Cobalt: Physiological Effects and Uptake Mechanisms in Plants

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CONTENTS

SUMMARY

This thesis describes an investigation into the mechanism of uptake of cobalt (Co) into plants and the physiological consequences of Co uptake and distribution within plants. The experiments were conducted with mung beans grown in solution culture but comparison was also made with Co uptake in a giant alga, *Chara corallina*.

Co has no known function in plant cells yet there appeared to be 3 possible uptake systems for Co; 1) a high affinