity effects between cotyledons and trifoliate leaves and/or alterations in the chlorophyll biosynthetic pathway between the two organs.

Chlorophyll fluorescence measurements indicate that the presence of metal pollutants affected the photosynthetic process itself. The Fv/Fm ratios in the presence of either metal pollutant tended to be depressed compared to control leaves. This parameter is an indication of the maximum potential of PSII (photosystem II) to become excited in dark-adapted leaves. Thus a lowering in this parameter indicates a decrease in the efficiency of the light-reactions of photosynthesis (Bolhar-Nordenkampf *et al.* 1989). Decreases in Fv/Fm have been reported in response to Al (Moustakas *et al.* 1996) and to Cd in wheat (Ouzounidou *et al.* 1997). Krupa, Oquist and Huner (1993) on the other hand found that short exposures (up to 7 days) of *Phaseolus vulgaris* seedlings to Cd had little effect on the Fv/Fm ratios. The above author used a lower concentration range of Cd (0-approximately 5 mg/litre Cd) however and a shorter exposure period than in the present experiments. Krupa *et al.* (1993b) also found that Ni supplied in concentrations up to 25 mg/litre had little effect on the Fv/Fm ratio in primary leaves of beans. The lack of reduction in Fv/Fm in some cases but not in others, may be explained by differences in the concentration of metal pollutant supplied, the nature (identity) of the metal pollutant as well as the length of exposure time. Maksymiec and Baszynski (1996) found that Cu had little effect on the Fv/Fm value of the primary leaves of bean seedlings up to 6 days after placing in a solution of the metal pollutant. Thereafter this parameter was significantly decreased. Lowering of the Fv/Fm ratio in the present study indicates altering of the functioning of PS II. Cadmium has been reported to inhibit PSII, possibly affecting the water splitting

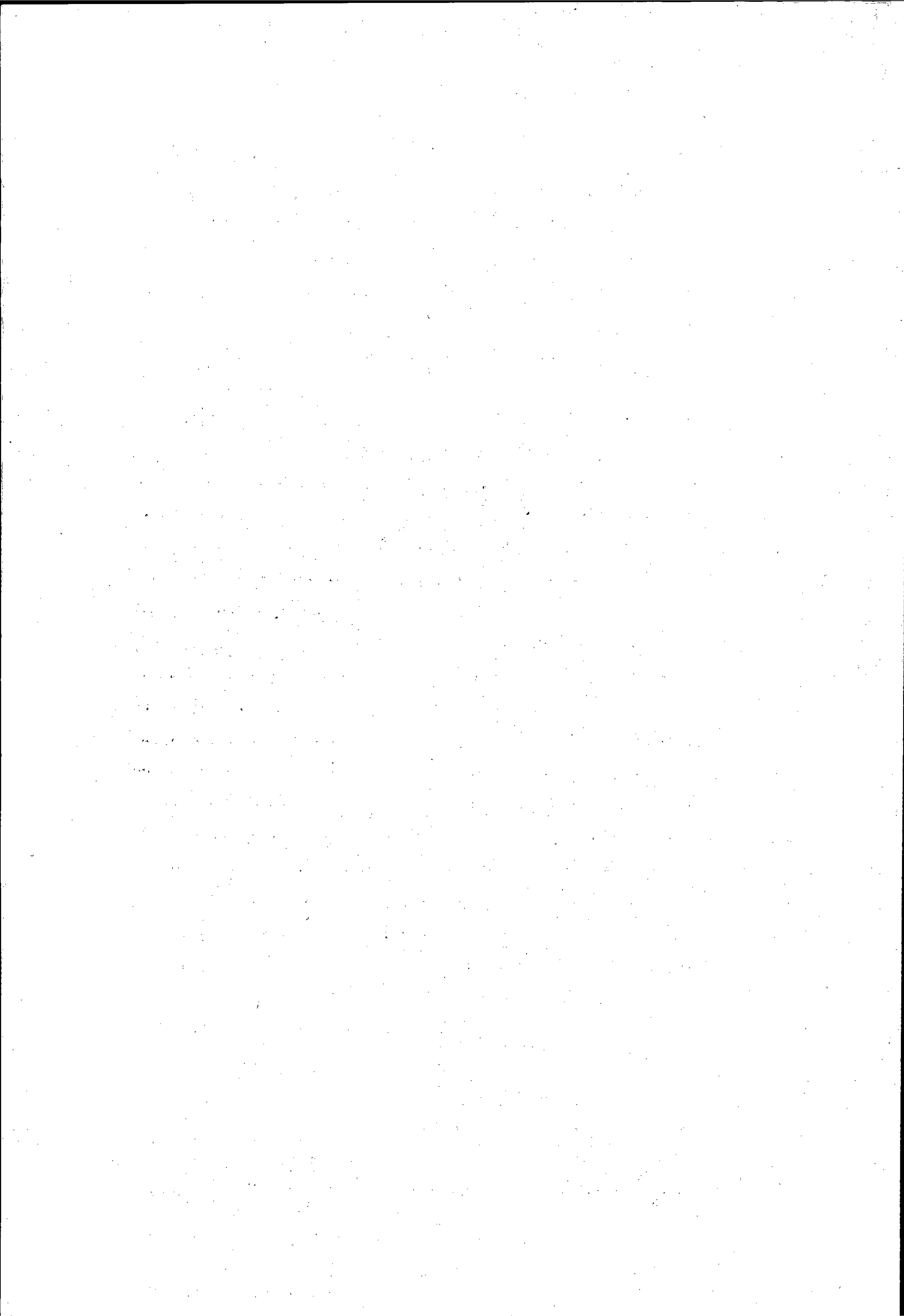
system (Prasad 1995b). In addition, shifts in the concentration of chlorophyll and thus antenna size can affect fluorescence (Siedlecka and Krupa 1996). The complex changes in chlorophyll content detailed previously may well confound the Fv/Fm ratio, obscuring any trends.

The Yield parameter (Fms-Fs/Fms), which indicates variable fluorescence whilst under steady-state light conditions, also showed a reduction in metal-treatment seedlings compared to control leaves. Changes in parameters associated with the slow phase of fluorescence induction kinetics are considered to be due to alterations in the dark reactions of photosynthesis (Bolhar-Nordenkampf and Oquist 1993). Cadmium has been reported to cause stomatal closure in some plants thus decreasing the availability of CO_2 (Huang *et al.* 1974). Krupa *et al.* (1993b) postulated that Ni exerted an indirect effect on photochemistry due to disturbances of the Calvin cycle reactions and feedback inhibition of electron transport by accumulation of ATP and NADPH due to non-efficient dark reactions. In the case of Yield as well as Fv/Fm, no clear correlation could be discerned with metal pollutant concentration and this might also be a result of changes in concentration of photosynthetic pigments. In addition, the fact that accumulation of Cd within the primary leaves did not always increase with ambient metal concentration may have contributed to this result.

The action of both metal pollutants on the photosynthetic process is likely to be complex. Firstly, because multiple sites of action may exist. Cadmium has been reported to cause a range of effects on photosynthesis, from stomatal closure, disruption of PSII functioning, inhibition of reactions of the Calvin cycle to causing changes in the ultra-structure of the thylakoid system (Prasad 1995b; Goshroy and Nadakavukaren 1990). Secondly, it was reported in section 3.3.4.5 that both Cd and Ni can also affect uptake of other nutritionally important ions including Fe and Mn. Secondary effects on photosynthesis, through effects on other metals, have been reported by Siedlecka and Baszynski (1993), as well as Siedlecka and Krupa (1996). Considering this complexity it is not surprising that the primary sites of toxicity action

of Cd and Ni with regards to the electron transport chain and the reactions of the Calvin cycle still require elucidation (Krupa *et al.* 1993a and b; Prasad 1995b).

Transfer of seedlings to the standard nutrient solution after imbibition in low concentrations of Cd or Ni (i.e. the recovery treatments) resulted in plants that appeared healthy (Tables 5.3 and 5.4). If the concentration of the metal pollutant was too high however, the plants were not able to recover and eventually died. An examination of leaf metal analyses, root biomass, chlorophyll content as well as fluorescence data indicates the extent to which physiological functioning of such seedlings was able to recover from a period of exposure to the metal pollutants. Although the recovery-treatment seedlings appeared healthy, in the case of both metals root biomass was reduced compared to the control treatments. In addition, although the Cd content of the primary leaves of the Cd-recovery treatments were lower than their metal-treatment counterparts, they were higher than control leaves. Despite this, total chlorophyll and total carotenoid contents of Cd-recovery seedlings showed no significant decrease compared to the values in the primary leaves of control seedling. Measurements of chlorophyll fluorescence revealed that significant reduction in photosynthetic functioning occurred mainly in the highest concentration recovery treatments (25 mg Cd/litre recovery treatment and 50 mg Ni/litre recovery treatment). Thus the primary effect of exposure to Cd or Ni appeared to be on root biomass. Titov, Talanova, Boeva *et al.* (1995) demonstrated that six days after a brief exposure to Pb (24 hrs), root mass had not been depressed at lower concentrations of the metal but was at higher metal pollutant concentrations. In the present study the effect of duration of metal pollutant exposure was not investigated. It is likely however that increased duration of exposure even at low concentrations of Cd or Ni may lead to more severe toxic effects.



CHAPTER 6

METAL POLLUTANTS AND SEED ULTRA-STRUCTURE

6.1 INTRODUCTION

The anatomy and ultra-structure of seeds from the different treatment groups used in this study was examined by means of light microscopy (LM) and transmission electron microscopy (TEM) respectively. Seed tissues that had been exposed to metal pollutants were compared with the appropriate controls and differences identified. The rationale for this strategy being that identification of such abnormalities or aberrations could potentially yield information concerning sites of metal pollutant action, as well as putative toxicity mechanisms. Seeds harvested from plants grown in the standard, Cd- and Ni-amended nutrient solutions (control, Cd- and Ni-*treatment* seeds) were examined. In addition, the ultra-structure of seeds germinated in Cd and Ni solutions as described in Chapter 5 (control, Cd- and Ni-*germinated* seeds) was also examined. Investigations were limited to studies of the cotyledons and radicle tip. Choice of these two tissues was made in the case of the former, because toxicity effects on storage reserves were of interest in this study and in the case of the latter, because growth of the radicle is essential for seedling development. Any deformities at the ultra-structural level in the radicle tip could potentially have serious implications for future seedling growth.

The anatomy of developing and mature soybean seeds has already been discussed (section 1.4.5). In addition, the organelles responsible for accumulation of the different seed storage reserves were described in section 1.4.3. Studies on the microstructure of developing soybean seeds have been carried out since at least the early 1960's (Bils and Howell 1963). Particular attention has been paid in the literature to the ultra-structure of the storage organelles of the cotyledons, especially

the protein bodies (Yoo and Chrispeels 1980; Saio, Kondo and Sugimoto 1985). The appearance of cotyledon cells at different seed development stages as described in the literature, is summarized in Table 6.1.

The ontogeny of these tissues is dominated by events concerning storage reserve accumulation. In brief, the small, thin-walled cotyledon cells gradually increase in size with age. The large central vacuole fragments into many small vacuoles, which become progressively filled with protein and are referred to as protein bodies. These organelles sometimes contain inclusions or globoids of various types, often containing crystals of phytate (Kondo *et al.* 1986). Plastids, containing starch grains gradually increase with DAF until just prior to maturity when they rapidly disappear. The number and size of lipid bodies increases steadily with cell development. The amount of rough endoplasmic reticulum (RER) in the cells also increases concomitant with protein body formation and then disappears towards the end of the development period. At maturity, cells of the cotyledons are large and packed with protein bodies, lipid bodies and free ribosomes with the virtual absence of other organelles.

As mentioned in section 1.4.2, although the sequence of events in seed development is the same, the length of time taken to reach each stage is likely to differ with growth conditions and cultivar. At the ultra-structural level, Kondo *et al.* (1986) also noted considerable seed-to-seed and cell-to-cell variability. Thus although the position of the growth stages determined in this study (IP, EP etc.) are also indicated in Table 6.1, the DAF shown for each developmental stage is approximate. Ontological events occurring at each growth stage will be discussed further in section 6.3.

Some discrepancies in the sequence of cytological events were noted between the different authors. For example Saio *et al.* (1985) reported that the tonoplasts of the large central vacuoles were first lined with protein before fragmentation into smaller vacuoles (Table 6.1). Adams, Norby and Rinne (1985) on the other hand, considered fragmentation of the central vacuole to be an early event that was

Table 6.1 *Changes in the ultra-structure of cotyledon cells with seed development as described in the literature. The stage of seed development is given as DAF (days after flowering) but is likely to be approximate. Growth stages used in this study (section 3.2.1) also indicated.*

DAF	Bils *et al.* (1963) cv "Chipewa"	Saio *et al.* 1985) cv "Enrei"	Adams *et al.* (1985) cv "Wells"	This study cv "Crawford"
< 15	Cell division completed in cotyledons by 14DAF (but not in radicle)			**VIP**
ca 15	Large central vacuole. Thin layer cytoplasm around cell edge. Nucleus, plastids, starch grains and mitochondria present.	Cells small, thin-walled, large central vacuole. Little ER. Many free ribosomes. A few LB and plastids with starch grains.	Large central vacuole. From 17DAF smaller peripheral vacuoles also present.	**IP**
ca 20		Cells larger. Starch grains, plastids and LB more numerous.	Fragmentation of central vacuole completed by 21DAF. Plastids with starch grains, free ribosomes now more numerous. A few LB present. No RER or PB present.	
ca 25	LB more numerous in cytoplasm. A few PB also present . Starch grains larger. Fewer mitochondria than at previous stages.	Tonoplast lined with protein. Fragmentation of central vacuole. Appearance of spherical protein-lipid-sugar bodies. Cells still enlarging.		**Early EP**
ca 30	**At 36DAF starch grains and PB** ± 4-5μm, LB 0.2-0.3 μm. Some cells contain only LB.	Accumulation of numerous LB, PB and starch. RER abundant. Transformation of Protein-lipid-sugar bodies to PB.	By 34DAF, distinct PB are recognizable in the cytoplasm. RER and LB increase. RER gives rise to protein-filled vesicles.	**EP**
ca 40		Cells maximum size. RER abundant and swollen. PB, LB, starch grains still increasing in size and number. Heterogeneity between cells. Plastids reach maximum in numbers and size.	34-36DAF, PB increasing rapidly in size. RER visible in close proximity, but not touching PB.	
> 40		Starch grains disappear. Plastids decrease in number and oil droplets appear in stroma. Staining of PB still not uniform.		**YP**
ca 55	Starch grains disappear just prior to maturity.	LB line plasmalemma at cell walls. Staining of PB homogeneous.	Cells packed with PB, LB and free ribosomes. Other organelles absent.	**BP**

PB= protein bodies; LB= lipid bodies, RER= rough endoplasmic reticulum. IP= immature pod, EP= expanded pod, YP= yellow pod, BP= brown pod.

subsequently followed by protein deposition and protein body formation. This and other variations in organelle ontogeny may result from examination of differing soybean cultivars or alternatively, from differing tissue preparation protocols. Dry plant tissue such as found in maturing seeds, can be difficult to prepare for electron microscopy. Preparation usually involves soaking the tissue in aqueous solvents, which may cause changes in cytological features (Chabot and Leopold 1985; Yaklich, Wergin and Erbe 1994).

Chabot and Leopold (1985), Yaklich *et al.* (1994) amongst others have carried out ultra-structural studies of soybean seed axes. Interesting differences were noted between the protein bodies of soybean cotyledons and axes by Monma *et al.* (1990 and 1992). According to these authors, protein bodies from the former tissue contain very few inclusions compared to protein bodies from the latter. These same authors also reported differences between inclusions of the cortex and of the stele.

Nothing could be found in the literature regarding the effects of metal pollutants on the ultra-structure of developing seeds. Numerous studies however have documented cellular aberrations brought about by the presence of Cd in other plant organs. Such studies include the roots of various plant species such as beans (Vazquez *et al.* 1992b) and *Agrostis* (Rauser and Ackerley 1987) as well as the leaves of maize (Rascio *et al.* 1993) and *Coriandrum* (De Pasquale, Rapisardo, Germano *et al.* 1995) amongst others. In addition, several studies have examined metal pollutant-germinated seeds and seedlings (Angelov *et al.* 1993; Corradi *et al.* 1993). A fairly wide range of ultra-structural malformations has been reported in the literature. This is perhaps not surprising considering the broad extent of effects brought about by metal pollutants on plant physiology and metabolism (section 1.3.6.3). Ultra-structural aberrations brought about by the presence of a metal pollutant may be either the cause, or consequence of physiological malfunctioning (Barcelo *et al.* 1988).

Ultra-structural deformities of chloroplasts, resulting from exposure to Cd, appear to be a common finding (Goshroy and Nadakavukaren 1990). Such studies have

frequently reported disruption of the thylakoid membranes (Ouzounidou *et al.* 1997) often accompanied by an increase in plastoglobuli within the stroma (Rascio *et al.* 1993; Stoyanova and Tchakalova 1997). According to Das *et al.* (1997) several authors have found aberrations concerning the structure of the nucleus and cell division is commonly inhibited by the presence of Cd. Margination of chromatin (Lindsey and Lineberger 1981) and invagination of the nucleus (Reese, McCall and Roberts 1986) have also been reported. Several authors have reported the presence of cellular granules as a result of exposure to Cd, which may or may not contain the metal pollutant. For example Neumann, Lichtenberger, Gunther *et al.* (1994) studied the effect of the metal on *Lycopersicon* cell cultures grown in 0.01M Cd. They noted the presence of small osmiophilic droplets of lipid, between the plasmalemma and the cell wall, as well as the presence of heat shock granules in the cytoplasm. Rauser and Ackerley (1987) found Cd-containing electron-dense granules in the parenchyma cells of *Agrostis* and *Zea mays* roots after exposure to the metal. Such granules were found in the cytoplasm and vacuoles of differentiating and mature cells and in the nuclei of undifferentiated cells.

Disruption of mitosis, resulting in abnormal cell division and disturbances in cell orientation, are the major cytological effects reported for Ni (Mishra and Kar 1974). These authors cite the findings of Glass (1955 and 1956) who examined the effect of $Ni(NO_3)_2$ on *Vicia faba* root tips. Root tip aberrations, including mitotic disturbances and cell enlargement, were dependent on the concentration of the solution used as well as on the duration of the treatment. The above results are supported by the work of L'Huillier *et al.* (1996), who also found that Ni inhibited cell division in the root tips of a non-tolerant Maize cultivar. Elongation of root cells was noted, accompanied by an increased number of vacuoles in these cells. The authors also noted cellular disjunctions and attributed this to possible increased fragility of the cell walls. Interesting changes in the metabolism of starch as a result of Ni toxicity were noted by the above authors as well as by Rauser (1978).

Reports of other ultra-structural aberrations, apart from those described above, are also to be found in the literature. It should be noted however that in many studies,

no obvious ultra-structural changes were apparent, or else these became evident only when the plants were subjected to very high metal levels. Another important point is that the extent of ultra-structural damage is frequently not directly proportional to the concentration of metal pollutant (Vazquez *et al.* 1989). For example Barcelo *et al.* (1988) found that the roots of bean plants exposed to Cd displayed little cytological malformation compared to other plant tissues, despite the fact that the highest metal pollutant concentrations were found in this organ. This is likely to be a consequence of the presence of tolerance mechanisms as well as differential sensitivity of various plant tissues to metal pollutants (section 1.3.8).

6.2 MATERIALS AND METHODS

Tissue samples from metal pollutant-treatment seeds were excised at each growth stage. Care was taken to ensure that the tissue was removed from the same region of each seed. In the case of the radicle tip, a block of tissue 2 mm x 1 mm x 1mm in size was excised. This was performed in such a manner that the long axis of the block was coincident with the long axis of the radicle and the middle portions of the root tip as well as the meristematic regions were included. In the case of the cotyledons, a $1mm^3$ block of tissue was excised from the centre of the abaxial side of the cotyledon. Tissue samples from metal-germinated seedlings were taken 48 hr after the initiation of imbibition (section 5.2.1). Segments were excised from the radicle tip and abaxial cotyledon surface as described above.

Tissue samples were processed for LM and TEM by first fixing overnight in 2.5% glutaraldehyde, prepared in 0.1M sodium phosphate buffer (pH 7.4) and containing 0.5% caffeine. After rinsing twice in phosphate buffer, samples were post-fixed in 1% OsO_4 for a period of 2 hr, followed by dehydration in a graded acetone series. To this end, samples were placed sequentially in 30, 50, 70, 80, 90 and 95% acetone solutions for 10 min each. The material was then infiltrated with Spurr's epoxy resin (Spurr 1969) by placing the material for 24 hr each in 50, 70 and 100%

resin solutions (diluted with acetone). To aid infiltration, samples were left in 100% resin for 4 days, fresh resin being used every day. On the last day, samples were placed under vacuum for 24 hr. The tissue segments were then embedded in resin and the blocks polymerised at 60° C for 16 hr.

Light microscope sections (1 μm thick) were prepared by cutting the resin blocks with a Reichert Ultracut-S ultra-microtome. Sections were mounted in drops of water on glass microscope slides and dried over low heat. For routine anatomical visualization, sections were stained using Toluidine Blue O according to the method of O'Brien, Feder and McCully (1964). In addition, stains were used to localize specific histochemicals in the tissues of Cd-, Ni- and control-treatment seeds. Protein, was localized using Coomassie Briliant Blue (CBB), carbohydrate, using periodic acid-Schiff's reagent (PAS) and lipid, with Sudan Black B. All histochemical localisations were according to the procedures recommended by Saio, Nakano and Uemoto (1983).

Sections for TEM were cut at 80 nm, collected on copper grids and post-stained for 15 min each in 2% aqueous uranyl acetate and lead citrate (Reynolds 1963). Observations were made using a Zeiss EM109 transmission electron microscope.

6.3 RESULTS

6.3.1 Metal-treatment and control seeds

6.3.1.1 Light microscopy (LM) and histochemistry

Preliminary studies were carried out at the LM level to examine root anatomy. In addition, cotyledons were also examined at this level to check for homogeneity between cells and regions with regard to distribution of storage reserves. Figure 6.1 shows the general anatomy of the radicle tip in IP seeds. The white starch grains

can be clearly discerned against the dark background of lipid and protein bodies stained with Sudan Black. The area examined with the TEM is demarcated. Starch grains in the root tip were viewed most clearly when sections were stained with either Sudan Black or PAS. Observations of the root tip at all growth stages for both Cd-, Ni- and control-treatment seeds showed that neither cell size nor the number of starch grains appeared to be affected by metal pollutant treatment.

In general, distribution of the storage reserves within cotyledons appeared to be reasonably homogeneous. On a smaller scale however, some areas of both control and metal-treatment seeds appeared to contain fewer protein bodies than others (Figs. 6.2 and 6.3). The reason for such heterogeneity is not clear and may have been the result of tearing of the cell walls that resulted in loss of protein bodies during preparation of the specimen. Alternately, certain areas of the cotyledons may naturally be deficient in this storage reserve. This phenomenon was not investigated further, but was kept in mind when interpreting TEM micrographs. In addition, no differences between control and metal pollutant-treatment seeds were apparent at the LM level.

6.3.1.2 <u>Transmission electron microscopy (TEM)</u>

i) Radicle apex cells

Cells from the radicle tip of control IP seeds were rectangular in shape with thin cell walls and several vacuoles (Fig. 6.4). The large nuclei each contained one prominent nucleolus as well as other areas of dark condensed material. The full complement of organelles was present, including starch grains. Dark spots were also observed in the mitochondria and were similar to those reported by Adams *et al.* (1985). Protein deposition on the inner tonoplast surface of several vacuoles was evident (Fig. 6.5). Small lipid bodies were visible in the cytoplasm.

The most distinctive feature of YP and BP radicle cells was the single layer of darkly stained lipid bodies lining the plasmalemma and surrounding the protein bodies (Fig. 6.6 as well as Figs. 6.8 and 6.9). These were similar to those shown by Monma *et al.* (1992). Although these cells were not as packed with storage reserves as the cotyledon cells (see below), the cytoplasm was dense and many rounded, protein-containing vacuoles were present. Starch grains were rare. Membranes and globoid inclusions

within the protein bodies were frequently visible. Such inclusions often consisted of an electron-translucent area containing dark crystals, possibly of phytate. The inclusions were usually bounded by a membrane although sometimes, no clear boundary was visible (Fig. 6.7). In other cases, the dark crystals were absent. At the most mature growth stage, nuclei usually exhibited a "halo" of electron-translucent nucleoplasm around the nucleolus (Fig. 6.6). Nuclei were less regular in shape compared to the IP stage. Despite the precautions taken to ensure adequate infiltration of the tissue with resin, protein bodies of mature seeds often showed areas where the section was discontinuous.

No qualitative differences could be discerned between metal-pollutant treatment seeds and control seeds i.e. there were no ultra-structural features that were present in Cd- or Ni-treatment seeds that were not present in control seeds (or vice versa). The "halo" around the nucleoli was present in BP seeds of all treatment groups. On the other hand, starch grains did appear to be more numerous in some of the cells of Ni-treatment seeds (Fig. 6.8). Crystals in protein body inclusions were also especially common in some cells of the same treatment group (Fig. 6.9) although not in others (Fig. 6.8).

ii) Cotyledon cells

Development of the microstructure of cotyledon cells appeared to be similar to that summarized in Table 6.1. By the IP growth stage (± 16 DAF) the vacuoles of many of the cells had already fragmented into smaller organelles, although cells with large central vacuoles were still quite common. There was variation in the extent to which protein was deposited. Figure 6.10 shows the small vacuoles of a cotyledon cell at the IP growth stage. Globules of protein are visible, arranged on the inside of the tonoplast. In the same figure (as well as Fig. 6.11), vacuoles containing more extensive protein deposits are visible. Occasionally large, rounded deposits of protein were seen, similar to those present in radicle apex cells (Fig. 6.5). In some cells with large central vacuoles, protein deposition had already been initiated (not shown). Spherical, electron-dense bodies were visible (Fig. 6.10), possibly the protein-lipid-sugar bodies (PLS) reported by Saio *et al.* (1985). Rough endoplasmic

reticulum (Fig. 6.12), starch grains, mitochondria as well as membranes within the vacuoles were also observed in cells at this growth stage.

By the YP growth stage (± 50 DAF), cotyledon cells were packed with protein bodies (Figs. 6.2 and 6.3) which were no longer darkly stained as in IP cells. There was still some variation between protein bodies with regard to homogeneity of the protein content. A few inclusions were noted within these organelles, although these were not numerous. Such inclusions usually took the form of electron-translucent areas or dark spots (Fig 6.13). The cytoplasm was compressed by the large protein bodies and was consequently very dense. Numerous lipid bodies were visible by this stage, often containing dark inclusions that appeared to be aligned in the same direction (Fig 6.13). Starch was still visible at this stage. Cotyledon cells of BP (mature) seeds appeared very similar to YP cells excepting that the contents of the protein bodies was virtually completely homogeneous. The most striking feature of these cells was the orderly arrangement of osmiophilic lipid bodies.

No marked ultra-structural features distinguished the cotyledons of metal-treatment seeds from control seeds, excepting that inclusions were noted in the nucleoplasm of some Cd-treatment seeds (Fig. 6.14). These appeared to be minute vesicles (Fig. 6.15) and exhibited an organized structure. They were present in approximately 20% of the nuclei observed from this treatment group. Whilst condensed areas were present in the nucleoplasm of YP control (Fig 6.13) and Ni-treatment seeds, these did not appear to contain vesicles. Due to the dense nature of the cytoplasm, nuclei at the YP and BP growth stages were not easily observed.

6.3.2 Metal-germinated seeds

6.3.2.1 Radicle apex cells

Examination of cells from the radicle tip of control seeds, 48 hr after initiation of imbibition revealed that whilst some cells still contained several small vacuoles others appeared to have coalesced to form large central vacuoles (Fig. 6.16). Protein deposits, in the form of condensed osmiophilic globules, were visible within these

deposits, in the form of condensed osmiophilic globules, were visible within these organelles, adhering to the inner surface of the tonoplast (Fig. 6.17). Starch grains were once again present but were small and grouped together (Fig. 6.18). A few dark lipid bodies were also visible in the cytoplasm. In general, nuclei were rounded in shape, contained a prominent nucleolus and occasionally, areas of condensed chromatin.

Seeds germinated in a solution of 3 mg/litre Cd, showed only a few ultra-structural aberrations compared to the controls (Fig. 6.19). Storage protein was present in the form of condensed globules and the nuclei were also similar to those of control seeds. Nonetheless, starch grains did appear to be less numerous compared to control cells. In addition, only in very few of the cells had the small protein-containing vacuoles coalesced to form large central organelles. It is therefore possible that this step was delayed in the apical cells of Cd-germinated seeds. The radicle tip cells of seeds germinated in 225 mg/litre Cd on the other hand, showed extensive cellular disruption (Figs. 6.20 and 6.21). The appearance of protein within the vacuoles, although darkly stained as in control seedlings, appeared to be more diffuse (Fig. 6.20). Starch grains were rare and when present were small and not grouped together. The most noticeable ultra-structural difference was apparent in the nucleoli. Electron-translucent areas, which were sometimes rounded in shape (Fig. 6.20) or (rarely) elongated (Fig. 6.21) were clearly visible in the nucleoli. Similar electron-translucent areas were not observed in the nucleoli of control cells.

Cells from the radicle tip of seeds germinated in up to 50 mg/litre Ni exhibited no marked micro-structural abnormalities. Proteolysis did not appear to be inhibited, since extensive protein deposits were not a feature of these cells (Fig. 6.22). In addition, plastids, containing clusters of starch grains, were also present in the cytoplasm. Nevertheless, in some of the cells, the marginal cytoplasm had formed invaginations, although the plasmalemma appeared to be intact (Fig. 6.23). This aberration was apparent only in limited regions of a few cells and was not observed in the controls. Figure 6.24 shows an apical cell from a seed germinated in 700 mg Ni/litre. Extensive condensed protein deposits were present in small vacuoles and

no large central vacuoles had formed. In addition, only a few, small starch grains were present. Nucleoli showed small electron-translucent areas and frequently exhibited a diffuse boundary.

6.3.2.2 Cotyledon cells

Forty-eight hours after the initiation of imbibition, the cotyledon cells of control seeds were still very densely packed with storage reserves and there was a complete absence of vacuoles in the tissues examined (Fig. 6.25). Protein bodies were frequently no longer rounded in shape and sometimes exhibited diffuse boundaries. In some cells, protein bodies contained many small black inclusions (Fig. 6. 26), whereas in others these were largely absent (Fig. 6.25). Although there was little evidence of storage reserve mobilization, starch grains, which had been absent at maturity, were once again common in the cytoplasm.

Few differences were apparent between the cotyledon cells of metal-germinated seedlings and those of controls. Furthermore, aberrations were apparent only at the highest metal concentrations. Cadmium appeared to exert little effect on cell ultra-structure apart from at very high concentrations when possible inhibition of storage reserve mobilization was observed. This was indicated by the fact that no protein bodies with diffuse boundaries were observed and starch grains were rare.

In the case of Ni, starch grain formation, as well as protein catalysis, appeared to be reduced. Preliminary studies showed that ultra-structural malformations were also possibly present in the nuclei (Fig. 6.27), where electron-translucent areas were visible in the nucleolus. Control nuclei on the other hand, did not contain electron-translucent areas. Confirmation of these phenomena was not possible however, since very few nuclei could be found in any cotyledonary tissues.

Fig. 6.1

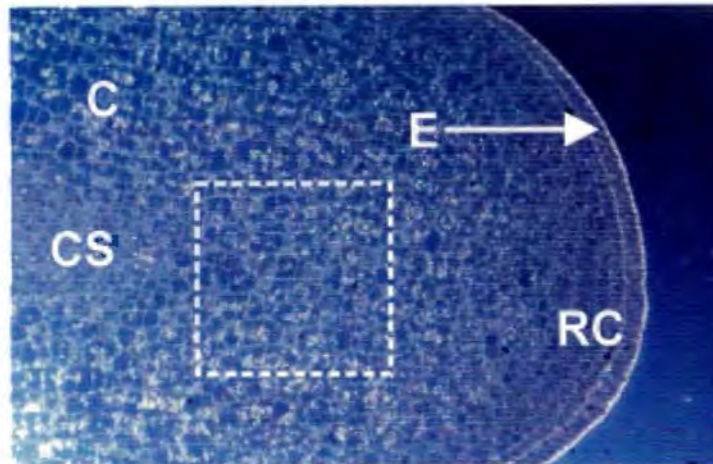


***Fig. 6.1** LM image of the radicle tip of control IP seed showing the general anatomy as well as numerous white starch grains. The area examined using TEM is demarcated. (Mag. X 16. Stain = Sudan Black B). C = cortex, CS = central stele, E = epidermis, RC = root cap.*

Fig. 6.2

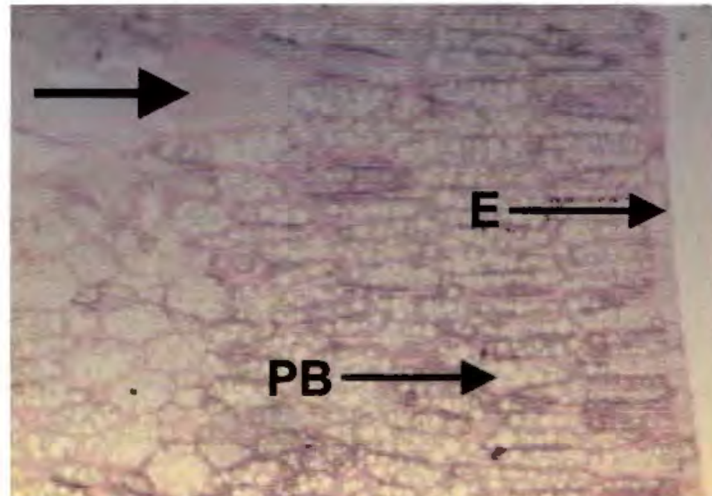


Fig. 6.3

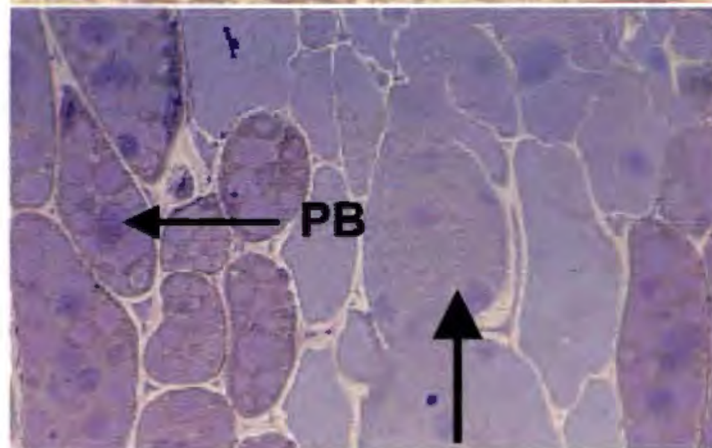
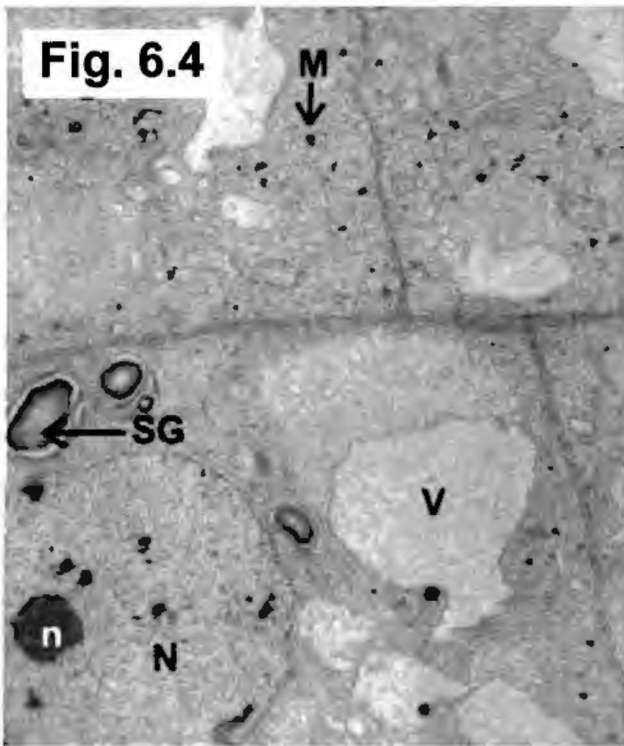


***Fig. 6.2 and 6.3** LM images of control YP seed cotyledon cells showing the distribution of storage reserves. Protein bodies appear unstained (white) in the first figure and violet in the second. Some areas of tissue (indicated by arrow) appear to contain few protein bodies. (**Fig. 6.2**; Mag. X 16. Stain = PAS. **Fig. 6.3**; Mag. X 40. Stain = CCB). E = epidermis, PB = protein body.*



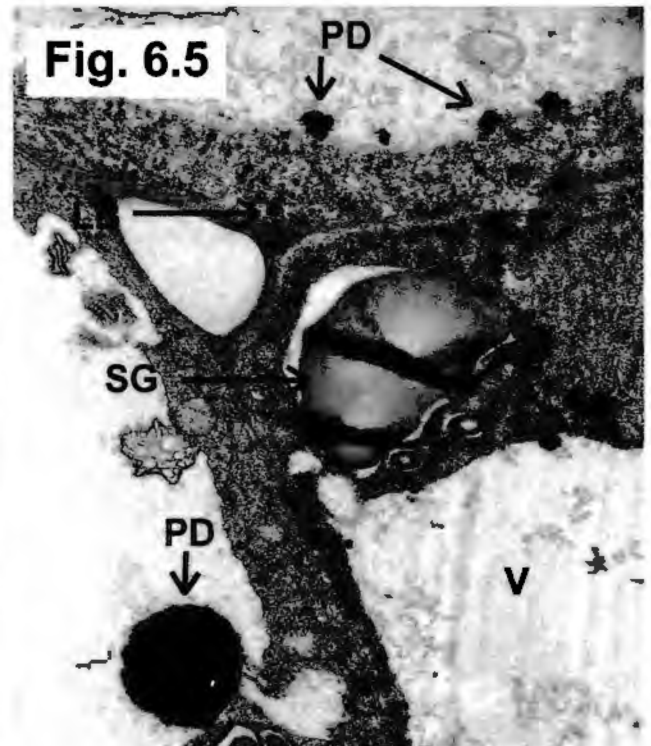
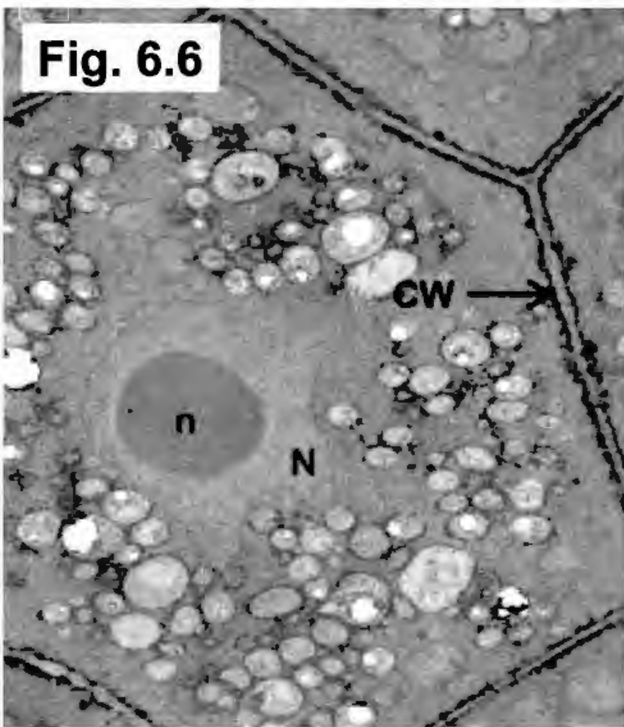


Fig. 6.4* and *Fig. 6.5 *TEM micrographs of radicle apex cells from IP control-treatment seed.* ***Fig. 6.4*** *Thin-walled cells can be seen as well as several vacuoles. A large nucleus with a single nucleolus and areas of condensed chromatin is visible in the cell on left-hand side. Starch grains and mitochondria are also present in the cytoplasm. (Mag. X 5 900).* ***Fig. 6.5*** *Protein has been deposited on the inside of the tonoplast of the vacuole. Note the large, round protein deposit in the bottom left-hand corner. (Mag. X 26 600). LB = lipid body, M = mitochondrion, N = nucleus, n = nucleolus, PD = protein deposit, SG = starch grain, V = vacuole.*



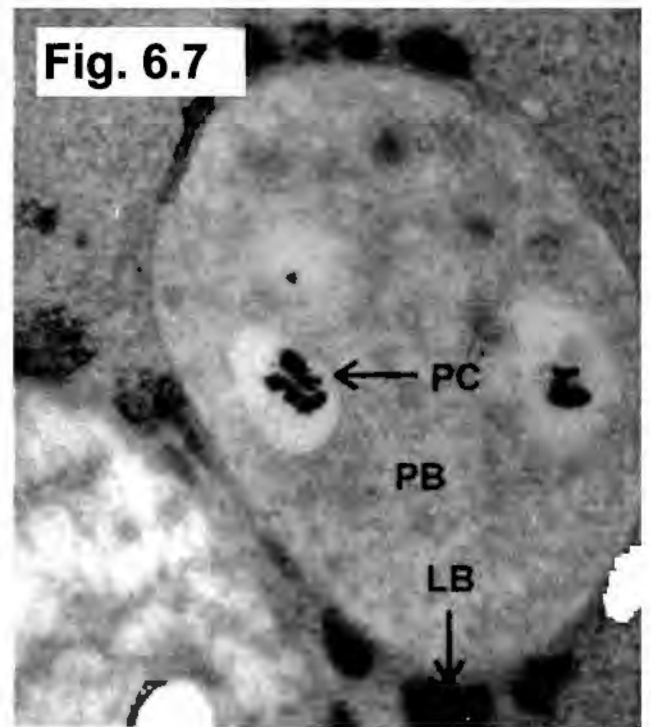
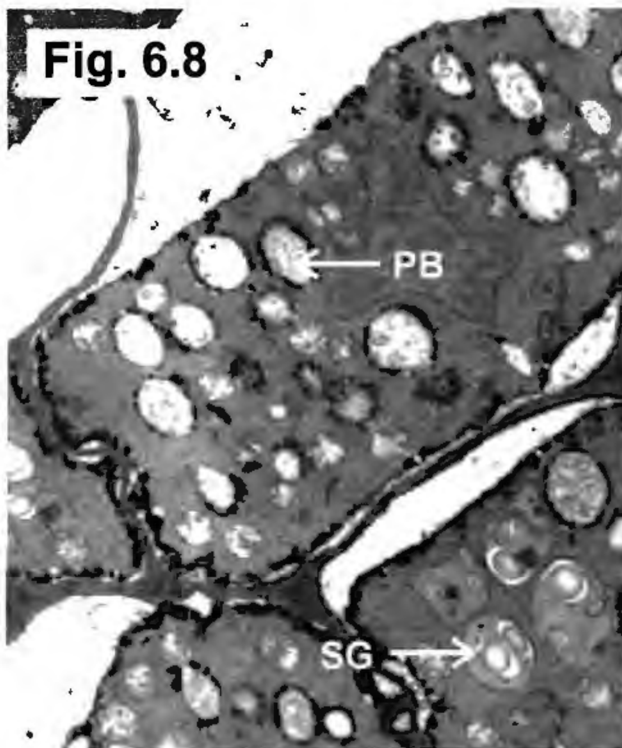


Fig. 6.6* and *Fig. 6.7 *TEM micrographs of radicle apex cells from BP control-treatment seed.* ***Fig. 6.6*** *Numerous small dark lipid bodies can be seen lining the cell walls and surrounding protein bodies. A "halo" of more electron-translucent nucleoplasm around the nucleolus is present. Protein and various inclusions are visible inside the protein bodies. (Mag. X 4 400).* ***Fig. 6.7*** *Phytate crystals within globoid inclusions of a protein body. Note, the left inclusion is possibly surrounded by a limiting membrane. (Mag. X 5 900). CW = cell wall, LB = lipid body, N = nucleus, n = nucleolus, PB = protein body, PC = phytate crystals.*



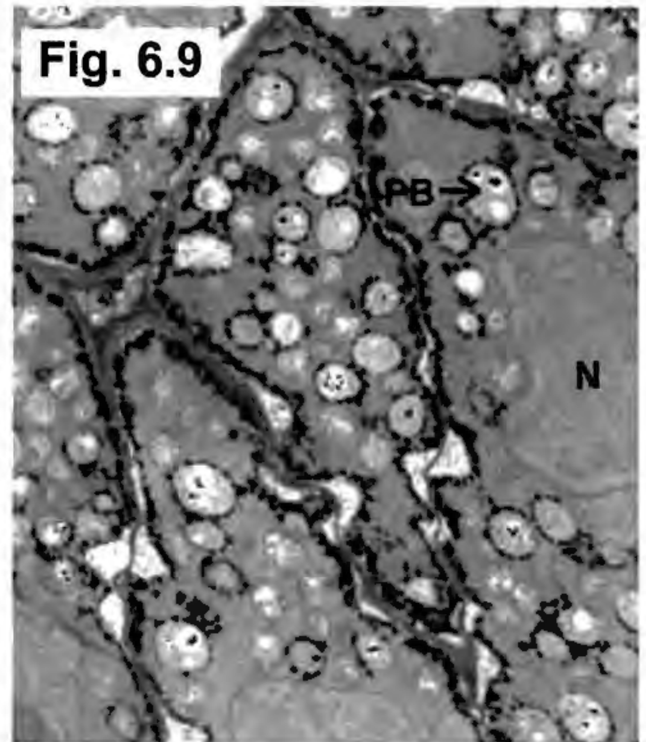
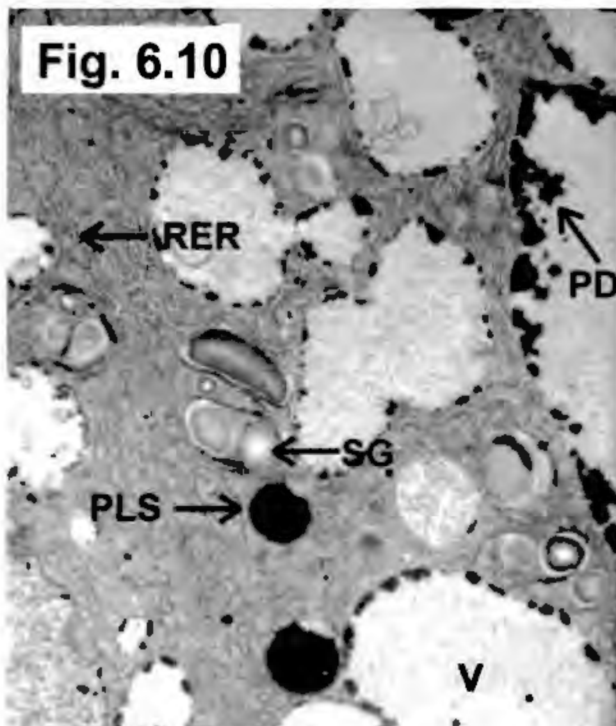


Fig. 6.8 and 6.9 TEM micrographs of radicle apex cells from BP Ni-treatment seed. **Fig. 6.8** Note the starch grains present in plastids. (Mag. X 5 800). **Fig. 6.9** Crystals are especially numerous within protein body inclusions. (Mag. X 5 900). N = nucleus, PB = protein body, SG = starch grain.



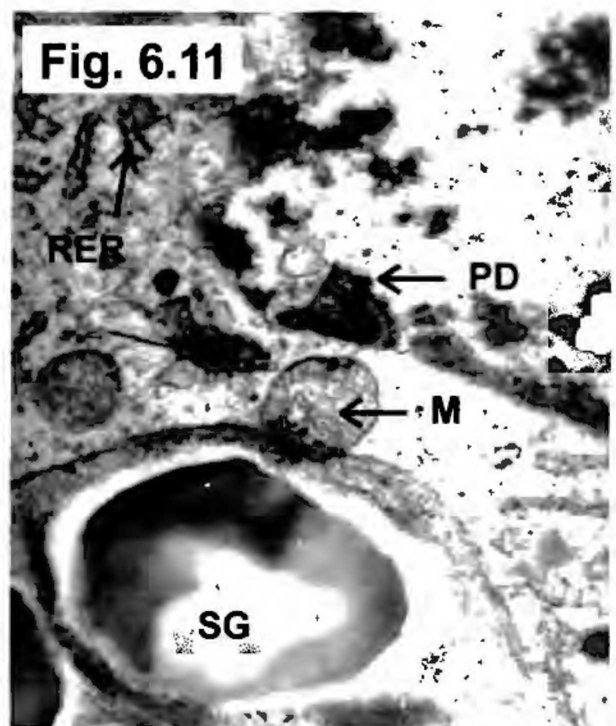
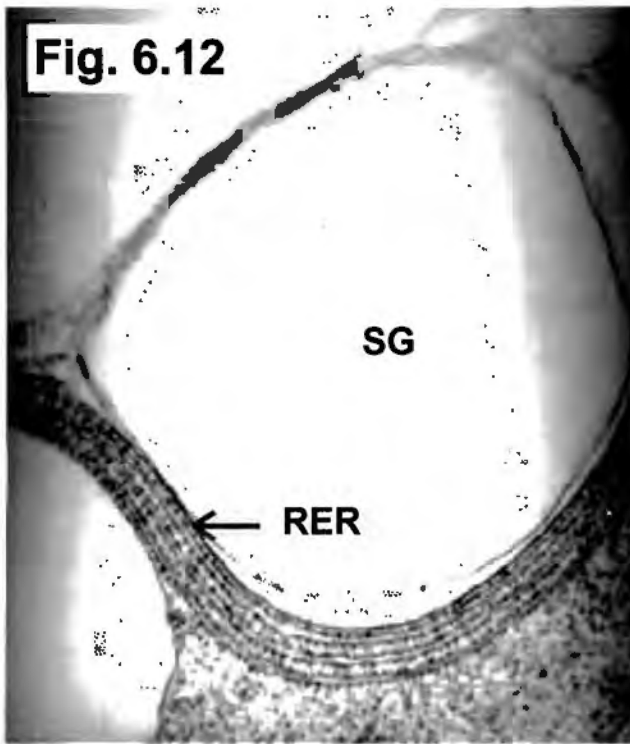


Fig. 6.10 and 6.11 TEM micrographs of cotyledon cells from IP control seeds. **Fig. 6.10** Note deposit of electron opaque protein on inner tonoplast surface as well as PLS bodies. (Mag. X 9 600) . **Fig. 6.11** Extensive deposition of storage protein is visible as well as mitochondria and RER. (Mag. X 26 600). M = mitochondrion, PB = protein body, PLS = PLS body, RER = rough endoplasmic reticulum, SG = starch grain, V = vacuole.



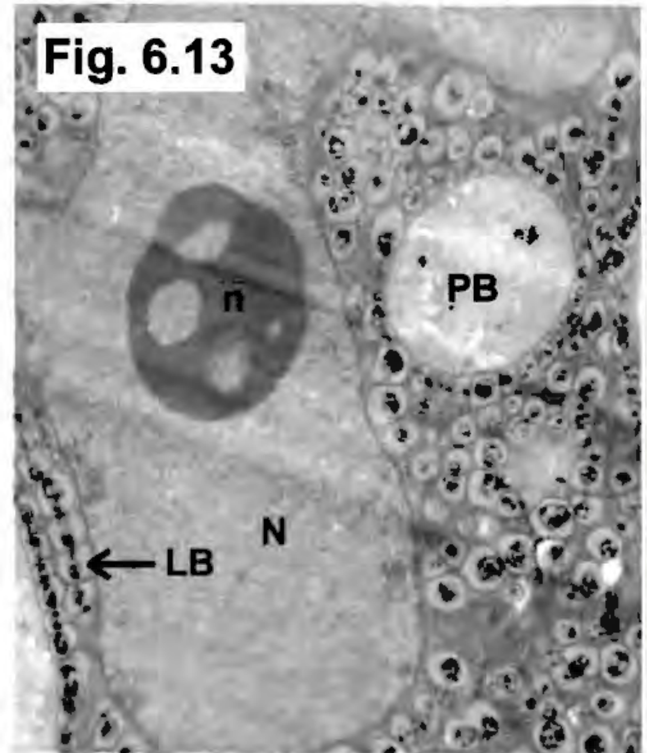
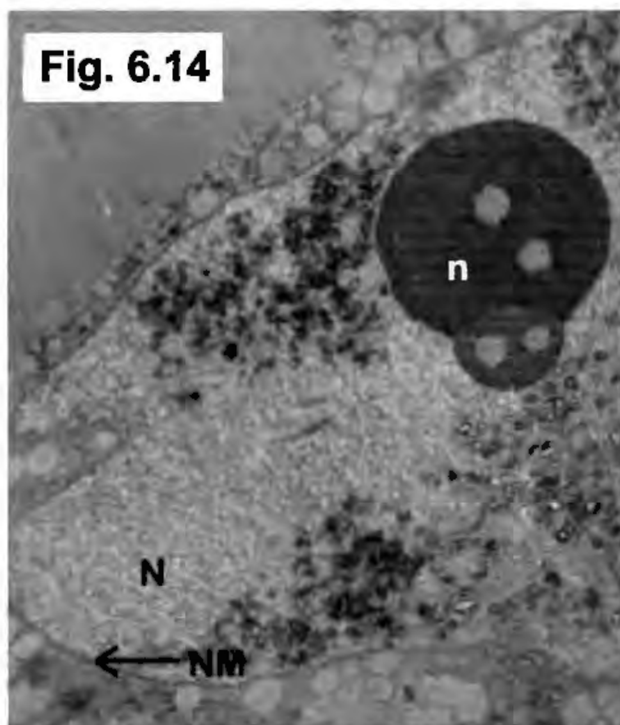


Fig. 6.12 TEM micrograph of cotyledon cell from IP control seed showing extensive RER. (Mag. X 26 000). SG = starch grain, RER = rough endoplasmic reticulum.

Fig. 6.13 TEM micrograph of cotyledon cell from a YP control seed. A large nucleus is shown, as well as numerous lipid bodies containing inclusions aligned in the same direction. A protein body is also indicated. (Mag. X 9 600). LB = lipid body, N = nucleus, n = nucleolus, PB = protein body.



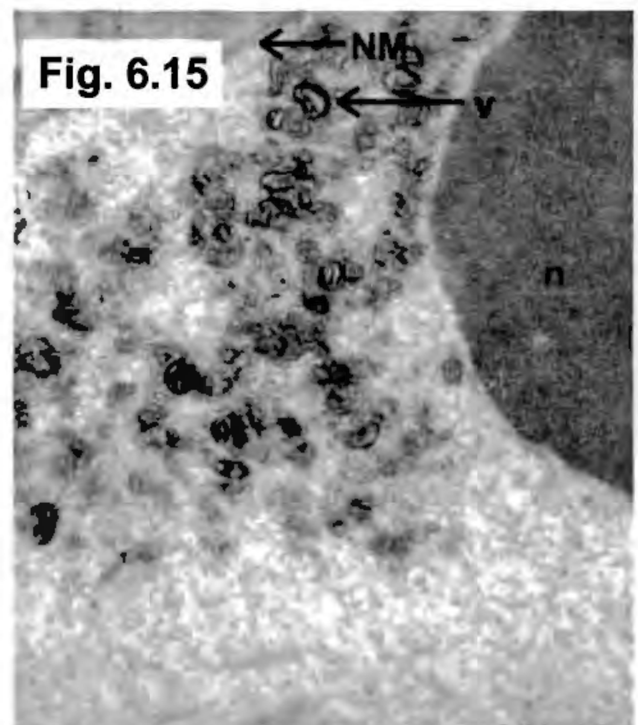
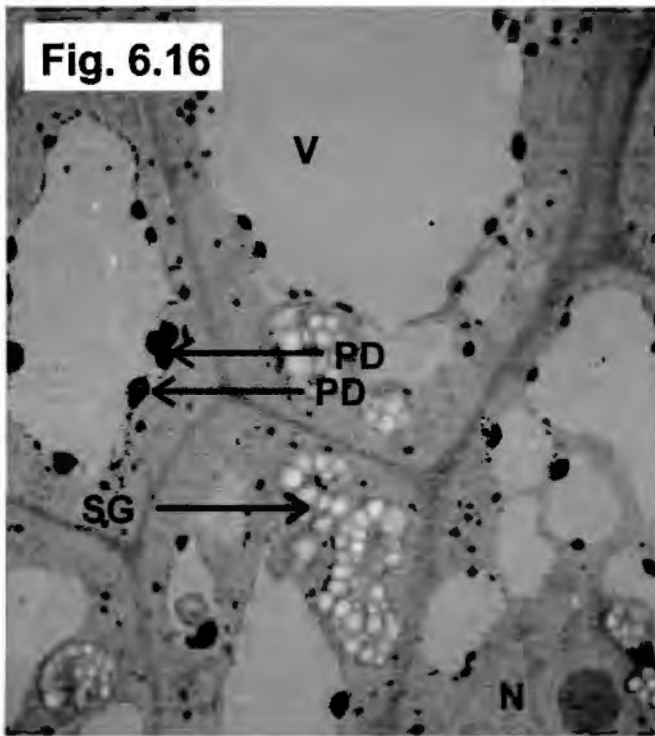


Fig. 6.14 and **Fig. 6.15** TEM micrographs of the nucleus of a Cd-treatment YP cotyledon cell. **Fig. 6.14** Dark vesicles in the nucleoplasm. (Mag. X 9 800). **Fig 6.15** The same vesicles at a higher magnification. Note the organised structure. (Mag. X 26 600). N = nucleus, NM = nuclear membrane, n = nucleolus, v = vesicle.



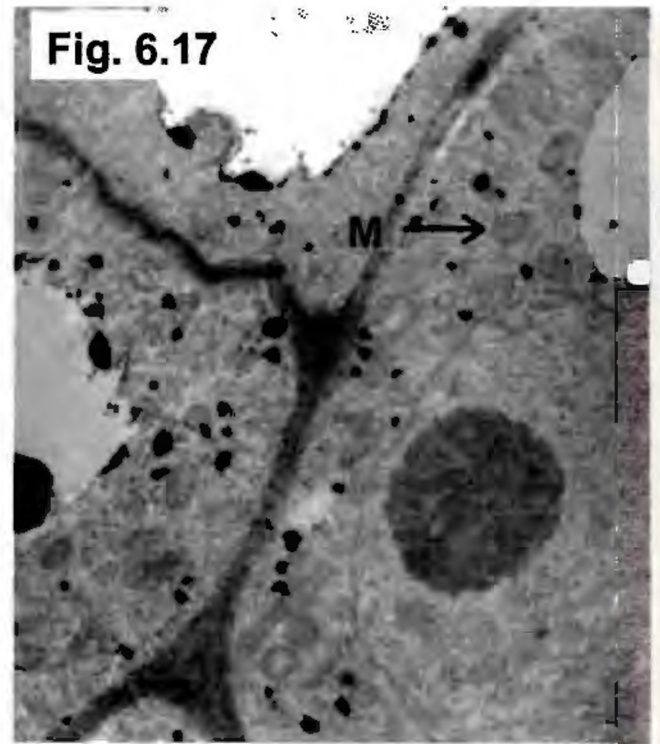
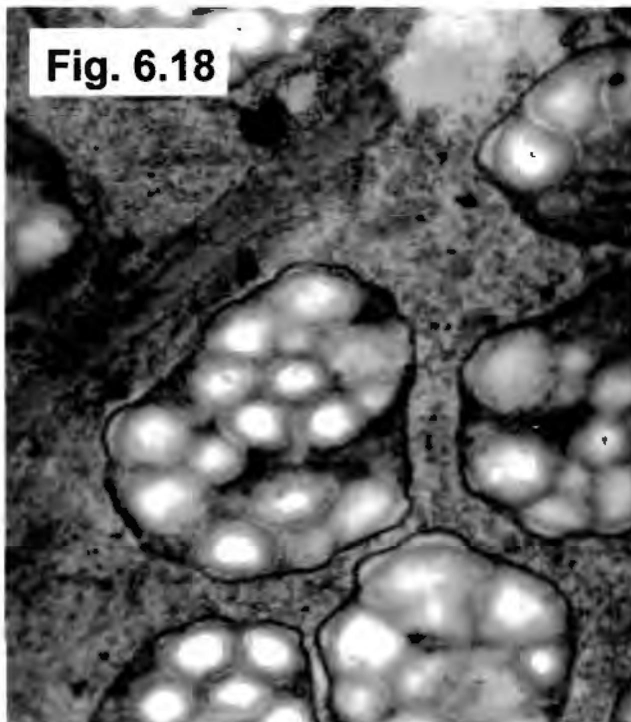


Fig. 6.16 and 6.17 TEM micrographs of radicle apex cells from control seedling, 48 hr after imbibition. In some cells the protein bodies have coalesced to form large, central vacuoles containing dark, condensed protein deposits. Groups of starch grains are visible in some cells. (Mag. X 5 100 and x 9 000 respectively). M = mitochondrion, N = nucleus, PD = protein deposit, SG = starch grain, V = vacuole.



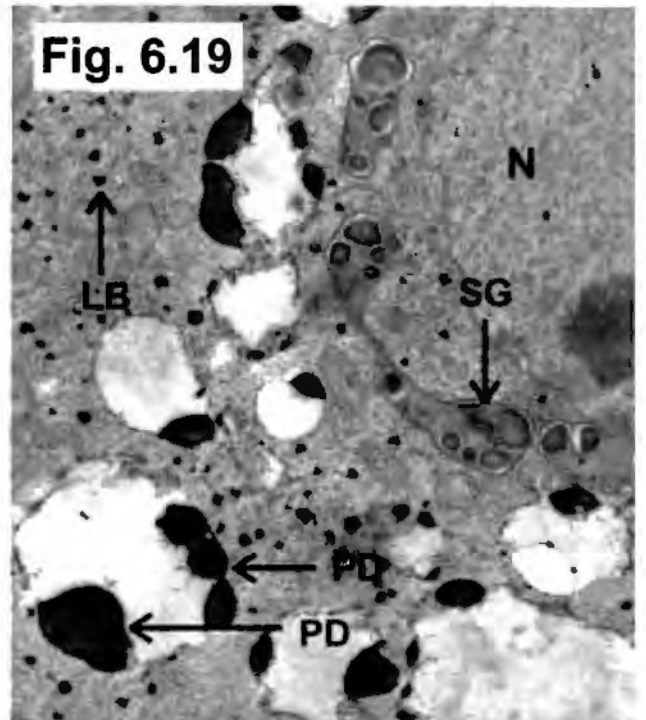
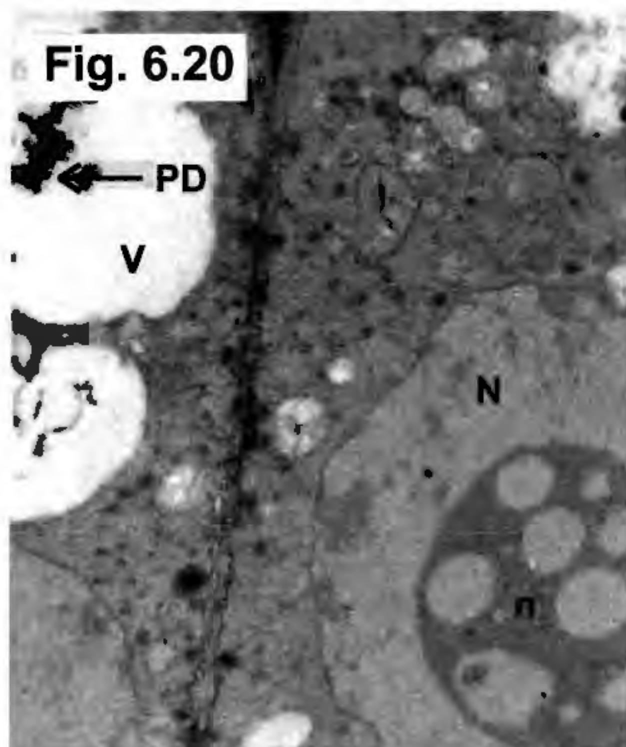


Fig. 6.18 TEM micrograph of radicle apex cells from control seedling showing the numerous small starch grains. (Mag. X 9 400). SG = starch grain.

Fig. 6.19 TEM micrograph of radicle apex cells from seedling germinated in 3 mg Cd/litre, 48 hr after imbibition. Note the reduced size of starch grains compared to **Fig. 6.18**. (Mag. X 9 700). LB = lipid body, N = nucleus, PD = protein deposit, SG = starch grain.



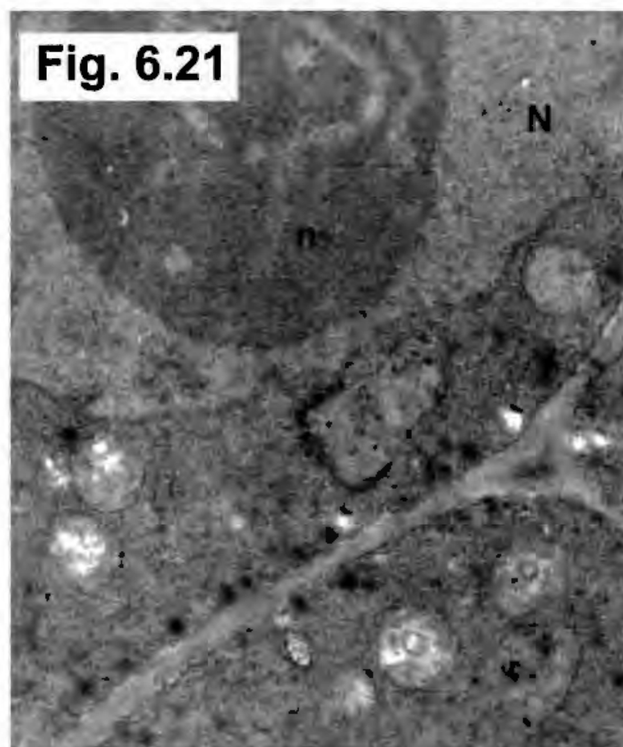
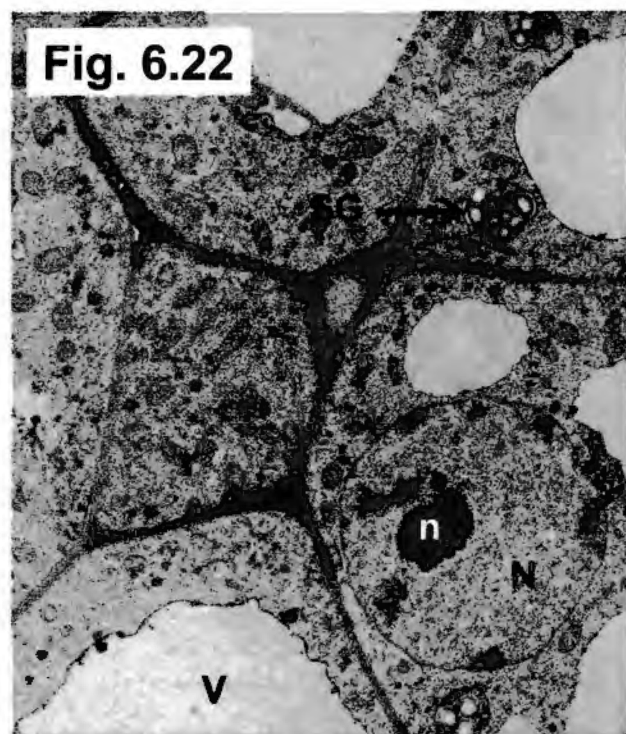


Fig. 6.20 and Fig. 6.21 TEM micrographs of radicle apex cells from seedling germinated in 225 mg Cd/litre, 48 hr after imbibition. **Fig. 6.20** Vacuole containing protein deposits. A nucleus is also present containing extensive rounded electron-translucent areas in the nucleolus. (Mag. X 9 700). **Fig. 6.21** Elongated electron-translucent areas in the nucleolus of another cell. (Mag. X 16 000). N = nucleus, n =nucleolus, V = vacuole, PD = protein deposit,.



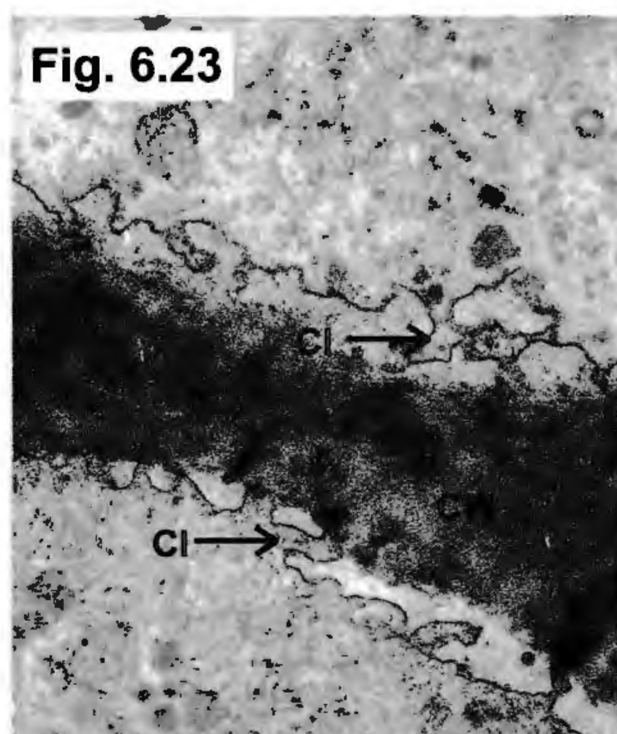
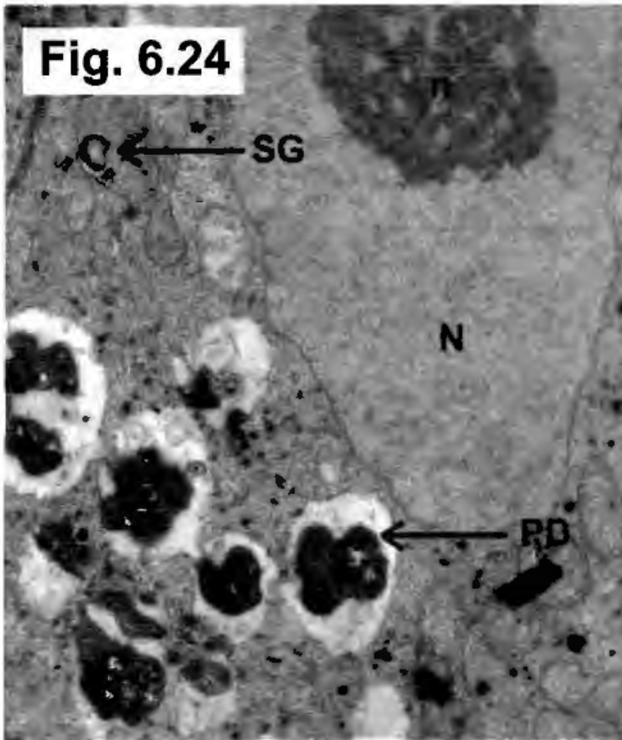


Fig. 6.22 and Fig. 6.23 TEM micrographs of radicle apex cells from seedling germinated in 50 mg Ni/litre, 48 hr after imbibition. **Fig. 6.22** General view, starch grains visible. (Mag. X 5 800). **Fig. 6.23** Cytoplasmic invaginations sometimes exhibited by these cells. (Mag. X 16 000). CI = Cytoplasmic invagination, CW = cell wall, N = nucleus, n = nucleolus, SG = starch grains, V = vacuole.



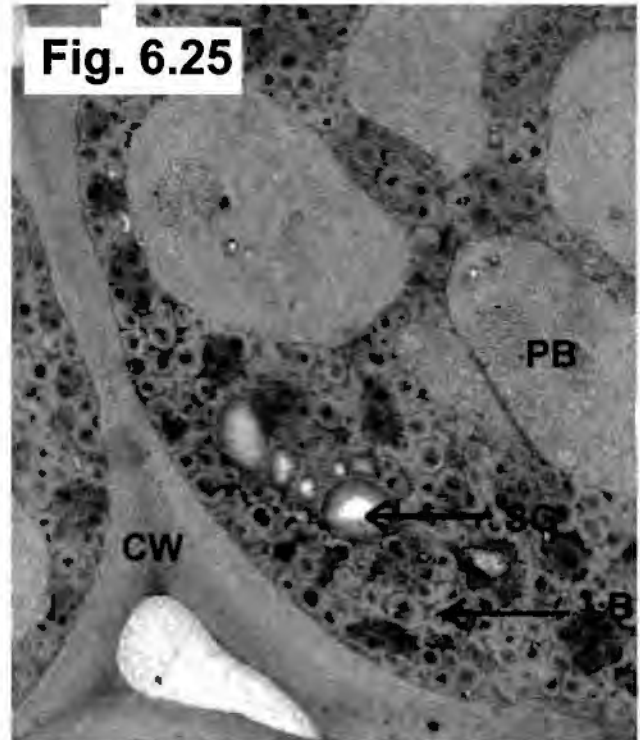
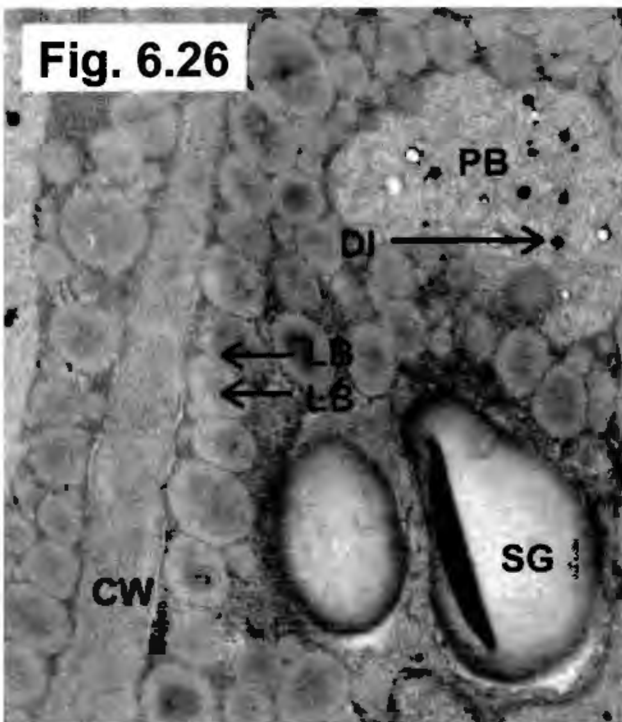


Fig. 6.24. TEM micrograph of radicle apex cell from seedling germinated in 700 mg/litre Ni solution, 48 hr after imbibition. Structural aberrations are visible in the nucleolus. No large vacuoles are present and protein is in a condensed form. (Mag. X 9 600). N = nucleus, n = nucleolus, PD = protein deposit, SG = starch grain.

Fig. 6.21 TEM micrograph of cotyledon cells from control seedling, 48 hr after imbibition. Storage reserves in the form of starch grains, protein bodies and lipid bodies are present. (Mag. X 5 900). CW = cell wall, LB = lipid body, PB = protein body, SG = starch grain.



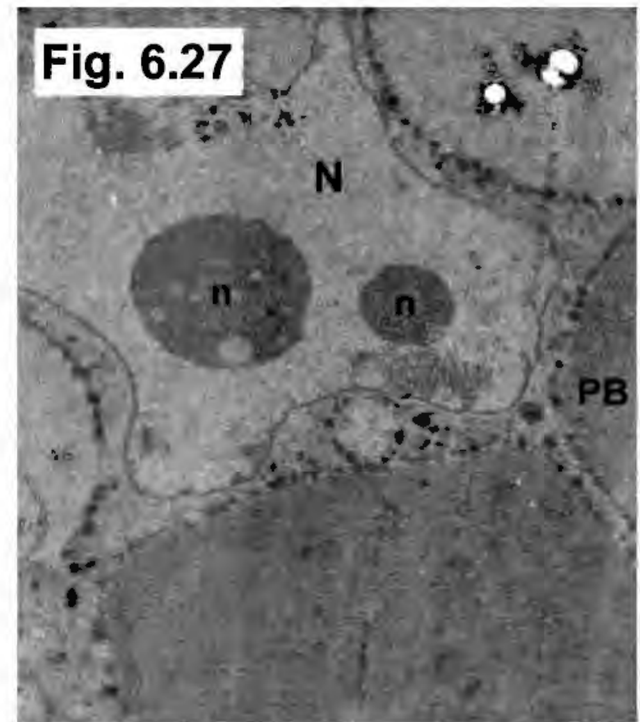


Fig. 6.26 TEM micrograph of cotyledon cells from control seedling, 48 hr after imbibition. Distinctive inclusions are visible in the protein bodies of some cells but (see **Fig. 6.25**) not others. (Mag. X 16 000). CW = cell wall, DI = distinctive inclusion, LB = lipid body, PB = protein body, SG = starch grain.

Fig. 6.27 TEM micrograph of cotyledon cells from seedlings, 48 hr after imbibition in 700 mg/litre Ni solution. Note the electron-translucent areas in the nucleolus. (Mag. X 9 600). N = nucleus, n = nucleolus, PB = protein body.

6.4 DISCUSSION

Development of control "Crawford" seeds appears to be in line with that described for other cultivars of this species. Fragmentation of the central vacuole is an early event and although not complete in all the cotyledon cells of IP seeds, is well underway by this growth stage. We agree with the findings of Saio *et al.* (1985) that protein is deposited on the inner surface of the large central vacuole. Furthermore, our findings support those of Adams *et al.* (1985); namely, that protein is also deposited within the small vacuoles. Although some limited deposition of protein did occur before fragmentation, the prime mechanism for the bulk of protein deposition appeared to be via the small vacuoles after fragmentation of the central organelle. This is a finding similar to that of Yoo and Chrispeels (1980). Whilst considering the topic of protein body formation, to avoid confusion, perhaps a brief comment should be made concerning the PLS bodies described by Saio *et al.* (1985) and also noted in this study. These organelles were termed PLS bodies by the above authors, as they stain positively for protein, lipid and carbohydrate (sugar). They are present early during development of cotyledon cells (Fig. 6.10) but gradually become indistinguishable from the protein bodies derived from vacuoles. According to Monma *et al.* (1992), PLS bodies have not been observed in developing axes, this is possibly attributable to the fact that the storage protein glycinin, a major constituent of the PLS bodies is not found in soybean axes. The role of PLS bodies is obscure but may be involved with shifts in the type of storage protein synthesized (Kondo *et al.* 1986). The above description of the ontogeny of PLS bodies is consistent with the observations made in this study, with the exception that Saio *et al.* (1985) reported the appearance of PLS bodies only after 20-25 DAF, whereas in this study they were already visible in IP cotyledon cells (± 15 DAF).

Two ultra-structural features noted in this study were not found described in the literature. The first feature was the "halo" of electron-translucent nucleoplasm around the nucleoli of mature radicle tip cells, the second, the inclusions frequently observed within lipid bodies. This second feature was notable in that the inclusions were frequently aligned in the same direction (Fig. 6.13). Whilst such phenomena

may possibly be artifacts arising from the preparation and staining methods used, they were observed consistently, even when seed samples were processed on differing occasions. Although worthy of research, since these features were recorded in both control and metal-treatment tissues they were not investigated further.

In general, the presence of metal pollutants in developing seeds appeared to have little definite effect on the ultra-structure of the tissues examined. This is consistent with the limited effects observed on storage reserve accumulation, membrane integrity as well as germination. In the light of the low concentration of Cd found in Cd-treatment seeds (1.5 μg/g.dm in the cotyledons and 0.04 μg/g.dm in the axis), this is perhaps understandable. It is less understandable if the accumulation of Ni in the radicle tip is considered (section 4.3.2). Concentrations of up to 100 μg Ni/g.dm were demonstrated to be present in the radicle tip of Ni-treatment seeds and the fact that no obvious ultra-structural damage was apparent emphasizes the low toxicity of this metal. In this regard, the possible occurrence of increased phytate deposits in the protein bodies of Ni-treatment seeds is of interest. Deposition of Ni within the globoid inclusions of protein bodies could conceivably be a possible tolerance mechanism and the reason for the lack of ultra-structural damage. As discussed in section 4.4, studies by Spitzer *et al.* (1980) and (1981) demonstrated that toxic metals, including Ni and Cd, did not accumulate in the globoid crystals of wheat, *Lycopersicon* or *Capsella*. Nevertheless, this requires further investigation, since seed species have been found to differ greatly in the mineral content of protein body inclusions (Lott *et al.* 1995) and Al has been reported to accumulate within these organelles in some seeds (Egerton-Warburton *et al.* 1995).

Examination of radicle apex tissues from Ni-treatment seeds suggested that starch grains, although not counted, were more numerous in these cells than those of the controls. No difference was noted between control and Ni-treatment cotyledon cells however. In Chapter 3, it was reported that Ni-treatment seeds had a similar starch content to that of their control counterparts. This represents the bulk seed content however and is largely dependent on the starch content of the cotyledons, since

these represent the major portion by mass. Re-distribution of carbohydrate could be a possible mechanism whereby the frequency of starch grains was increased in the axis compared to the cotyledons. Altered carbohydrate metabolism as a response to Ni toxicity has been reported in the literature. Rauser (1978) found that excess Ni, but not Cd, caused starch to accumulate in the unifoliate leaves of *Phaseolus vulgaris* seedlings. L'Huillier *et al.* (1996) made similar findings in a non-tolerant strain of maize grown in Ni-amended nutrient solution. He reported that large amounts of starch accumulated in the chloroplasts of bundle sheath cells. Furthermore, statoliths were absent from the root cap cells of the same plants. These findings led the above authors to postulate that transport of carbohydrate between root and leaves was impaired in these plants. The entire question of altered carbohydrate metabolism in seeds, as a response to Ni-phytotoxicity requires further investigation.

Cadmium-containing electron-dense granules, were found by Rauser and Ackerley (1987) in the nuclei of undifferentiated cells of *Agrostis* and maize roots. In the present study, condensed material was observed in the nucleoplasm of cotyledon cells from all treatment groups. Only in the nuclei from cotyledons of Cd-treatment seeds, did the condensed material appear to have a definite structure however. The appearance of the granules in the work referred to above, was very different compared to the vesicles observed in this project. Whereas those in the other study appeared as a dark, round deposits, the vesicles found in this study exhibited an organised ultra-structure. It is possible that such vesicles are a feature of a particular stage of the cell cycle, rather than an effect caused by Cd *per se*. This is another aspect that requires further investigation.

With the possible exception of the nucleoplasmic vesicles mentioned above, no absolute differences were noted between control and metal-treatment seeds, although as discussed above, possible quantitative differences were apparent. Detailed developmental studies are needed to confirm that Ni-treatment promoted phytate deposition and starch grain formation. Furthermore, chemical analyses are also required to confirm that the crystalloid inclusions in protein bodies are

composed of phytate. Investigation of these aspects is complicated by several factors. Firstly, seed development is a continuous process and hence it is essential to compare treatment and control tissues at exactly the same developmental stage. This is especially important when considering the effect of Ni on starch accumulation, which under natural conditions, first increases and then decreases again. Classification of seeds using the growth stages employed in this study was possibly too course for detailed ontological studies and may explain some of the inconsistencies. Secondly, quantitative studies are required in which the above features (starch grains, putative phytate crystals and nucleoplasm vesicles) are counted and compared in a large number of treatment and control samples. Thirdly, the radicle apex is an area of complex anatomy. Cells close together have very different developmental destinies depending on whether they eventually differentiate to become root cap, cortex, or cells of the vascular system. Although great care was taken during selection of the area for TEM, it is possible that variations in ultra-structural features may well be due to observations on the different cell types. For example, some of the Ni-treatment radicle apex cells in which increased starch and globoid inclusions were seen appeared to exhibit a different shape to those of the control (Figs. 6.8 and 6.9). The most likely reason for this is that the sections were cut tangentially rather than parallel to the long axis of the root. The possibility that for example, developing vascular tissue or cortical tissue was observed, rather than undifferentiated cells, cannot be ruled out however. Finally, investigations into the effect of metal-pollutants on the nuclei of mature cotyledon cells were hampered by the fact that due to the profusion of storage reserves, very few of these organelles were visible. Owing to time constraints, the extensive studies required to confirm cellular aberrations in metal-pollutant seeds were not undertaken, but do deserve further investigation.

As discussed in the previous chapter, the "metabolic direction" of germinating seeds is opposite to that of developing seeds, i.e. directed towards storage reserve catabolism rather than accumulation. This was reflected in the ultra-structure of control seeds, 48 hr after the initiation of imbibition. In both radicle apex cells and cotyledon cells, break down of protein was evident and starch grains were present

once more in the cytoplasm. According to Webster and Leopold (1977) starch grains were observed in the cotyledons of germinating soybean seeds within 20 min of imbibition. As in the case of developing soybean seeds (section 1.4.3.3), starch represents a temporary storage phase of carbon, which in germinating seeds, originates from the break-down of protein and lipid reserves (Adams, Broman and Rinne 1981; Briarty and Pearce 1982).

In comparison to observations on metal-treatment seeds, cells from the radicle tips of seeds germinated in solutions of the two pollutants did show ultra-structural changes. In the case of both metals, the extent of these malformations was roughly proportional to the concentration employed. One of the primary effects exerted by Cd appeared to be inhibition of both protein catabolism as well as its subsequent conversion to starch. Whilst in seeds germinated in dilute Cd-containing solutions, the number of starch grains appeared to be slightly reduced (Fig. 6.19), this effect was very noticeable in the 225 mg Cd/litre (highest) treatment. Furthermore, protein deposits in the highest Cd treatment were diffuse compared to control cells and the fusion of small vacuoles to form large organelles appeared to be impaired. No ultra-structural alterations were found in the apical cells of seeds germinated in solutions containing low concentrations (3 mg/litre) of Ni. At levels of 50 mg Ni/litre, minor disruption of the nucleoli was observed and the matrix appeared to be more diffuse. Although such dissipation of the chromatin of the nucleoli was a common symptom of Ni toxicity, this was never as severe as in Cd-germinated seeds. Furthermore, it was only at the highest levels of Ni (700 mg/litre) that inhibitory effects on proteolysis were noted. Thus once again, Cd appeared to be more phytotoxic than Ni. Germination however, like seed development, is a continuum of events and it is essential to compare seeds at exactly the same stage. Differential rates of water uptake may have caused seeds to be at different stages of the germination process and thus quantitative studies of starch grain formation and proteolysis are required.

Distinctive regions of cytoplasmic invagination were exhibited by some radicle apex cells as a response to germination in Ni (Fig. 6.23). A perusal of the relevant literature yielded no comparable reports. Corradi *et al.* (1993) noted that immersion

of *Salvia sclarea* seedling roots in Cr solution caused plasmolysis of cotyledon cells. Although it is conceivable that this effect is due to plasmolysis, the appearance of the peripheral cytoplasm is not entirely consistent with such a hypothesis. Furthermore, the appearance of the plasmalemma appeared normal and no discontinuities were observed. Germination in Cd-solutions did not appear to elicit a similar effect and no aberrations of the plasmalemma or peripheral cytoplasm were observed in samples from this treatment group.

The principal toxic effects of Cd and Ni on germinating seeds appear to be inhibition of proteolysis and impairment of nuclear functioning. Both processes are vital for normal seedling development and growth. Ultra-structural aberrations in the nuclei of radicle apex cells were elicited by both Cd and Ni. The presence of translucent areas in the nucleolus may indicate malfunctioning of rRNA formation and although not examined directly, it is likely that cell division may be inhibited. Such nuclear aberrations were most obvious in the high concentration treatments and indeed, seedlings imbibed in concentrations of 225 mg Cd/litre or higher than 125 mg Ni/litre did not survive for longer than three weeks, even if transferred to standard nutrient solution (Tables 5.3 and 5.4). Inhibition of proteolysis was also observed in apical cells of seeds germinated in the metal pollutants. The cotyledons on the other hand, the major storage area of accumulated reserves appeared to be much less sensitive to the presence of metal pollutants. Changes at the ultra-structural scale were noted only at the very highest concentrations and were consistent with an inhibition of storage reserve mobilization. It is likely that impairment of nuclear functioning within the cells of the radicle tip is potentially a more serious threat to seed germination, than inhibition of storage reserve mobilization. Whilst the presence of structural abnormalities in various tissues may give clues to possible effects of the metal pollutants on seed functioning, results from these findings should be interpreted with caution however. As mentioned previously, such aberrations may be either the consequence or cause of metabolic malfunction (Barcelo *et al.* 1988), making the identification of the primary toxicity mechanism difficult.

CHAPTER 7

CHEMICAL SPECIATION MODELLING OF THE NUTRIENT SOLUTION

7.1 INTRODUCTION

In previous chapters of this work, quantification and localization of the metal content of seeds, in regard to the metal pollutants Cd and Ni, as well as in regard to the nutritionally important metals, Fe, Mn, Zn and Mg was examined. With the possible exception of trace quantities of minerals that may have originated from the parent seed (Hewitt 1966), all of these elements entered the plant from the growth medium in which the plants were cultivated. Thus the chemical composition of the nutrient solution in which the roots were immersed is critical in determining the ultimate chemical composition of the various plant organs, including seeds. As discussed in Chapter 1, it is now well known that for a given element in a biological system, the nature of the chemical species in which that element occurs, in addition to the total analytical concentration, is of prime importance. In the case of plant growth media (soil, nutrient solution etc.), plant uptake is positively correlated with the proportion of a given element in the bioavailable form. In this section of the study, chemical speciation of the nutrient solution under different conditions will be examined, in order to ascertain if this could explain the observed changes in seed metal content.

The speciation of a particular chemical element in a given system can be defined as the identity and abundance of each and every physico-chemical form in which that element occurs (Murray and May 1990). Because many chemical species occur in only trace amounts, quantification is difficult and most analytical techniques (atomic absorption spectroscopy, ICP-AES) determine total elemental content (Phipps 1981). Relatively few methods (e.g. anodic-stripping-voltammetry, polarography) are able to distinguish between closely related chemical species (Henze 1989; Wallman, Petersen and Pinglin

1989). At present, computational equilibrium modelling is capable of producing more detailed speciation patterns than experimental techniques (Duffield, Marsicano and Williams 1991). Furthermore, in many cases experimental analysis can result in shifts in the chemical equilibria, changing the speciation of the solution under consideration without the researcher being aware of this (Murray and May 1990). It is only with the advent of commercially available computer programs within the last decade or so, that multi-element speciation studies of complex aqueous equilibria have become relatively common. This has resulted in a wide range of aqueous systems being modelled including natural water bodies (rivers, sea, lakes), urine, milk, xylem exudates, soil solutions and plant nutrient solutions (Little 1984; Chaney 1991; Morgan and Stumm 1991). In addition to being a powerful tool for modelling complex systems that contain many trace species, computational speciation is also useful for making predictions of responses to changes in various system parameters such as pH, ionic strength or addition of other components (Murray and May 1990). The technique does however suffer from several limitations. Firstly, it is dependent on the use of accurate stability constants, a factor that can be especially critical for trace components (Phipps 1981). A comprehensive database of experimentally determined thermodynamic constants is under continual revision (NIST 1993). Secondly, the method can only compute concentrations of species defined either directly by the user, or indirectly by the software package. Thirdly, speciation models assume that the chemical system is at equilibrium. According to Murray and May (1990) although most reactions between chemical species in aqueous solutions establish equilibrium within milliseconds, reactions involving solids can take up to hundreds of years. Knowledge of the kinetics of the reactions under consideration is also required therefore.

Computational speciation studies have been carried out on plant nutrient solutions using a range of software packages including MINEQL (Voye′ 1985), the fore-runner of MINTEQA2, as well as ECCLES, NUTRIENT and GEOCHEM (Laurie, Tancock, McGrath *et al.* 1991 a and b; Parker, Chaney and Norvell 1995). Aluminum toxicity in soybeans has been investigated using nutrient solutions and computational speciation (Noble, Fey and Sumner 1988). Other work concerned with plant uptake and chemical speciation in nutrient solutions include, a study of the effect of pH on Cu uptake in citrus

(Zhu and Alva 1993) and another, investigating the effect of Cl on Cd uptake in Swiss chard (Smolders and McLaughlin 1996). Especial interest in the literature has been directed towards the question of metal chelation and plant uptake (Srivastava and Appenroth 1995; Laurie *et al.* 1991a and b). This aspect will be discussed further on in this chapter (section 7.3).

The speciation package used in this study was MINTEQA2 Version 3.11 (Allison, Brown and Novo-Gradac 1991), a geochemical equilibrium model capable of computing equilibria amongst the dissolved, adsorbed, solid and gas phases in an environmental setting. This model was developed by US EPA and has been used extensively {e.g. phosphorus speciation studies in Inanda dam (Pillay, Kerr, Buckley *et al.* 1993); metal toxicity studies on a wide range of organisms (Pretorius 1993)}. The aim of a speciation model is to provide detailed information on the identities of the predominant species under certain conditions and to examine the effect of changing conditions on this speciation (Murray and May 1990). The underlying principles as well as the methods employed in computational speciation modelling have been discussed elsewhere (Lumsdon and Evans 1995; Holm, Christensen, Tjell *et al.* 1995) and will only be considered briefly here. The following discussion is taken largely from Phipps (1981).

The tendency for a metal-ligand interaction to occur is measured by the equilibrium constant for the reaction:

$$x\,\mathbf{M}^{n+} + y\,\mathbf{L}^{m-} \leftrightharpoons [\mathbf{M}_x\mathbf{L}_y]^{(nx-my)}$$

$$K_{stability} = \frac{[\mathbf{M}_x\mathbf{L}_y]^{(nx-my)+}}{[\mathbf{M}^{n+}]^x_{free}\,[\mathbf{L}^{m-}]^y_{free}}$$

Where:

M = free metal ion

L = ligand (any chemical entity binding to the metal)

$\boldsymbol{K}_{stability}$ = equilibrium (stability) constant[1]

x, y = stoichiometric coefficients of the reactants

m, n = appropriate electrical charge numbers

And the terms in brackets refer to the molar equilibrium concentrations.

The above is often called the *mass action equation*. In addition it is possible to define the *conservation equations* (referred to as mass balance equations in Allison *et al.* 1991) such that the total dissolved concentration of a chemical component (M_{total}, L_{total}), is equal to the sum of the concentrations of that component in all its chemical forms. For example, the total dissolved concentration of a metal ($[M^{n+}]_{total}$) is equal to the sum of the concentration of metal present as the free aqueous ion ($[M^{n+}]_{free}$) added to that of the metal in all the other chemical forms present in the system. In a similar fashion the conservation equation can be defined for all the other chemical components.

In a complex solution containing many chemical components, ligands compete with each other for access to the metal ions. Which one actually succeeds in binding the most metal depends on the relative strengths of binding (i.e. the stability or equilibrium constants) as well as the concentration of the components. Computational speciation packages are able to consider all competing equilibria, for all possible chemical reactions, in the specified solution at the same time. In addition, since the stability constants and starting concentrations of the components are known, it is possible to solve the mass action equations and conservation equations simultaneously, in order to calculate the concentrations of each species at equilibrium. In MINTEQA2, this is carried out by means of a least squares optimization algorithm. This package also takes precipitation into account, by computing the saturation index (SI) for each possible solid and adjusting the concentration of dissolved species accordingly (Allison and Brown 1995).

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[1]Whilst it is recognized that strictly speaking, thermodynamic equilibrium constants are applicable when reactant and product activities are given rather than concentration, in the interest of simplicity concentration terms are used here.

It was found in this study that one of the major effects brought about in mature soybean seeds by metal pollutants, was a shift in the content of nutritionally important metals (section 3.3.4.5). The presence of Cd in the nutrient solution decreased seed Fe and Mn levels and increased Zn and Mg compared to control seeds. Addition of Ni to the nutrient solution on the other hand, resulted in lowered levels of all elements except for Zn, which showed a slight increase. Differences in seed metal composition between control and metal-treatment seeds could possibly result from internal changes in metal transport within the plant. Alternatively, this may be a consequence of external changes in the nutrient solution. Addition of $CdCl_2$ or $NiCl_2$ to the nutrient solution may cause changes in the speciation of the relevant elements, altering bioavailability and hence plant uptake. Thus changes in seed metal levels as a response to metal pollutant treatment, may merely reflect shifts in metal availability in the growth medium rather than changes in translocation or element partitioning within the plant.

Monitoring of the nutrient solution in which the plants were grown (section 2.3.3) revealed that during the initial stages of cultivation, pH either decreased slightly or remained constant. Thereafter it always increased, reaching a peak at approximately 9-10 weeks after germination. At this stage, the daily change in pH (ΔpH) was approximately 0.5 pH units. This was attributed to uptake of nitrate ions by the plant with a concomitant efflux of hydroxyl ions to maintain electro-neutrality. Changes in pH have been reported to exert a profound effect on speciation in aqueous systems (Parker *et al.* 1995). Thus bioavailability of the elements of interest in fresh nutrient solution (pH 6.0) as well as at higher pH levels was examined.

In this chapter the following questions concerning nutrient solution speciation will be addressed:

1. What percentage of Cd or Ni added to the nutrient solution is in a bioavailable form:

- **a)** At the concentrations used for routine seed production (i.e. 0.05 mg/litre Cd or 1 mg/litre Ni).
- **b)** At the concentrations used to identify optimum pollutant levels. (i.e. 0.05, 0.1, and 0.5 mg/litre Cd, as well as 1, 2, 5, 10 and 15 mg/litre Ni).

Throughout the modelling exercise, the following assumptions were made:

1. The system was at equilibrium.
2. The bioavailable form of all metals except Fe was the aqueous free ion: Cd^{+2}, Ni^{+2}, Mg^{+2}, etc. The bioavailable form of Fe (III) was FeEDTA (Fe-ethylene-diamine-tetra-acetic acid; section 7.3).
3. The system was kept at a constant temperature of 25°C (i.e. the fact that at night the growth cabinet temperatures were reduced to 20°C was ignored).
4. The partial pressures of CO_2 and O_2 were equal to atmospheric (Voye' 1985; Woolard 1994).
5. Adsorption of any chemical species on the internal surfaces of the circulating nutrient solution apparatus was negligible.
6. Due to the large volume of nutrient solution and its frequent renewal, the concentration of plant or bacterial exudates in the system was negligible.

The effect of various factors on chemical speciation of the nutrient solution system was examined by modelling the solutions listed below.

i) Control (standard) nutrient solution both before and after the addition of NaOH.
ii) The nutrient solution after addition of various concentrations of $CdCl_2$.
iii) The nutrient solution after addition of various concentrations of $NiCl_2$.
iv) The above nutrient solutions at a range of different pH values.

Particular attention was paid to the proportion of the total metal content in the bioavailable form, especially with regard to the elements of interest, namely: Cd, Ni, Fe, Zn, Mg and Mn.

7.3 RESULTS AND DISCUSSION

7.3.1 Modelling of the control nutrient solution

7.3.1.1 Model development

The molybdate ion (MoO_4^{-2}), a constituent of Hoagland's nutrient solution, was not listed in the MINTEQA2 component database and required addition. Although the values of stability constants are adjusted automatically by the software according to the computed ionic strength of the test solution, the values supplied in the MINTEQA2 thermodynamic database are referenced to 25° C and ionic strength (μ) of zero (Serkiz, Allison, Perdue *et al.* 1996). The relevant protonation constants for MoO_4^{-2} given in the standard NIST (1993) thermodynamic database however, are derived for solutions with μ = 0.1. Thus the stability constants used by Voye' (1985), which are appropriate for conditions of zero ionic strength were employed. The relevant reactions are given below:

$$H^+ + MoO_4^{-2} \rightleftharpoons HMoO_4^- \qquad \log K = 4.04$$

And

$$2H^+ + MoO_4^{-2} \rightleftharpoons H_2MoO_4 \qquad \log K = 8.03$$

MoO_4^{-2} was added to the component database (COMP.DBS file) and H_2MoO_4 and $HMoO_4^-$ added to the species database (Thermo.dbs file) according to the prescribed procedure recommended in the MINTEQA2 manual (Allison *et al.* 1991). The third protonation of the molybdate ion was disregarded since protonation of H_2MoO_4 would only occur under very acidic conditions not likely to occur in the system under consideration (Heyns 1976). Other reactions likely to involve MoO_4^{-2} (Voye' 1985) are given below and were also added to the software database:

$$Fe^{+3} + MoO_4^{-2} \rightleftharpoons FeMoO_4^+ \qquad \log K = 8.73$$

$$Fe^{+3} + 3MoO_4^{-2} \rightleftharpoons Fe(MoO_4)_3^{-3} \qquad \log K = 17.33$$

$$Ca^{+2} + MoO_4^{-2} \rightleftharpoons CaMoO_4 \qquad \log K = 8.36$$

Further possible reactions were ignored since MoO_4^{-2} was supplied in trace quantities (Table 7.1) and it was considered that any other chemical entities formed would have a minor effect on the speciation profile of the system.

The stability constants for reactions involving EDTA were also modified since those in the MINTEQA2 database were reported to be unreliable (Pretorius *pers. comm.* 1997). Only the log K values for Fe-EDTA complexation reactions as well as the EDTA protonation constants were altered as these were considered to be the most important. The values used were taken from Voye' (1985) and are listed in Table 7.2 along with the appropriate reaction. The log K values listed in MINTEQA2 as well as the values given in the NIST (1993) database for critical stability constants of metal complexes are also shown. Changing the above log K values appeared to have little effect on the speciation pattern obtained however. The only difference noted was that the computed ionic strength was reduced marginally from 2.299E–02 to 2.297E –02.

Redox reactions between Fe^{+3}/ Fe^{+2}, Mn^{+3}/Mn^{+2} and Cu^{+2}/ Cu^{+} were taken into account by modelling the nutrient solution in equilibrium with oxygen. The partial pressure of O_2 was set at atmospheric (0.2095 atm) and the pE (negative log of the electron concentration), pH and ionic strength computed by the model.

The model was validated by comparing computed pH, with the experimentally measured value of this variable. The pH of one litre of freshly prepared basal, control nutrient solution at exactly 25°C was found to measure 5.02. If supersaturated solids were allowed to precipitate during the modelling exercise (discussed below) the correspondence between computed pH and actual pH was poor (4.63 versus 5.02). If the system was constrained to preclude precipitation of potential solids however, a much better correspondence between the final computed equilibrium pH and the actual value was obtained (4.93 versus 5.02).

***Table 7.2** Comparison of EDTA stability constants listed in the MINTEQA2 database, the NIST database and those used in this study for the EDTA complexation reactions given below.*

Reaction	MINTEQA2 Log K	NIST* Log K	Log k used
$EDTA^{-4} + H^+ \leftrightharpoons HEDTA^{-3}$	9.96	11.014	10.91
$EDTA^{-4} + 2H^+ \leftrightharpoons H_2EDTA^{-2}$	16.21	17.334	17.55
$EDTA^{-4} + 3H^+ \leftrightharpoons H_3EDTA^-$	18.86	20.02 (μ = 0.1)	20.69
$EDTA^{-4} + 4H^+ \leftrightharpoons H_4EDTA$	20.93	22.02 (μ = 0.1)	22.96
$EDTA^{-4} + 5H^+ \leftrightharpoons H_5EDTA^+$	18.785	20.54	18.785
$EDTA^{-4} + Fe^{+3} \leftrightharpoons FeEDTA^-$	27.7	25.1 (μ = 0.1)	26.97
$EDTA^{-4} + Fe^{+3} + H^+ \leftrightharpoons FeHEDTA$	29.2	26.4 (μ = 0.1)	28.34
$EDTA^{-4} + Fe^{+3} + 2H^+ \leftrightharpoons FeH_2EDTA^+$	19.8	-	19.22
$EDTA^{-4} + Fe^{+3} + 3H^+ \leftrightharpoons FeH_3EDTA^{+2}$	9.7	-	9.44

*NIST = National Institute of Standards (1993).

According to Voye' (1985) validation of nutrient solutions by comparison of computed with measured pH is adequate for examination of major chemical species. Because pH is governed largely by macronutrients however, it is difficult to validate the micronutrient concentrations. Validation using computed ionic strength and measured conductivity is therefore likely to suffer from the same problem. Thus although the model is likely to be correct with respect to the major ions there may be inconsistencies between computed and actual concentrations of the micronutrients. Whilst direct measurement of some of these species may have been possible, due to time constraints and technical difficulties (section 7.1) this avenue of research was not pursued further.

If precipitation were allowed to take place, the following solids were predicted to be present in the solution (MINTEQA2 identification numbers given in parenthesis):

Hematite $Fe_2(OH)_6.3\ H_2O$ (3028100)
Pyrolusite $Mn(OH)_4.\ H_2O$ (2047000)

Little could be found in the literature concerning the rate of formation of hematite and pyrolusite. The oxide and hydroxide K_{sp} (solubility constant) values for Fe however, can range over several orders of magnitude depending on the degree of crystallinity and especially on particle size effects (Macalady 1990; Nordstrom, Plummer, Langmuir *et al.* 1990). Inaccurate solubility constants in the database may therefore result in the artefact of precipitation. Furthermore, it is well known that whilst precipitation of some solids may be predicted from equilibrium considerations, the rate of formation can be so slow that a detectable level is never formed (Little 1984). Indeed, this is one of the limitations of computational speciation and was mentioned in the introduction to this chapter. On centrifugation of the nutrient solution for an extended length of time at low temperature, no precipitates could be detected. In addition, agreement between computed and observed pH was improved if no solids were allowed to form. On the basis of the above considerations therefore it was concluded that the model should be constrained so as to disallow precipitation.

7.3.1.2 Addition of NaOH

The model was developed further by simulating addition of NaOH. It was found that to raise the pH of the nutrient solution to 6.0 required exactly 1.9114E-04 moles of NaOH per litre of nutrient solution. The concentrations of Na^+ and OH^- given as input in PRODEFA were therefore adjusted accordingly. In addition, the pH of the system was constrained to remain at exactly 6.0. Apart from the above two modifications, the parameters and input data were exactly the same as for the previous model.

If precipitation was allowed to occur, it was found that the solids at equilibrium were:

Hematite $Fe_2(OH)_6.3\ H_2O$ (3028100)
Pyrolusite $Mn(OH)_4.\ H_2O$ (2047000)
Hydrapatite $Ca_5(OH)(PO_4)_3.H_2O$ (7015003)

The first two solids have already been discussed since they were predicted to form in the nutrient solution at pH 5.02. Hydrapatite (also referred to as hydroxyapatite) has been reported in the literature to have a low solubility. Nonetheless it has also been reported to exhibit a slow rate of crystallization out of solution (Burr 1925; Brauer 1963; McDowell, Gregory and Brown 1977). Since no precipitation was detected after addition of alkali and ultracentrifugation, once again it was decided that on kinetic grounds, precipitation could be ignored. Consequently the input parameters were set to preclude precipitation. The above model (referred to as the "control" model) was used in all further simulations.

7.3.1.3 <u>Speciation profile of the control nutrient solution</u>

The speciation profile of the nutrient solution before addition of alkali is not shown since this was not used in the experimentation but was rather used as described above, to develop and validate the model. The speciation profile of the control nutrient solution (pH 6.0) as used to cultivate control plants is given in Figure 7.1. The MINTEQA2 output for this model is given in Appendix 1.

Figure 7.1 shows the percentage of each component that is predicted by MINTEQA2 to be present as the free ion. The major metals of interest are shown, as well as other important plant nutrients such as nitrate. Most of the essential ions were largely present in the form of the free metal ion and were therefore available for uptake by the plant roots, exceptions being the Fe (II)/Fe (III) redox couple as well as Cu^{+2}. In the case of Fe, the model predicted that at equilibrium very little would be present in the +2 oxidation state (4.724E-15 mol/kg) compared to the +3 oxidation state (8.990E-05 mol/kg) and that of the Fe^{+3}, approximately 98% would be bound to EDTA. The majority of Fe in the nutrient solution was in the chelated form therefore and was bioavailable. Copper was

largely present in the +2 oxidation state (3.200E-07 Cu^{+2} compared to 1.093E-19 Cu^{+}) and therefore the latter is omitted from Fig 7.1. Of the Cu^{+2}, 42% was in the free metal ion form and 54% bound to EDTA. No free $EDTA^{-4}$ was predicted to be present at equilibrium and this was true for all simulations carried out on the various nutrient solution systems. This is consistent with the chelating role of this ligand. The question of EDTA-metal chelation and its effect on bioavailability is discussed later.

7.3.2 Effect of cadmium on speciation in the nutrient solution

The effect of $CdCl_2$ on speciation in the nutrient solution was examined by adding Cd^{+2} to the list of components in the PRODEFA input file used to generate the control model. The system was first modelled after addition of 0.05 mg/litre (4.5E-07M) Cd^{+2} to the system, which was the concentration of this metal pollutant used in the Cd-treatment experiments. The total dissolved concentration of Cl^{-} in the system was also adjusted accordingly.

The speciation profile of this system is shown in Figure 7.2, which represents the percentage of selected components predicted to be present as the free metal ion. It can be seen that 87% of the total dissolved Cd in the system would be in this form. The detailed speciation profile is summarised in Table 7.3, in which a comparison of control, Cd-treatment and Ni-treatment nutrient solutions is given. MINTEQA2 predicted that any Cd present in solution, other than as the free metal ion, would be in the form of $CdSO_4$ (11%) and $CdNO_3^{+}$ (1.4%). It can also be seen from the table that addition of this metal pollutant appeared to have little effect on the speciation pattern of the nutrient solution at least in regard to the metals of interest namely: Fe, Mg, Mn and Zn.

The model was then re-run using incremental additions of Cd^{+2} (and appropriately adjusted Cl^{-} input values). The simulated Cd^{+2} concentrations were the same as those employed in Chapter 2 during preliminary experiments to establish the optimal metal pollutant concentrations for experimentation (section 2.2.2).

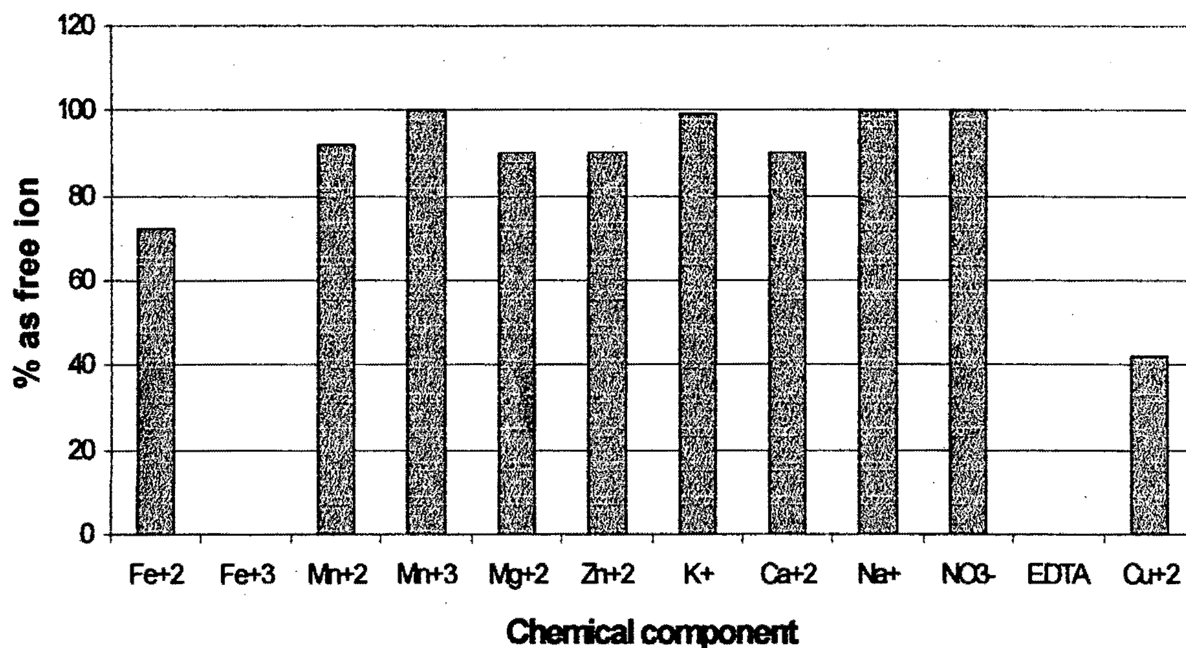


Fig. 7.1 *Speciation profile of the control nutrient solution showing the percentage of each component predicted by MINTEQA2 to be present as the free/bioavailable ion.*

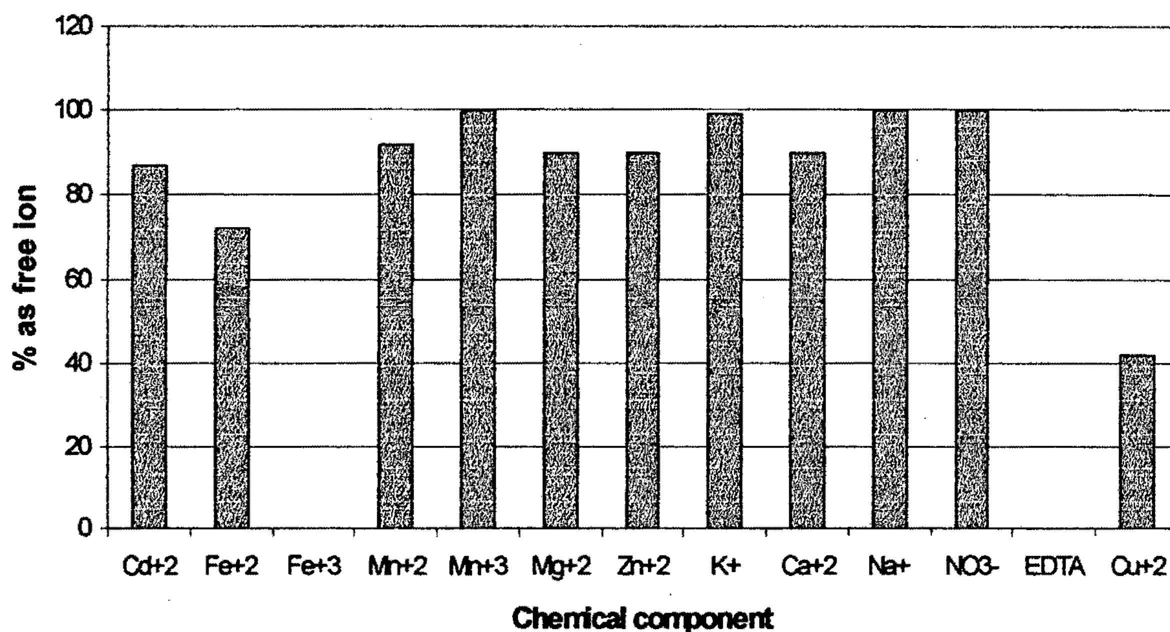


Fig. 7.2 *Speciation profile of the Cd-treatment nutrient solution showing the percentage of each component predicted by MINTEQA2 to be present as the free/bioavailable ion.*

***Table 7.3** Speciation profile of control, Cd- and Ni-treatment nutrient solutions as predicted by MINTEQA2 for components deemed to be of especial relevance to this project. The percentage of each component as the free ion is given, as well as the computed concentration of each chemical species at equilibrium.*

Component	**Starting conc (M)**	**Control N.S.**		**Cd-treatment N.S.**		**Ni-treatment N.S**	
		% as free ion	**Conc. at equil. (M)**	**% as free ion**	**Conc. at equil. (M)**	**% as free ion**	**Conc. at equil. (M)**
Cd	(4.5E-07)	0	0	87 (11%as $CdSO_4$)	Cd^{+2}= 3.896E-07 $CdSO_4$=5.10E-08	0	0
Ni	(1.7E-05)	0	0	0	0	49 (46as NiEDTA, 4% as $NiSO_4$)	Ni^{+2}=8.75E-06 $NiEDTA^{-2}$=7.872E-06 $NiSO_4$=7.404E-07
Mg	2.0E-03	90	Mg^{+2}= 1.798E-03	90	Mg^{+2}= 1.798E-03	90	Mg^{+2}=1.799E-03
Fe(II) **Fe(III)**	0 8.99E-05	72 0 (98%as FeEDTA)	Fe^{+2}= 3.392E-15 Fe^{+3}= 2.703E-13 $FeEDTA^-$= 8.800E-05	72 0 (98%as FeEDTA)	Fe^{+2}= 3.412E-15 Fe^{+3}= 2.719E-13 $FeEDTA^-$=8.8E-05	72 0 (90% as FeEDTA, 9%as $Fe(OH)_2^+$)	Fe^{+2}=1.501E-13 Fe^{+3}=1.197E-11 $FeEDTA^-$ =8.044E-05 $Fe(OH)_2^+$ =7.763E-06
Mn(II) **Mn(III)**	9.1E-06 0	92 100	Mn^{+2}= 8.403E-06 Mn^{+3}= 2.243E-16	92 100	Mn^{+2}= 8.403E-06 Mn^{+3}= 2.243E-16	92 100	Mn^{+2}=8.403E-06 Mn^{+3}=2.244E-16
Zn(II)	7.6E-07	90	Zn^{+2}= 6.818E-07	90	Zn^{+2}= 6.818E-07	90	Zn^{+2}=6.854E-07
EDTA	8.99E-05	0 (98%as FeEDTA)	$EDTA^{-4}$= 2.281E-18 $FeEDTA^-$=8.800E-05	0 (98%as FeEDTA)	$EDTA^{-4}$=2.268E-18 $FeEDTA^-$=8.799E-05	0 (90% as $FeEDTA^-$, 9%$NiEDTA^{-2}$)	$EDTA^{-4}$=4.724E-20 $FeEDTA^-$ =8.044E-05 $NiEDTA^{-2}$=7.872E-06
Cu(II)	3.2E-07	42 (54%as CuEDTA)	Cu^{+2}= 1.328E-07 $CuEDTA^{-2}$= 1.722E-07	42 (54%as CuEDTA)	Cu^{+2}= 1.333E-07 $CuEDTA^{-2}$=1.717E-07	88 (2%$CuEDTA^{-2}$)	Cu^{+2}= 2.809E-07 $CuEDTA^{-2}$=7.526E-09

These concentrations were: 0.1, 0.5, 1, and 5 mg/litre Cd (9.0E–7M, 4.5E-6M, 9.0E-6M and 4.5E-5M Cd respectively).

Results from these simulations are not shown, since even at the highest level of Cd (5 mg/litre), the proportion of the total Cd present as free metal ion was the same as for the lowest concentration (87%) and no changes in the speciation pattern of the other metal ions were apparent. This is possibly a result of the fact that the concentration of this metal pollutant (Cd_{total}) in the system was very low.

7.3.3 Effect of nickel on speciation in the nutrient solution

Simulations using the control model were run again, excepting that Ni^{+2} was included as a component. Incremental additions of this metal pollutant were made, ranging from the concentration routinely used for plant cultivation (1 mg Ni/litre; 1.7E-7M) to 15 mg Ni/litre (2.6E-4M). The input levels of Cl^{-} were also adjusted appropriately.

The speciation profile of the Ni-treatment nutrient solution is shown in Figure 7.3. Only 49% of the added Ni was predicted to be present as the free metal ion. By comparing Figures 7.1 (control nutrient solution) and 7.3 (Ni-treatment nutrient solution), it can be seen that the speciation profile for Mn, Mg and Zn was unchanged on addition of the metal pollutant to the system. Table 7.3 shows more detailed speciation profiles for control and Ni-treatment nutrient solutions, including the computed concentration of each chemical species at equilibrium. For each component the percentage predicted to be present as the free metal ion (and hence bioavailable) is shown, as is the percentage bound in any other significant (in terms of amount) chemical species. From this table it can be seen that any Ni, not in the form of the free metal ion, was either bound to EDTA (46%) or to $NiSO_4$ (4%). Compared to the control nutrient solution, the FeEDTA concentration was decreased by 8%. Whether this was enough to reduce the Fe content of seeds however is not easy to deduce. The other major difference in the speciation profiles of the control and Ni-treatment nutrient solutions, was the increased free Cu (II) in the latter. This appeared to be a result of competition with Ni for binding

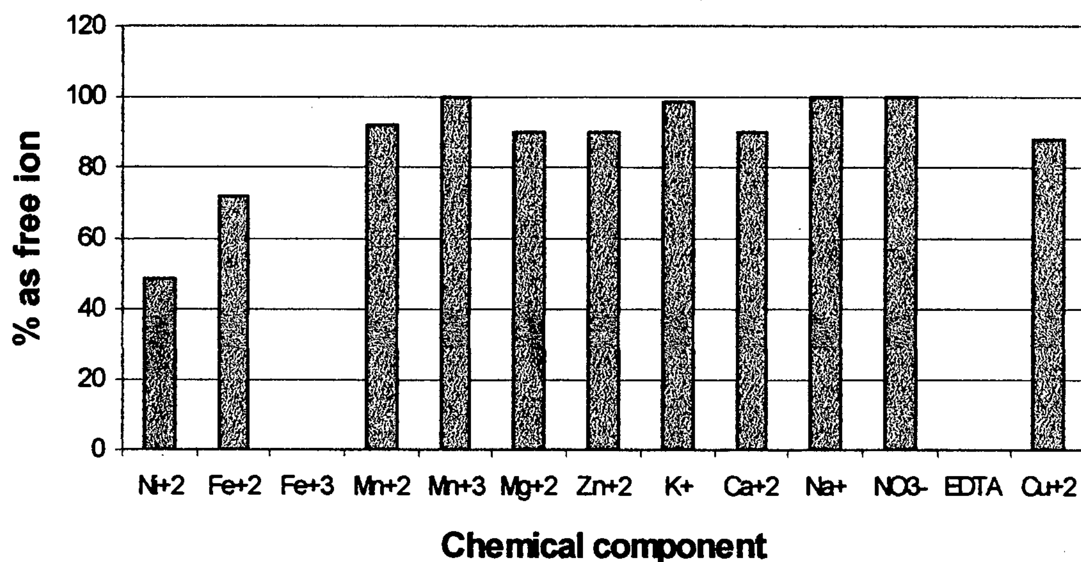


Fig. 7.3 *Speciation profile of the Ni-treatment nutrient solution showing the percentage of each component predicted by MINTEQA2 to be present as the free/bioavailable ion.*

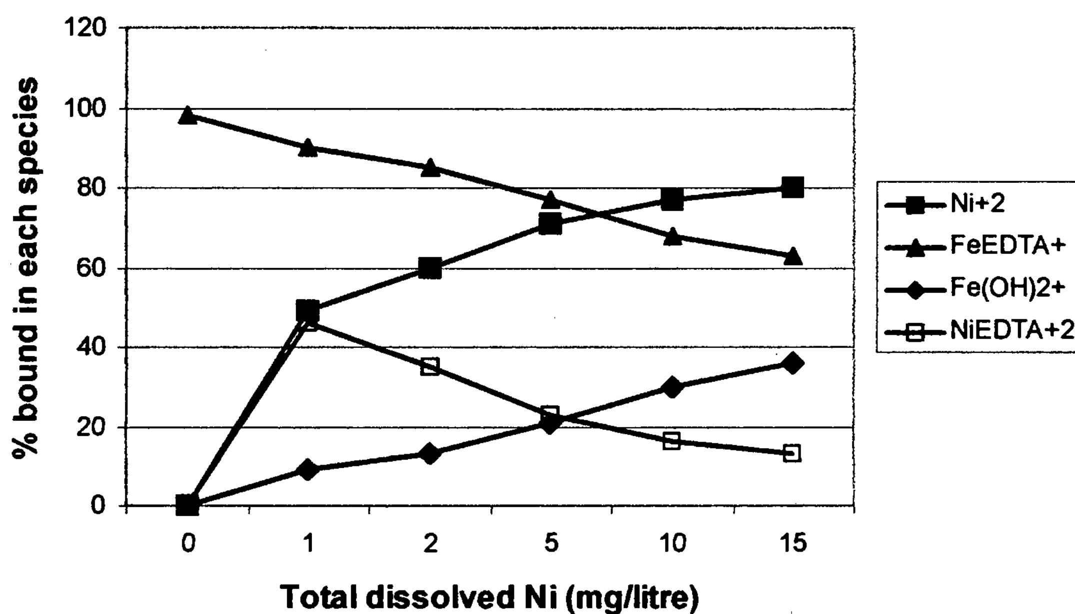


Fig. 7.4 *Effect of increasing Ni concentration on the speciation of selected components as predicted by MINTEQA2.*

to the EDTA ligand. As in the control solution, levels of free Fe(II), Fe(III) and $EDTA^{-4}$ were minimal.

The model was then re-run after increasing the total Ni concentration in the system incrementally to 15mg/litre. Results from these simulations are given in Figure 7.4. which shows the effect of increasing $NiCl_2$ concentration on the speciation of the nutrient solution system. Only chemical species that were affected by the amendment are shown. In the case of Fe (II), Mg, Mn and Zn, addition of the metal pollutant even at high concentrations appeared to have little or no effect on the speciation of these components and these are omitted. As the total Ni concentration in the system increased, the percentage present as the free metal ion (Ni^{+2}) also increased. This appeared to be largely due to the fact that the proportion of Ni bound to EDTA decreased from 46% in the 1 mg Ni/litre solution to 13% in the solution containing the highest level of metal pollutant. Nonetheless despite the fact that the *percentage* bound to EDTA decreased, the total *amount* of Ni chelated to this ligand increased from 6.9E-06 in the first solution to 3.38E-05 in the highest Ni solution. This is a reflection of the fact that the total concentration of all other components except Ni and Cl remained the same in all test solutions. Due to competition between Ni and Fe(III) ions for the EDTA ligand, the proportion of Fe(III) bound to EDTA decreased with increasing metal pollutant concentration. In addition, since the ratio of Fe(II):Fe (III) remained constant throughout the simulations, the amount of bound Fe(III) also decreased as the Ni concentration was increased. Iron (III) displaced from binding with EDTA was predicted by the model to be present as $Fe(OH)_2^+$ and was thus potentially unavailable for plant uptake. At high levels of Ni in this system therefore, Fe may become limiting to plant growth.

7.3.4 Effect of pH on speciation

The effect of pH on speciation in the nutrient solution was examined by increasing the pH of the system in increments of 0.25 pH units. Although during experimentation the pH of the nutrient solution was adjusted every second day, it was found that if this

precaution was omitted, during the fastest portion of the plant growth curve, pH values of up to 7 were attained after only a few days. It was considered that modelling the system from pH = 6 to pH =7 therefore, would be a realistic range for this parameter. The effect of pH on the speciation patterns of control, Cd-treatment and Ni-treatment nutrient solutions was examined and the results presented below.

7.3.4.1 Control nutrient solution

The effect of pH on chemical speciation of the control nutrient solution is shown in Figures 7.5 and 7.6. At the initial pH of 6, almost 98% of the total Fe(III) was predicted to be in the form of Fe(III)EDTA and the remaining 2% as Fe(III)OHEDTA. With increasing pH (and hence increasing hydroxyl ion concentration) the relative proportion of these two species was predicted to change so that at pH=7, only 83% of Fe(III) was present as Fe(III)EDTA and 16% as the hydroxylated Fe-EDTA form. Thus depending on the bioavailability of the latter species, Fe uptake by the plants may be depressed under conditions of high pH.

Copper was not one of the nutritionally important metals identified to be of especial interest in this study. Nonetheless, copper is also an important micronutrient in plants. Furthermore pH appeared to have a profound effect on the speciation of this element, and for that reason it is included. Figure 7.5 shows that as pH increased, an increasing proportion of the free metal ion became chelated to EDTA. At the highest pH value of 7, only 2% of Cu was available as the free metal ion. In contrast to Cu, pH appeared to have no effect on the speciation patterns of Mn(II) and Mn(III) (results not shown). This system variable also had a limited effect on Zn, Mg and Fe (II) (Fig 7.6). Increasing pH tended to slightly diminish the free metal ion concentrations of these three components.

7.3.4.2 Cadmium-treatment nutrient solution

The effect of increasing pH on the speciation of Cd is shown in Figure 7.7. The percentage of Cd as the free metal ion decreased slightly (from 87% to 78%) as pH increased. This appeared to be due to enhanced chelation of this metal with EDTA. Nevertheless, even at the highest pH, the proportion of Cd bound to this ligand was only 10% of the total metal pollutant concentration. Small amounts of Cd were also bound to

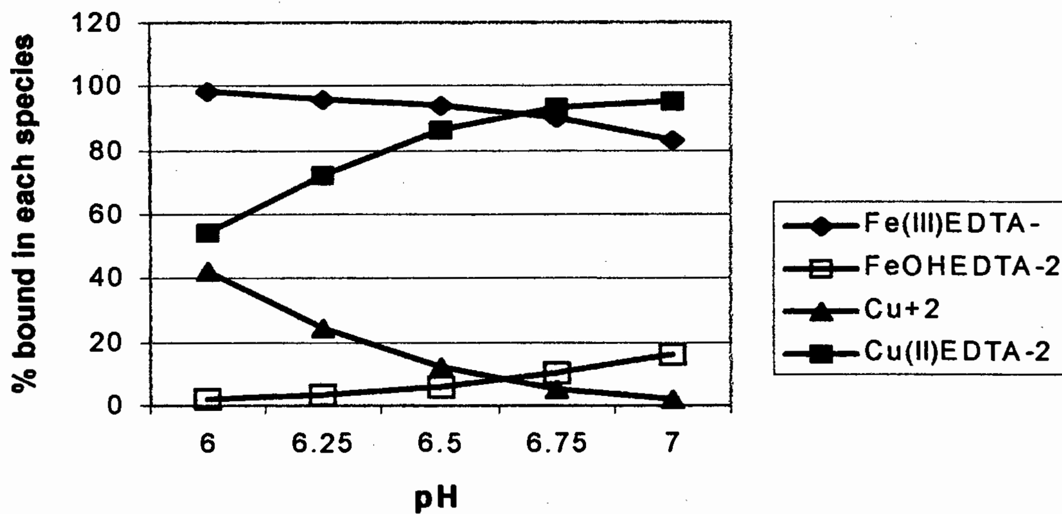


***Fig. 7.5** Effect of pH on the speciation of Fe (III), Cu(II) and EDTA in the control nutrient solution as predicted by MINTEQA2. The percentage of the three elements bound in each species is shown.*

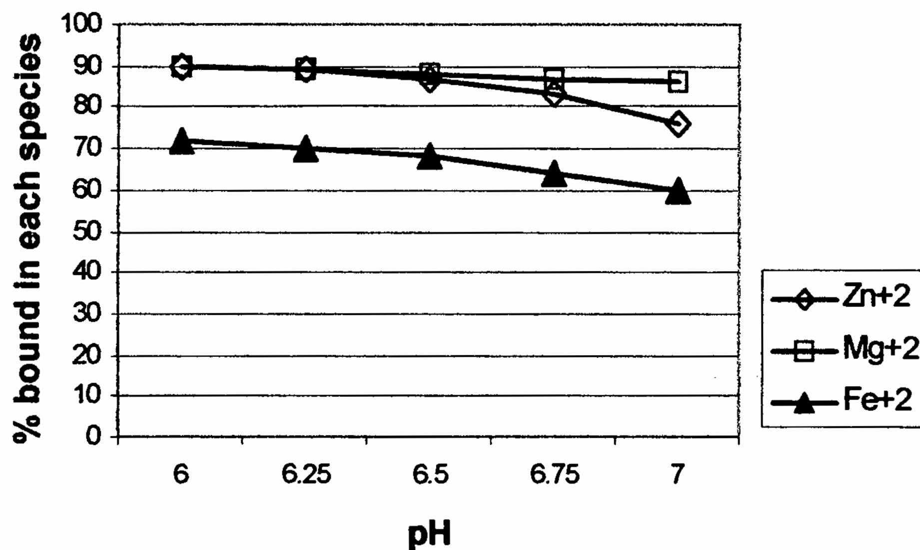


***Fig. 7.6** Effect of pH on Zn, Mg and Fe (II) speciation in the control nutrient solution. The percentage of the elements present as the free ion is indicated.*

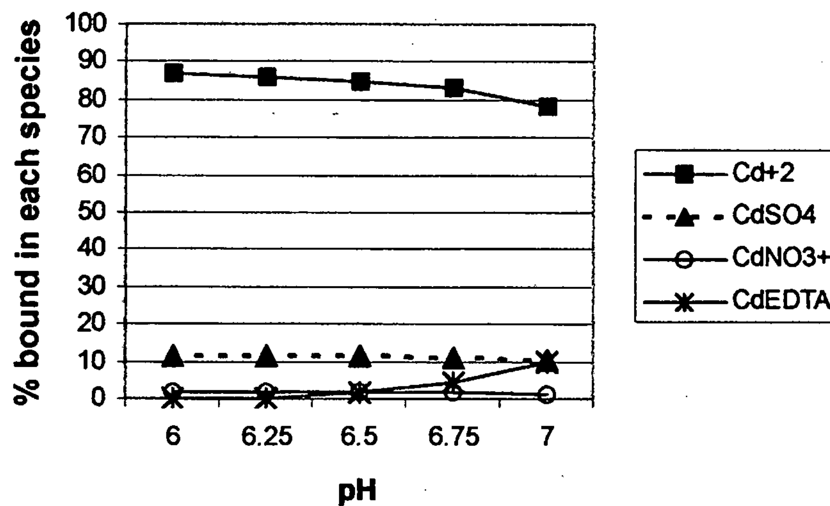


***Fig. 7.7** Effect of pH on the speciation of Cd as predicted by MINTEQA2. The percentage of Cd bound in each chemical species is given.*

SO_4^{-2} and NO_3^-, although these were a minor portion of the total metal concentration and remained approximately constant with increasing pH. Speciation of the other relevant components was virtually identical to that of the control nutrient solution at the equivalent pH and thus will not be discussed further.

7.3.4.3 Nickel-treatment nutrient solution

The effect of increasing pH on the speciation of Ni is shown in Figure 7.8. Changing pH was predicted to exert a profound effect on the speciation of Ni. Whilst 49% was present as the free metal ion at pH = 6, this percentage had decreased to less than 3% at pH = 7. This effect appeared to be largely due to enhanced chelation of the metal pollutant with EDTA at higher pH levels. Whilst some of the total Ni was also bound to the SO_4^{-2} ion, this represented only a minor portion and did not change markedly with pH. The implications of metal-chelate interactions with regard to plant uptake will be discussed in the next section.

The effect of pH on the speciation of other components in the Ni-treatment nutrient solution is shown in Figures 7.9 and 7.10. Increasing pH was predicted to result in higher proportions of Fe(III) bound to OH^- in various forms e.g. $FeOHEDTA^{-2}$, $Fe(OH)_2^+$. The resulting effect was a decrease in Fe(III) chelated to EDTA, and hence a possible decrease in bioavailability of this critical element. The effect of pH on Mg, Mn and Zn was either absent or very slight and so is not reported here. Once again however, pH exerted a profound effect on the speciation of Cu, leading to a marked decrease in the free metal ion as pH increased (Fig. 7.10).

7.3.5 Implications of speciation modelling to the current study

Central to this entire chapter is the question of metal chelation and bioavailability. It was mentioned in Chapter 1 that in general for divalent metals, uptake and toxicity are directly proportional to the concentration of the free metal ion. Possible exceptions to this rule have been noted, for example, Smolders and McLaughlin (1996) reported the possible uptake by Swiss chard roots of $CdCl_n^{2-n}$ species, in addition to the free metal ion. Parker and Pedler (1997) have recently critically reassessed the free-ion activity model (FIAM) of metal bioavailability. They used computer simulations to examine metal speciation in the presence of various chelating ligands (including EDTA). Cell wall binding of metal ions was included in the model by representing this effect as a competing ligand in the system. These authors concluded that the FIAM is likely to be most applicable in solution culture experiments in which a wide ratio of nutrient solution volume to root biomass is maintained and one in which strong chelators, such as EDTA are employed. The experimental system used in the current project did satisfy the above criteria and it will be assumed in this study therefore, that only the free metal ion is bioavailable. Parker and Pedler (1997) hypothesised that the lack of correlation found by Smolders and McLaughlin (1996) between Cd^{+2} and total plant Cd content may have been a result of discontinuities in the endodermis and subsequent uptake of complexed Cd. An alternative hypothesis put forward was that the weak Cl ligand could not compete with the binding effect of the cell wall.

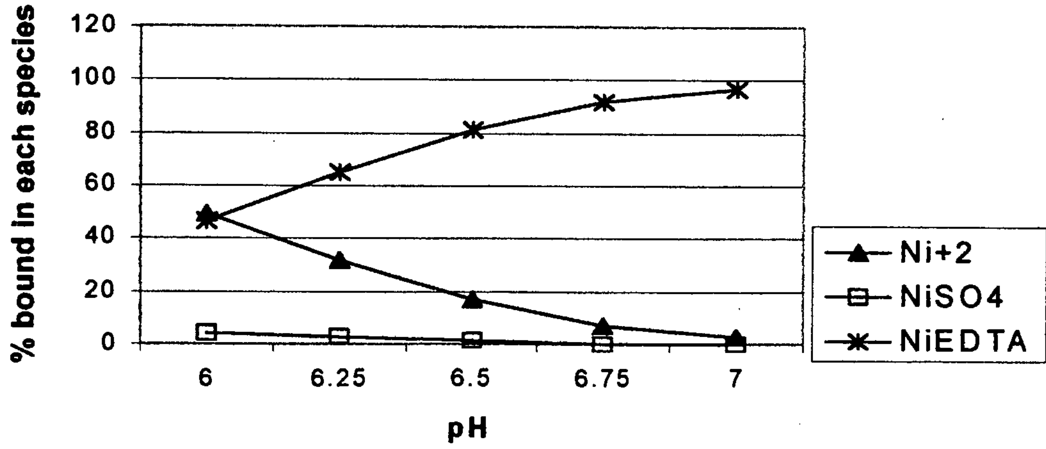


Fig. 7.8 Effect of pH on the speciation of Ni in the Ni-treatment nutrient solution.

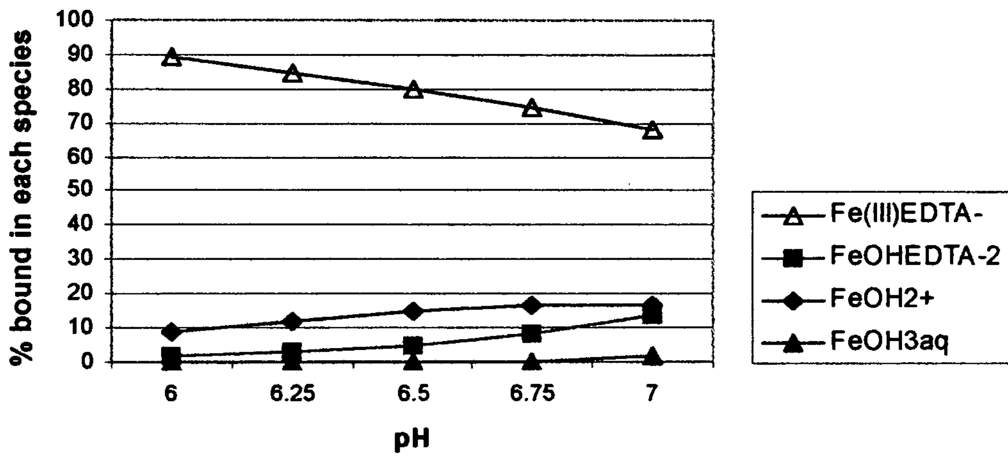


Fig. 7.9 Effect of pH on the speciation of Fe(III) in the Ni-treatment nutrient solution.

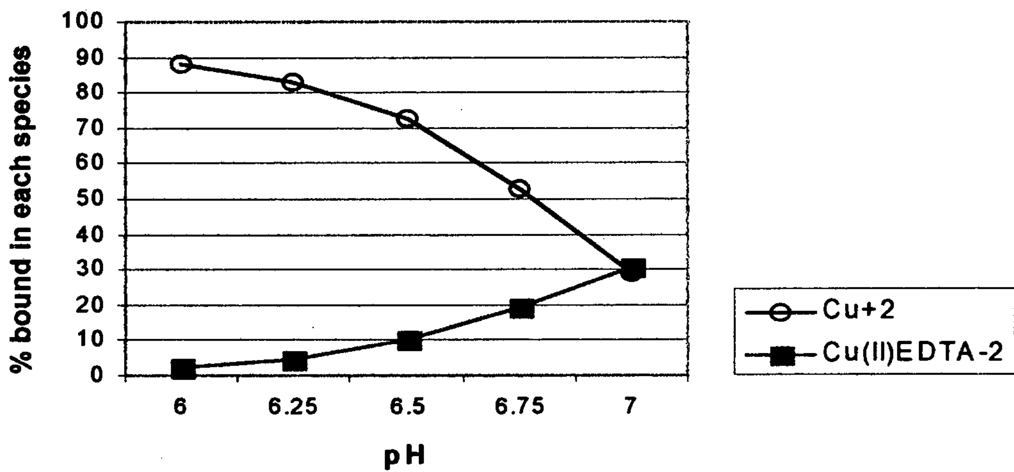


Fig. 7.10 Effect of pH on the speciation of Cu (II) in the Ni-treatment nutrient solution.

Considerable research had been directed towards an understanding of Fe uptake in plants since this element is often limiting in soils and unless bound to a suitable chelator in nutrient solutions, readily precipitates out as the hydroxide or phosphate (Parker *et al.* 1995). It has now been established that for Strategy I plants such as soybean, Fe (III) is first reduced to Fe (II) at the cortical cell membrane, prior to transport across this structure (Romheld and Marschner 1983). Uptake of this element is then dependent on the ability of the chelate to bind Fe (II). The reduction of Fe (III) to Fe (II) decreases the ability of most synthetic chelators to compete with the roots for Fe and the free chelate is released into solution (Parker *et al.* 1995; Srivastava and Appenroth 1995). Uptake of Fe by Strategy I plants is thus unrelated to free Fe^{+3} activities (Parker *et al.* 1995) and it thus differs from most other elements. Concentrations of free Fe in nutrient solution are usually very low (Chaney 1991) – a finding that was supported by the results of this present study. Uptake of intact Fe (III)-chelate in plants has been reported, but only when these are supplied at unrealistically high concentrations. Uptake at more modest solution concentrations has not been demonstrated unequivocally (Parker *et al.* 1995). Addition of chelators such as EDTA, to hydroponic solutions has generally been found to depress uptake of other divalent metals including Ni (Dunemann *et al.* 1991). Accumulation of Cd in duckweed was also found to decrease as chelation with EDTA increased (Srivastava and Appenroth 1995). Although it is generally considered that uptake of the complexed metal does not occur (Chaney 1991) the experimental evidence in the literature is conflicting (Wolterbeek, van der Meer and de Bruin 1988; Laurie *et al.* 1991a and b; Lorenz *et al.* 1997). Thus the behavior of metal ions in the presence of chelates should be interpreted with caution (Parker *et al.* 1995).

On consideration of the speciation profile of the control Hoagland's nutrient solution, it appears that with the possible exception of Cu, the bioavailability of all the essential nutrients was high. With regard to the two metal pollutants, in the treatment nutrient solutions used for routine cultivation, 87% of Cd_{total} and 49% of Ni_{total} were available for uptake. The resulting equilibrium concentration of the free metal ions in the solutions was 3.9E-07M Cd^{+2} and 8.33E-06 M Ni^{+2}. Thus although less than half the added Ni was bioavailable, the level of this toxicant in the nutrient solution was still higher than that of Cd and the statement made earlier in this work that Cd appears to be more toxic

than Ni is still valid.

The effect of pH on the speciation of especially Ni, Fe and Cu was marked. The percentage of Ni_{total} present as the free metal ion was reduced from 49% to 3% by an increase of one pH unit, whilst Cd did not appear to be particularly sensitive to this system parameter. The bioavailability of both Fe and Cu were reduced in the control, Cd- and Ni-treatment systems on increasing pH. Similar effects have been reported for Cd, Cu and Zn in various other nutrient solutions (Chaney 1991; Parker *et al.* 1995). Without a detailed speciation study of a given system it is difficult to predict which elements may be affected by this factor. These results emphasise the need for vigilant monitoring of pH levels when uptake or toxicity experiments are conducted in nutrient solution systems.

Virtually immediately plants are added to a growth medium, uptake of nutrients as well as exudation of chemical species (e.g. OH^-, complex organic molecules) by the roots result in changes in the composition of the nutrient solution. Secretion of plant exudates by plant roots can have a marked effect on speciation (section 1.3.6.1). Thus the plant roots experience the speciation environment resulting from fresh nutrient solution for only a limited period of time and predictions of plant uptake based on the above scenario may possibly lead to erroneous conclusions. Measurement of the qualitative and quantitative nature of compounds exuded by plant roots can be difficult (Hodge *et al.* 1996). Thus other than the question of pH, changes in the speciation pattern of the growth medium with time were not considered in this study. The system employed in these experiments constituted a relatively large volume of nutrient solution per plant which this was replaced frequently. Thus due to dilution, the concentration of root exudates in the system was probably very low and omission of this factor from the model was not unreasonable.

One of the main aims of this chapter was to ascertain whether shifts in seed metal content could be explained by changes in speciation of the growth medium resulting from addition of Cd or Ni. Bioavailability of Mn, Mg and Zn was only marginally affected by pollutant amendment of the nutrient solution. Thus changes in seed levels of these

metals are unlikely to result from speciation changes. Decreases in seed metal levels were reported in Cd-treatment seeds for Fe and Mn and in Ni-treatment seeds for Fe, Mn and Mg (section 3.3.4.5). Such reductions in treatment seeds compared to their control counterparts may possibly be explained by ion competition effects (section 3.4). Increases in Zn and Mg levels were reported in Cd-treatment seeds and Zn accumulation also occurred to a limited extent in Ni-treatment seeds. Such increases are difficult to explain in terms of speciation or ion competition effects. To find a satisfactory explanation, a radically different approach may be necessary, for example through investigating changes in patterns of metal distribution within the plant. The fact that Cd can act as a Zn analogue (section 1.3.41) could conceivably result in displacement of the latter from binding sites in the main portion of the plant and its accumulation within seeds.

In contrast to the other three nutritionally important metals, bioavailability of Fe was reduced on addition of Ni to the growth medium. The predicted reduction however, was only 8% less than in the control nutrient solution. The question then arises, "what magnitude must the change in percentage bioavailability be in order to firstly, affect the amount of an element taken up by the plant and secondly, to result in a depletion/accumulation within the seeds?" The minimum concentrations of bioavailable ions required for normal plant growth have been estimated for several metals. The critical concentration of Zn^{+2} appears to be $10^{-10.6}$ M (Halvorson and Lindsay 1977, cited by Chaney 1991; Laurie *et al.* 1991a). That of Cu^{+2} has been estimated to be approximately 10^{-13} M in corn and somewhat lower in barley, whilst the minimum Mn^{+2} level has been found to be in the region of 10^{-7}-10^{-8} M (Laurie *et al.* 1991a; Parker *et al.* 1995). According to Chaney (1991) the value for Cu^{+2} should be viewed with some caution since it was derived for a member of the Poaceae which are known to exude phytosiderophores, or chelators (section 1.3.6.1). This same author found that in tomatoes, Cu^{+2} levels of 10^{-15} –10^{-16} M caused reductions in yield (Chaney 1991). In the current study, even when only 2% Cu^{+2} was predicted to be bioavailable, the concentration of this element was 6.4^{-9} M, well above the required minimum limit. The concentrations of the other metals in the bioavailable form were also not likely to result in deficiencies. The probable reason for this is due to the fact that the concentrations

of ions in nutrient solutions are usually much higher than in the soil (section 2.1). The question of whether the bioavailable form of Cu or any other chemical species may have become limiting in the nutrient solution requires further investigation. In the general field of plant nutrition, extensive research is required to define critical nutrient levels more accurately and to assess their constancy or variation across plant species (Parker *et al.* 1995).

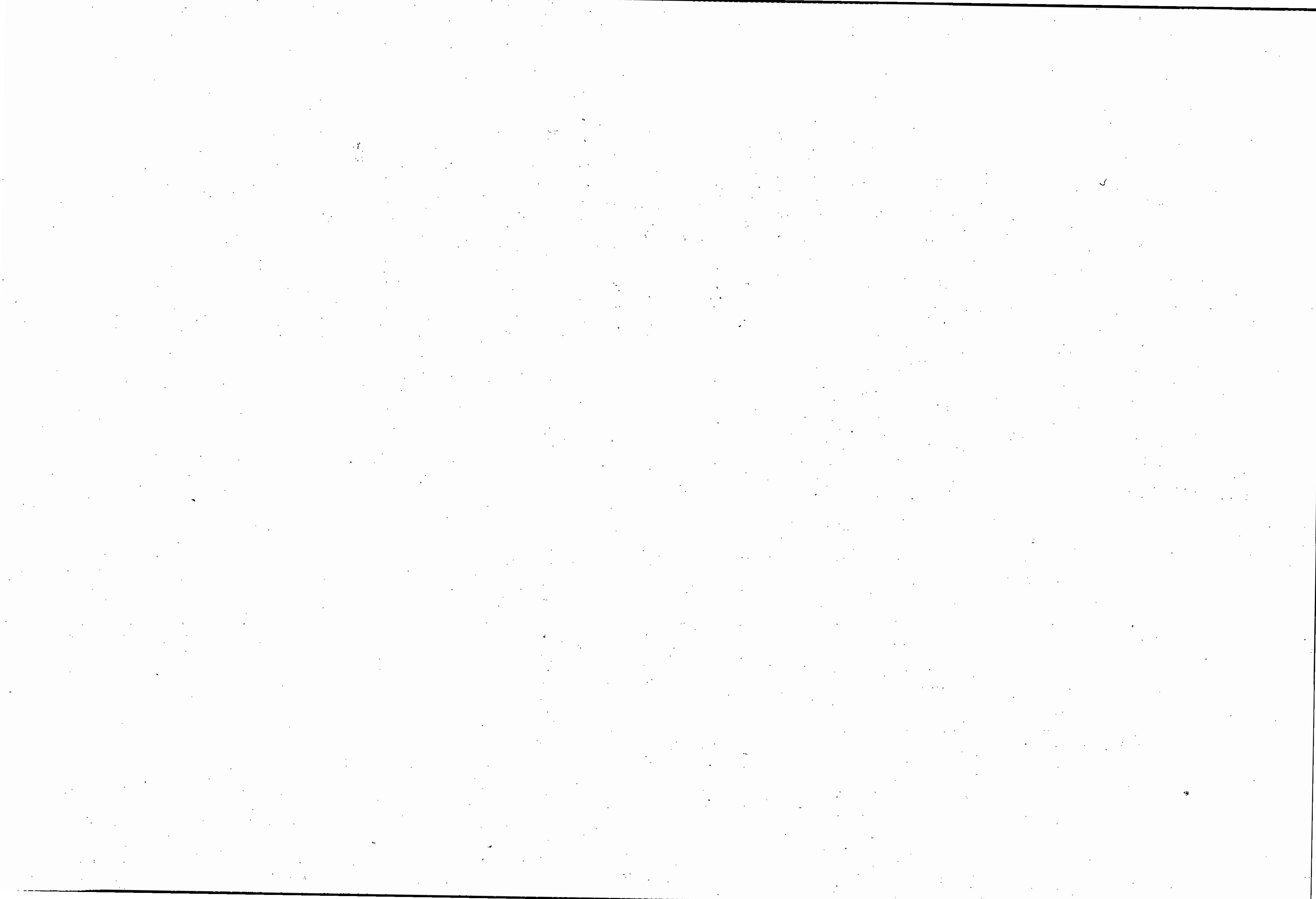
7.4 CONCLUSIONS

Results from this study have shown that interpretation of changes in speciation patterns with regard to uptake of metal ions is not straightforward. Uptake of both nutrients and other ions by plants is a complex phenomenon (Marschner 1983; Chaney 1991). Metal ions can be taken up by active, passive and facilitated mechanisms and uptake of a single metal species may occur by means of more than one mechanism simultaneously (Phipps 1981). Different carrier molecules are thought to be involved in the uptake of differing metal ions (Marschner 1986). The situation has been summarised succinctly by Parker *et al.* (1995), who states that "Even if chemical speciation in the bulk solution is understood, this knowledge alone is unlikely to fully explain the observed responses of plants to changes in solution composition. For example it also is necessary to consider the characteristics of the ion uptake process, the interaction of the uptake of one ion with another, transport of ions to the membrane surface and the physical structure of the root." The implications of speciation changes with regard to bioavailability and uptake can be difficult to interpret. Yet, whilst conducting toxicity or nutrition experiments, in order to fully assess the situation it is vital to take cognizance of chemical speciation in the growth medium.

Addendum

Subsequent to completion of the speciation modelling, the following reference was found in the literature:- "Rapid communication: Correcting errors in the thermodynamic database for the equilibrium speciation model MINTEQA2" by Serkiz *et al.* (1996). The above paper outlines possible errors in the MINTEQA2 database for complexes between

metals and organic ligands such as EDTA. The errors result from incorrect formulation of species in terms of MINTEQA2 components as well as the inclusion of equilibrium formation constants in the thermodynamic database that were derived for conditions other than zero ionic strength and temperature of 25°C. The authors contrast current MINTEQA2 log K values with the corrected values for a selected set of reactions involving CuEDTA complexes. These values were corrected in the present study and the model rerun on the control nutrient solution. Although there was a marked effect on the speciation of Cu^{+2}, making even less bioavailable, there was no effect on the speciation of the metals of interest to this project. It was not clear from the above paper however, exactly which other equilibrium constants were incorrect and what the new values should be. The protonation constants for EDTA as well as for FeEDTA complexation reactions had already been adjusted (section 5.3.1). Furthermore, since speciation changes due to addition of Cd and Ni to the nutrient solution did not appear to be responsible for the shifts in seed metal content and since time was limited, this line of research was not pursued further. According to Serkiz *et al.* (1996) the latest version of MINTEQA2 (Version 3.12) has been corrected for the above faults. It is possible that modelling of the nutrient solution with the updated version of MINTEQA2 may reveal different speciation patterns to those found in this study. As of 3/12/98 however, the updated version of this program was still not available (Stancil, Centre for Exposure Assessment Modelling - US EPA, *pers. comm.* 1998).



CHAPTER 8

CONCLUSIONS

The main conclusions arising from this study are summarised below:

1. pH was found to exert a profound effect on the speciation profile of metal pollutants, as well as on nutrients. Bioavailability of Cd^{+2}, Ni^{+2}, Fe (III), and Cu (II) was reduced on increasing the pH of the nutrient solution from 6.0 to 7.0. A marked effect was noted on Ni^{+2}. The proportion of the metal in this form (the bioavailable form) decreased from 49% to 3% over the same pH range. Maintenance of constant pH is therefore important when conducting uptake or toxicology experiments in nutrient solution systems.

2. In the respective treatment solutions, 87% of Cd, but only 49% of Ni, was bioavailable. Despite this, the former metal was still found to be more phytotoxic to soybean plants than the latter. This was reflected in the lower concentrations of Cd required to elicit a toxicity response (e.g. reduction in seed production or ultra-structural defects) as well as in the lower LC_{50} and EC_{50} values recorded for germination and radicle extension in the presence of this metal.

3. Visual toxicity symptoms exhibited in response to both Cd and Ni were very similar and this was apparent in both metal-germinated seedlings as well as in the mature, metal-treatment plants. Exceptions to this were the presence of terminal deformed pods, as well as red spots in the interveinal areas of leaves, which were both symptomatic of Ni toxicity.

4. Both Cd and Ni accelerated plant senescence in metal-treatment plants. Leaf abscission was promoted and in the case of the older growth stages, the rate of pod development was increased relative to that of control pods.

5. The major effect exerted by both metal pollutants was reduction in biomass and this was applicable to roots, shoots and seeds. The presence of either Cd or Ni in the nutrient solution decreased pod production. Such a reduction was not a result of enhanced pod abscission during the developmental period investigated, but was due to an earlier disruption in the reproductive process. We postulate that this disruption was abortion of flowers prior to fertilization and/or abortion of pods post-fertilization, but prior to the earliest development stage examined in this study. Furthermore, Cd decreased seed size relative to control seeds but the number of seeds per pod was not affected. Nickel had no effect on seed size, but the number of seeds produced per pod was decreased. In a similar manner, the major effect during the early growth phase of seedlings germinated in metal pollutants was also a reduction in growth. Lateral root production was especially affected in this regard.

6. The presence of Cd in the nutrient solution reduced the lipid, starch and total N content of seeds harvested from soybean plants grown in such a medium. This was a result of reduced mass, since significant reduction was noted only when the results were expressed on a per seed basis. Nickel had little effect on the quantity of storage reserves accumulated within seeds.

7. Mature seeds harvested from Cd-treatment plants had lower Fe and Mn, but higher Zn and Mg contents than control seeds. Nickel-treatment seeds also exhibited reduced Fe, Mn and elevated Zn contents, but Mg levels were also reduced. Shifts in seed concentrations of the nutritionally important metals noted above, were also reflected in the metal assays for pods. The most significant change was the decrease in Fe content

in pods at all growth stages, which was elicited by both metal pollutants. Shifts in seed contents of Mg, Mn and Zn, in response to amendment of the nutrient solution with metal pollutants, cannot be explained by changes in chemical speciation in the growth medium. The decrease in Fe content in Ni-treatment seeds may possibly be a consequence of decreased bioavailability of this ion in the nutrient solution but this requires further research. Decreases in Fe content of both Cd- and Ni-treatment seeds were the result of reduced Fe levels in the testa and the axis but, not in the cotyledons, which contained the same levels of Fe as in the controls.

8. Pollutant loads in the roots were much higher than in shoots. This was found in be the case in both metal-treatment plants and in metal-germinated seedlings. The Cd and Ni contents of the reproductive parts harvested from metal-treatment plants were lower than either roots or shoots. Pods did not appear to exclude entry of the metal pollutants into seeds. Lower concentrations (Ni) or similar concentrations (Cd) were found in pods compared to seeds.

9. The concentration of both metal pollutants in seeds (when expressed as μg/g.dm) was highest in the youngest growth stages and then decreased with seed age. The concentration of Cd in mature seeds (i.e. the bulk content for the entire seed) was ≅ 1μg/g.dm and that of Ni was ≅ 50 μg/g.dm. Thus accumulation of Cd within the seeds was approximately 20 times that in the nutrient solution, whereas accumulation of Ni was approximately 50 fold. We postulate that because high levels of the enzyme urease in which Ni is the obligatory metal co-factor are found in legume seeds, exclusion mechanisms that are operative against other metal pollutants (eg. Cd) are not as stringent against entry of Ni into the seed. This results in accumulation of the metal in these tissues.

10. Within the seeds, if expressed as (μg/g.dm), Cd was found to be largely confined to the testa and cotyledons, with very low concentrations in the embryo axis. Nickel on the other hand, reached the highest levels in the axis, specifically in the tissues of the cortex and apical meristem of the radicle, although the metal was largely excluded from the region of the root cap and central stele. On consideration of the total metal pollutant load in mature seeds, because the cotyledons form the bulk of seed mass and the axis relatively little, in the case of Cd, 17% was located in the testa, 83% in the cotyledons and 0.14% in the axis. Forty two percent of the total Ni content of mature seeds was localised in the testa, 43% in the cotyledons and 15% in the axis.

11. Seedlings germinated in metal pollutant and then placed in the standard nutrient solution were able to recover from a period of exposure, up to a critical concentration of metal pollutant. At concentrations higher than this they died. Recovery seedlings appeared relatively healthy. Nonetheless, although little reduction in the concentration of photosynthetic pigments or the efficiency of photosynthetic functioning was recorded, two weeks after exposure to the metal pollutants, root biomass was still reduced relative to that of control seedlings.

12. The total chlorophyll content of metal pollutant-germinated seedlings decreased at low concentrations of the metal pollutants, but then increased at higher concentrations. We suggest that this was the result of the combined effects of inhibition of photosynthetic pigment synthesis, coupled to reduced leaf expansion.

13. In both metal pollutant-treatment seeds as well as control seeds, two novel ultra-structural features were noted that have not to our knowledge been previously reported in the literature. These were, halos of translucent nucleoplasm around the nucleoli of mature radicle tip cells, as well as inclusions in the lipid bodies. The latter were frequently aligned in the same direction.

14. Metal pollutant-treatment seeds did not differ in external appearance from their control counterparts. Similarly no differences were noted at the LM level, although slight differences were noted at the ultra-structural level. The following possible ultra-structural aberrations in metal pollutant-treatment seeds were noted, but require further investigation:

i) Vesicles in the nucleoplasm of YP Cd-treatment cotyledon cells.

ii) An increase in the number of phytate inclusions in the protein bodies of Ni-treatment radicle tip cells.

iii) An increased number of starch grains in the radicle tip cells of Ni-treatment seeds. We postulate that if this was indeed a true effect, it resulted from redistribution of starch from other parts of the seed, since Ni-treatment did not increase the total starch content of seeds.

15. Significant ultra-structural changes in metal pollutant-germinated seedlings were noted compared to the controls. The extent of damage appeared to be roughly proportional to the concentration of metal pollutant. From examination of the ultra-structure of metal pollutant-germinated seeds, the principal toxic effect of Cd appeared to be on nuclear functioning as well as protein catabolism. Such deductions were made from the fact that structural aberrations were noted in the nucleoli of metal pollutant-germinated seeds, as well as because the contents of protein bodies in these tissues appeared to be in a condensed form. This is a tentative conclusion however as marked structural changes were apparent only at high concentrations of Cd and this is not necessarily the principal toxic effect. Nickel also seemed to affect nuclear functioning and proteolysis to a lesser extent. In addition, aberrations to the peripheral cytoplasm adjacent to the cell wall were also noted in seedlings germinated in the presence of this metal.

16. The extent of germination in metal pollutant-treatment seeds (as given by mean daily germination) was not impaired by the presence of either Cd or Ni within the tissues. The rate of germination relative to control seeds however, was depressed slightly in both treatments.

17. Germination of seeds imbibed in solutions of exogenous pollutants was much less sensitive to the metals than radicle extension or growth. This is possibly a result of impermeability of the testa to the metal pollutants. Alternatively it may be due to sensitivity of cell division to toxic effects of the metal pollutants and thus was demonstrated only during radicle extension.

In section 1.5, the five major objectives of the study were outlined. The first aim was to determine what proportion of Cd or Ni would accumulate in the seeds harvested from plants grown in a contaminated growth medium. This value was determined for seeds cultivated in nutrient solution amended with 0.05 mg/litre Cd or 1 mg/litre Ni. The second, third and fourth objectives were concerned with an examination of the effect that metal pollutant stress exerted on basic physiological and biochemical processes during seed development, germination and seedling establishment. Thus deposition of storage reserve in seeds, the rate and extent of germination as well as photosynthetic functioning in seedlings were examined, in addition to other physiological processes. It is acknowledged however, that because of the wide spectrum of toxic effects that metal pollutants have been reported to exert, many other aspects, for example respiration and flower initiation, to name but two, also require investigation. With regard to the third objective, which entailed examination of the quantity and quality of seed storage reserves, the quality of the storage reserves (protein, lipids, and carbohydrates) was not examined. The mineral content of such seeds however, was investigated with regard to four nutritionally important metals (Mg, Mn, Fe and Zn). The final aim of the project was to describe the chemical environment to which the plant roots were exposed to in

the nutrient solution. This was carried out successfully, including deriving the proportion of the two metal pollutants likely to be in the bioavailable form and investigating the effect of pH thereon. The correctness of these simulations however, is constrained by the accuracy of the formation constants used by the model MINTEQA2.

One clear message emerges from this study, namely, that remarkably little research has been directed towards examining the effects of metal pollutants on seed development and functioning. It is true that to a large extent plants seem to be able to exclude metal pollutants from these parts and when very high concentrations of metal pollutants are present in the growth medium, seeds are simply not produced. If plants are exposed to lower levels of metal pollutants, they are able to exclude them to a large extent from the seed tissues. Metal pollutants do still enter the seeds however, albeit in relatively low concentrations compared to other plant parts. There is an awareness of the danger of metal pollutants entering the food chain via seeds, but in a world where pollution is increasing, the more indirect effects of metal pollutants on the development and metabolism of these tissues need to be examined. This study represents an initial attempt to examine those effects, although much work still remains to be done. In particular, attention needs to be directed towards the effect of metal pollutants on nutritional quality. It was found in this study that the presence of Cd and Ni within the seeds did not have a profound effect on the amount of storage reserves accumulated per gram dry mass of seed. Due to financial and time restraints we were not able to examine the quality of storage reserves laid down. It is important that any deleterious changes in nutritional quality, particularly of protein, resulting from soil pollution be examined. Investigations into changes in the amino acid profile of metal pollutant-treatment seeds should be carried out. In addition, further attention should be paid to the effects of metal pollutants on the mineral content of these tissues. Soybeans are likely to play an increasing role in the nutrition of poor countries in the future. Furthermore, approximately 70% of all food for human consumption comes directly from

seeds and a large proportion of the remainder is derived from animals that are fed on these plant parts (Bewley and Black 1994). Thus it essential that attention be directed to assess the effects of metal pollutants on economically important seed crops in general.

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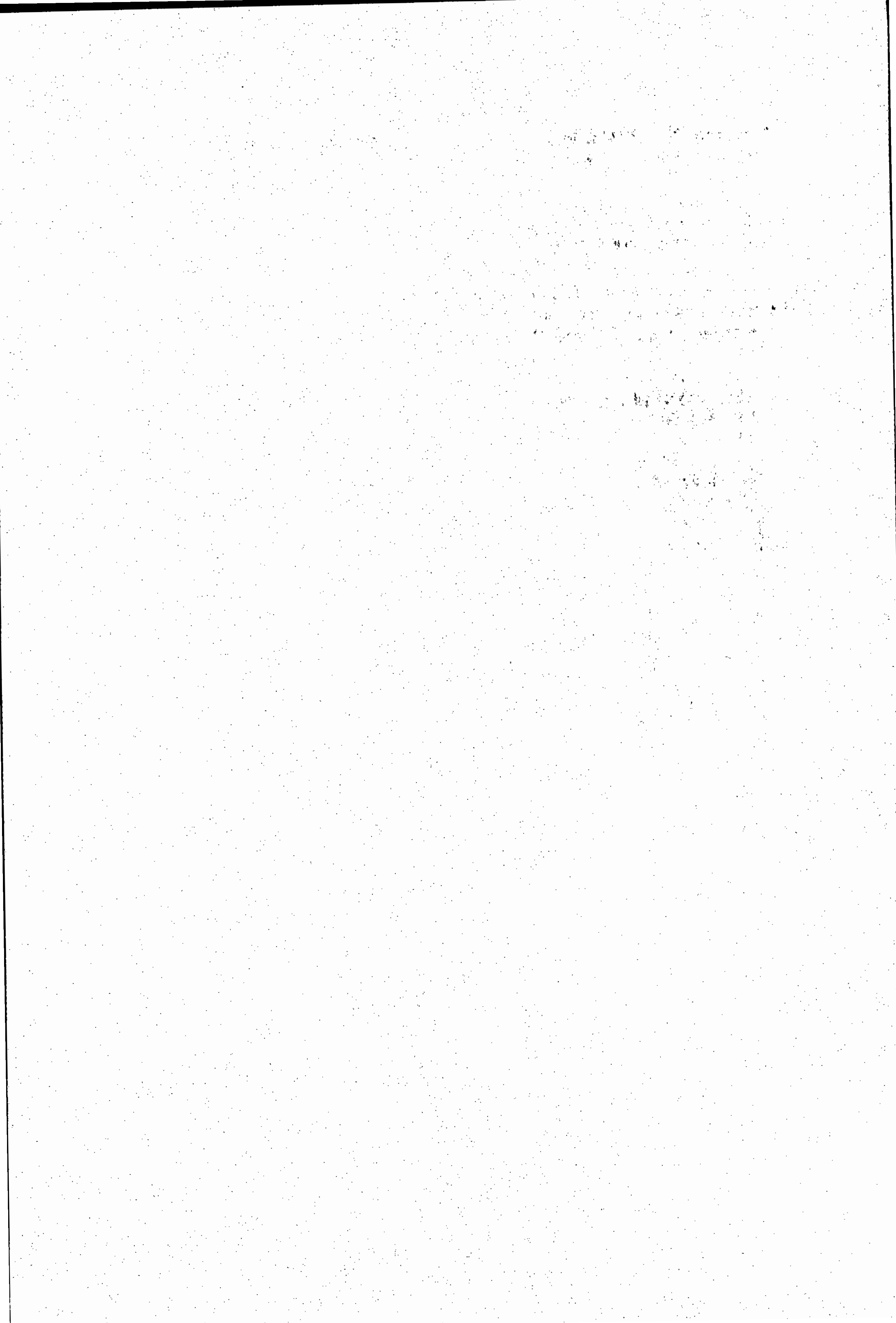

APPENDIX

The computer output (in an abbreviated form) from speciation modelling of the following systems is presented:

A. Control nutrient solution, pH = 6.0.
B. Cadmium-treatment nutrient solution, pH = 6.0.
C. Nickel-treatment nutrient solution, pH = 6.0.

A. CONTROL NUTRIENT SOLUTION

```
________________________ PART 1 of OUTPUT FILE

______________________________
  PC MINTEQA2 v3.10   DATE OF CALCULATIONS: 10-NOV-97   TIME: 14:13:29

BestB
pure NS, pH=6.0
--------------------------------------------------------------------
---
Temperature (Celsius):  25.00
Units of concentration: MOLAL
Ionic strength to be computed.
If specified, carbonate concentration represents total inorganic
Carbon.
Do not automatically terminate if charge imbalance exceeds 30%
Precipitation is allowed only for those solids specified as ALLOWEDin
the input file (if any). The maximum number of iterations is: 200
The method used to compute activity coefficients is: Davies equation
Intermediate output file
--------------------------------------------------------------------
---
    330  0.000E-01   -6.00 y
    410  5.000E-03   -2.30 y
    460  2.000E-03   -2.70 y
    150  4.000E-03   -2.40 y
    281  8.990E-05   -4.05 y
    500  9.181E-05   -4.05 y
    470  9.100E-06   -5.04 y
    950  7.600E-07   -6.12 y
    231  3.200E-07   -6.49 y
    580  1.000E-03   -3.00 y
    732  2.001E-03   -2.70 y
    492  1.200E-02   -1.92 y
    969  8.990E-05   -4.05 y
     90  4.600E-05   -4.34 y
    180  1.820E-05   -4.74 y
    140  0.000E-01  -16.00 y
    480  1.100E-07   -3.96
      1  0.000E-01  -15.60
    280  0.000E-01  -16.00
    471  0.000E-01  -16.00 y
    230  0.000E-01  -16.00 y

 H2O has been inserted as a COMPONENT
  3   6
3301403     21.6600     -0.5300
3300021    -82.4412    133.8300
2802810     13.0320    -10.0000
4704710     25.5070     25.7600
2302310      2.7200      1.6500
    330      5.9990      0.0000
```

```
 6   1
     1      0.0000      0.0000
----------------------------------------------------------------------
---
INPUT DATA BEFORE TYPE MODIFICATIONS
   ID        NAME        ACTIVITY GUESS     LOG GUESS    ANAL TOTAL
    330  H+1                  1.000E-06        -6.000    0.000E-01
    410  K+1                  5.012E-03        -2.300    5.000E-03
    460  Mg+2                 1.995E-03        -2.700    2.000E-03
    150  Ca+2                 3.981E-03        -2.400    4.000E-03
    281  Fe+3                 8.913E-05        -4.050    8.990E-05
    500  Na+1                 8.913E-05        -4.050    9.181E-05
    470  Mn+2                 9.120E-06        -5.040    9.100E-06
    950  Zn+2                 7.586E-07        -6.120    7.600E-07
    231  Cu+2                 3.236E-07        -6.490    3.200E-07
    580  PO4-3                1.000E-03        -3.000    1.000E-03
    732  SO4-2                1.995E-03        -2.700    2.001E-03
    492  NO3-1                1.202E-02        -1.920    1.200E-02
    969  EDTA-4               8.913E-05        -4.050    8.990E-05
     90  H3BO3                4.571E-05        -4.340    4.600E-05
    180  Cl-1                 1.820E-05        -4.740    1.820E-05
    140  CO3-2                1.000E-16       -16.000    0.000E-01
    480  MoO4-2               1.096E-04        -3.960    1.100E-07
      1  E-1                  2.512E-16       -15.600    0.000E-01
    280  Fe+2                 1.000E-16       -16.000    0.000E-01
    471  Mn+3                 1.000E-16       -16.000    0.000E-01
    230  Cu+1                 1.000E-16       -16.000    0.000E-01
      2  H2O                  1.000E+00         0.000    0.000E-01
_________________________ PART 4 of OUTPUT FILE
_________________________
PC MINTEQA2 v3.10   DATE OF CALCULATIONS: 10-NOV-97  TIME: 14:13:30

                 PERCENTAGE DISTRIBUTION OF COMPONENTS AMONG
              TYPE I and TYPE II (dissolved and adsorbed) species

Cu+2              41.5      PERCENT BOUND IN SPECIES #     231   Cu+2
                   3.8      PERCENT BOUND IN SPECIES #2317320   CuSO4 AQ
                  53.8      PERCENT BOUND IN SPECIES #2319691   CuEDTA

K+1               99.4      PERCENT BOUND IN SPECIES #     410   K+1

Mg+2              89.9      PERCENT BOUND IN SPECIES #     460   Mg+2
                   7.3      PERCENT BOUND IN SPECIES #4607320   MgSO4 AQ
                   1.3      PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+

                   1.5      PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ

Ca+2              89.6      PERCENT BOUND IN SPECIES #     150   Ca+2
                   8.3      PERCENT BOUND IN SPECIES #1507320   CaSO4 AQ
                   1.1      PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ

Fe+2              71.8      PERCENT BOUND IN SPECIES #     280   Fe+2
                   5.8      PERCENT BOUND IN SPECIES #2807320   FeSO4 AQ
                  15.6      PERCENT BOUND IN SPECIES #2805800   FeH2PO4
+
                   6.6      PERCENT BOUND IN SPECIES #2805801   FeHPO4
AQ

Na+1              99.6      PERCENT BOUND IN SPECIES #     500   Na+1

Mn+2              92.3      PERCENT BOUND IN SPECIES #     470   Mn+2
                   7.6      PERCENT BOUND IN SPECIES #4707320   MnSO4 AQ
Zn+2              89.7      PERCENT BOUND IN SPECIES #     950   Zn+2
                   9.5      PERCENT BOUND IN SPECIES #9507320   ZnSO4 AQ
```

```
MoO4-2          99.4    PERCENT BOUND IN SPECIES #     480   MoO4-2

PO4-3            2.5    PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+
                 3.1    PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ
                 4.5    PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ
                 4.0    PERCENT BOUND IN SPECIES #1505802   CaH2PO4
+
                 7.6    PERCENT BOUND IN SPECIES #3305800   HPO4 -2
                78.3    PERCENT BOUND IN SPECIES #3305801   H2PO4 -

SO4-2           74.7    PERCENT BOUND IN SPECIES #     732   SO4-2
                 7.3    PERCENT BOUND IN SPECIES #4607320   MgSO4 AQ
                16.6    PERCENT BOUND IN SPECIES #1507320   CaSO4 AQ
                 1.4    PERCENT BOUND IN SPECIES #4107320   KSO4 -

NO3-1          100.0    PERCENT BOUND IN SPECIES #     492   NO3-1

EDTA-4          97.9    PERCENT BOUND IN SPECIES #2819690   Fe EDTA
                 1.9    PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

H3BO3           99.9    PERCENT BOUND IN SPECIES #      90   H3BO3

Cl-1           100.0    PERCENT BOUND IN SPECIES #     180   Cl-1

Cu+1           100.0    PERCENT BOUND IN SPECIES #     230   Cu+1

CO3-2           33.7    PERCENT BOUND IN SPECIES #3301400   HCO3 -
                65.2    PERCENT BOUND IN SPECIES #3301401   H2CO3 AQ

H+1              2.7    PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+
                 1.7    PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ
                 2.4    PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ
                 4.2    PERCENT BOUND IN SPECIES #1505802   CaH2PO4
+
                 1.1    PERCENT BOUND IN SPECIES #3301401   H2CO3 AQ
                 4.0    PERCENT BOUND IN SPECIES #3305800   HPO4 -2
                83.6    PERCENT BOUND IN SPECIES #3305801   H2PO4 -

E-1            100.0    PERCENT BOUND IN SPECIES #4700020   MnO4 -

H2O             16.7    PERCENT BOUND IN SPECIES #2813301   FeOH2 +
                82.1    PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

Fe+3            97.9    PERCENT BOUND IN SPECIES #2819690   Fe EDTA
                 1.9    PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

Mn+3           100.0    PERCENT BOUND IN SPECIES #     471   Mn+3
```

B. CADMIUM-TREATMENT NUTRIENT SOLUTION

```
______________________ PART 1 of OUTPUT FILE
 PC MINTEQA2 v3.10   DATE OF CALCULATIONS: 10-NOV-97   TIME: 13:39:50

bestcd1
Cd, pH fixed 6.0
-----------------------------------------------------------------------
    330  0.000E-01   -6.00 y
    410  5.000E-03   -2.30 y
    460  2.000E-03   -2.70 y
```

```
    150  4.000E-03   -2.40 y
    281  8.990E-05   -4.05 y
    500  9.181E-05   -4.05 y
    470  9.100E-06   -5.04 y
    950  7.600E-07   -6.12 y
    231  3.200E-07   -6.49 y
    580  1.000E-03   -3.00 y
    732  2.001E-03   -2.70 y
    492  1.200E-02   -1.92 y
    969  8.990E-05   -4.05 y
     90  4.600E-05   -4.34 y
    180  1.920E-05   -4.74 y
    140  0.000E-01  -16.00 y
    480  1.100E-07   -3.96
      1  0.000E-01  -15.60
    280  0.000E-01  -16.00
    471  0.000E-01  -16.00 y
    230  0.000E-01  -16.00 y
    160  4.500E-07   -6.35

 H2O has been inserted as a COMPONENT
  3    6
3301403     21.6600     -0.5300
3300021    -82.4412    133.8300
2802810     13.0320    -10.0000
4704710     25.5070     25.7600
2302310      2.7200      1.6500
    330      5.9990      0.0000
  6    1
      1      0.0000      0.0000
```

PART 4 of OUTPUT FILE

```
 PC MINTEQA2 v3.10   DATE OF CALCULATIONS: 10-NOV-97  TIME: 13:39:51

                 PERCENTAGE DISTRIBUTION OF COMPONENTS AMONG
               TYPE I and TYPE II (dissolved and adsorbed) species
Cd+2              86.6     PERCENT BOUND IN SPECIES #     160   Cd+2
                   1.4     PERCENT BOUND IN SPECIES #1604920   CdNO3 +
                  11.3     PERCENT BOUND IN SPECIES #1607320   CdSO4 AQ

K+1               99.4     PERCENT BOUND IN SPECIES #     410   K+1

Mg+2              89.9     PERCENT BOUND IN SPECIES #     460   Mg+2
                   7.3     PERCENT BOUND IN SPECIES #4607320   MgSO4 AQ
                   1.3     PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+
                   1.5     PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ

Ca+2              89.6     PERCENT BOUND IN SPECIES #     150   Ca+2
                   8.3     PERCENT BOUND IN SPECIES #1507320   CaSO4 AQ
                   1.1     PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ

Fe+2              71.8     PERCENT BOUND IN SPECIES #     280   Fe+2
                   5.8     PERCENT BOUND IN SPECIES #2807320   FeSO4 AQ
                  15.6     PERCENT BOUND IN SPECIES #2805800   FeH2PO4
+
                   6.6     PERCENT BOUND IN SPECIES #2805801   FeHPO4
AQ

Na+1              99.6     PERCENT BOUND IN SPECIES #     500   Na+1

Mn+2              92.3     PERCENT BOUND IN SPECIES #     470   Mn+2
                   7.6     PERCENT BOUND IN SPECIES #4707320   MnSO4 AQ
Zn+2              89.7     PERCENT BOUND IN SPECIES #     950   Zn+2
```

```
                9.5      PERCENT BOUND IN SPECIES #9507320   ZnSO4 AQ

Cu+1          100.0      PERCENT BOUND IN SPECIES #    230   Cu+1

PO4-3           2.5      PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+
                3.1      PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ
                4.5      PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ
                4.0      PERCENT BOUND IN SPECIES #1505802   CaH2PO4
+
                7.6      PERCENT BOUND IN SPECIES #3305800   HPO4 -2
               78.3      PERCENT BOUND IN SPECIES #3305801   H2PO4 -

SO4-2          74.7      PERCENT BOUND IN SPECIES #    732   SO4-2
                7.3      PERCENT BOUND IN SPECIES #4607320   MgSO4 AQ
               16.6      PERCENT BOUND IN SPECIES #1507320   CaSO4 AQ
                1.4      PERCENT BOUND IN SPECIES #4107320   KSO4 -

NO3-1         100.0      PERCENT BOUND IN SPECIES #    492   NO3-1

EDTA-4         97.9      PERCENT BOUND IN SPECIES #2819690   Fe EDTA
                1.9      PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

H3BO3          99.9      PERCENT BOUND IN SPECIES #     90   H3BO3

Cl-1          100.0      PERCENT BOUND IN SPECIES #    180   Cl-1

MoO4-2         99.4      PERCENT BOUND IN SPECIES #    480   MoO4-2

CO3-2          33.7      PERCENT BOUND IN SPECIES #3301400   HCO3 -
               65.2      PERCENT BOUND IN SPECIES #3301401   H2CO3 AQ

Cu+2           41.6      PERCENT BOUND IN SPECIES #    231   Cu+2
                3.9      PERCENT BOUND IN SPECIES #2317320   CuSO4 AQ
               53.7      PERCENT BOUND IN SPECIES #2319691   CuEDTA

H+1             2.7      PERCENT BOUND IN SPECIES #4605801   MgH2PO4
+
                1.7      PERCENT BOUND IN SPECIES #4605802   MgHPO4
AQ
                2.4      PERCENT BOUND IN SPECIES #1505800   CaHPO4
AQ
                4.2      PERCENT BOUND IN SPECIES #1505802   CaH2PO4
+
                1.1      PERCENT BOUND IN SPECIES #3301401   H2CO3 AQ
                4.0      PERCENT BOUND IN SPECIES #3305800   HPO4 -2
               83.6      PERCENT BOUND IN SPECIES #3305801   H2PO4 -

E-1           100.0      PERCENT BOUND IN SPECIES #4700020   MnO4 -

H2O            16.8      PERCENT BOUND IN SPECIES #2813301   FeOH2 +
               82.0      PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

Fe+3           97.9      PERCENT BOUND IN SPECIES #2819690   Fe EDTA
                1.9      PERCENT BOUND IN SPECIES #2819692   FeOHEDTA

Mn+3          100.0      PERCENT BOUND IN SPECIES #    471   Mn+3
```

C. NICKEL-TREATMENT NUTRIENT SOLUTION

PART 1 of OUTPUT FILE

PC MINTEQA2 v3.10 DATE OF CALCULATIONS: 10-NOV-97 TIME: 13:55: 1

```
bestNi1
Ni added, pH=6
---------------------------------------------------------------------------
---------------------------------------------------------------------------
  330 0.000E-01  -6.00 y
  410 5.000E-03  -2.30 y
  460 2.000E-03  -2.70 y
  150 4.000E-03  -2.40 y
  281 8.990E-05  -4.05 y
  500 9.181E-05  -4.05 y
  470 9.100E-06  -5.04 y
  950 7.600E-07  -6.12 y
  231 3.200E-07  -6.49 y
  580 1.000E-03  -3.00 y
  732 2.001E-03  -2.70 y
  492 1.200E-02  -1.92 y
  969 8.990E-05  -4.05 y
   90 4.600E-05  -4.34 y
  180 5.220E-05  -4.74 y
  140 0.000E-01 -16.00 y
  480 1.100E-07  -3.96
    1 0.000E-01 -15.60
  280 0.000E-01 -16.00
  471 0.000E-01 -16.00 y
  230 0.000E-01 -16.00 y
  540 1.700E-05  -4.77

H2O has been inserted as a COMPONENT
 3  6
3301403   21.6600   -0.5300
3300021  -82.4412  133.8300
2802810   13.0320  -10.0000
4704710   25.5070   25.7600
2302310    2.7200    1.6500
    330    5.9990    0.0000
 6  1
    1    0.0000    0.0000
```

PART 4 of OUTPUT FILE

PC MINTEQA2 v3.10 DATE OF CALCULATIONS: 10-NOV-97 TIME: 13:55: 2

```
          PERCENTAGE DISTRIBUTION OF COMPONENTS AMONG
          TYPE I and TYPE II (dissolved and adsorbed) species

Ni+2        49.3  PERCENT BOUND IN SPECIES #    540  Ni+2
             4.4  PERCENT BOUND IN SPECIES #5407320  NiSO4 AQ
            46.3  PERCENT BOUND IN SPECIES #5409691  NIEDTA

K+1         99.4  PERCENT BOUND IN SPECIES #    410  K+1

Mg+2        89.9  PERCENT BOUND IN SPECIES #    460  Mg+2
             7.3  PERCENT BOUND IN SPECIES #4607320  MgSO4 AQ
             1.3  PERCENT BOUND IN SPECIES #4605801  MgH2PO4 +
             1.5  PERCENT BOUND IN SPECIES #4605802  MgHPO4 AQ
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Component	Percent		Species #	Species
Ca+2	89.6	PERCENT BOUND IN SPECIES #	150	Ca+2
	8.3	PERCENT BOUND IN SPECIES #	1507320	CaSO4 AQ
	1.1	PERCENT BOUND IN SPECIES #	1505800	CaHPO4 AQ
Fe+2	72.0	PERCENT BOUND IN SPECIES #	280	Fe+2
	5.8	PERCENT BOUND IN SPECIES #	2807320	FeSO4 AQ
	15.6	PERCENT BOUND IN SPECIES #	2805800	FeH2PO4 +
	6.6	PERCENT BOUND IN SPECIES #	2805801	FeHPO4 AQ
Na+1	99.6	PERCENT BOUND IN SPECIES #	500	Na+1
Mn+2	92.3	PERCENT BOUND IN SPECIES #	470	Mn+2
	7.6	PERCENT BOUND IN SPECIES #	4707320	MnSO4 AQ
Zn+2	90.2	PERCENT BOUND IN SPECIES #	950	Zn+2
	9.6	PERCENT BOUND IN SPECIES #	9507320	ZnSO4 AQ
Cu+1	99.9	PERCENT BOUND IN SPECIES #	230	Cu+1
PO4-3	2.5	PERCENT BOUND IN SPECIES #	4605801	MgH2PO4 +
	3.1	PERCENT BOUND IN SPECIES #	4605802	MgHPO4 AQ
	4.5	PERCENT BOUND IN SPECIES #	1505800	CaHPO4 AQ
	4.0	PERCENT BOUND IN SPECIES #	1505802	CaH2PO4 +
	7.6	PERCENT BOUND IN SPECIES #	3305800	HPO4 -2
	78.3	PERCENT BOUND IN SPECIES #	3305801	H2PO4 -
SO4-2	74.7	PERCENT BOUND IN SPECIES #	732	SO4-2
	7.2	PERCENT BOUND IN SPECIES #	4607320	MgSO4 AQ
	16.5	PERCENT BOUND IN SPECIES #	1507320	CaSO4 AQ
	1.4	PERCENT BOUND IN SPECIES #	4107320	KSO4 -
NO3-1	100.0	PERCENT BOUND IN SPECIES #	492	NO3-1
EDTA-4	8.8	PERCENT BOUND IN SPECIES #	5409691	NIEDTA
	89.5	PERCENT BOUND IN SPECIES #	2819690	Fe EDTA
	1.8	PERCENT BOUND IN SPECIES #	2819692	FeOHEDTA
H3BO3	99.9	PERCENT BOUND IN SPECIES #	90	H3BO3
Cl-1	100.0	PERCENT BOUND IN SPECIES #	180	Cl-1
MoO4-2	99.4	PERCENT BOUND IN SPECIES #	480	MoO4-2
CO3-2	33.7	PERCENT BOUND IN SPECIES #	3301400	HCO3 -
	65.1	PERCENT BOUND IN SPECIES #	3301401	H2CO3 AQ
Cu+2	87.8	PERCENT BOUND IN SPECIES #	231	Cu+2
	1.0	PERCENT BOUND IN SPECIES #	2313301	Cu(OH)2 AQ
	8.1	PERCENT BOUND IN SPECIES #	2317320	CuSO4 AQ
	2.4	PERCENT BOUND IN SPECIES #	2319691	CuEDTA
H+1	2.7	PERCENT BOUND IN SPECIES #	4605801	MgH2PO4 +
	1.7	PERCENT BOUND IN SPECIES #	4605802	MgHPO4 AQ
	2.4	PERCENT BOUND IN SPECIES #	1505800	CaHPO4 AQ
	4.3	PERCENT BOUND IN SPECIES #	1505802	CaH2PO4 +
	1.1	PERCENT BOUND IN SPECIES #	3301401	H2CO3 AQ
	4.1	PERCENT BOUND IN SPECIES #	3305800	HPO4 -2
	84.3	PERCENT BOUND IN SPECIES #	3305801	H2PO4 -
E-1	100.0	PERCENT BOUND IN SPECIES #	4700020	MnO4 -

H2O	89.2	PERCENT BOUND IN SPECIES	#2813301	FeOH2 +
	1.3	PERCENT BOUND IN SPECIES	#2813302	FeOH3 AQ
	9.1	PERCENT BOUND IN SPECIES	#2819692	FeOHEDTA
Fe+3	8.6	PERCENT BOUND IN SPECIES	#2813301	FeOH2 +
	89.5	PERCENT BOUND IN SPECIES	#2819690	Fe EDTA
	1.8	PERCENT BOUND IN SPECIES	#2819692	FeOHEDTA
Mn+3	100.0	PERCENT BOUND IN SPECIES	# 471	Mn+3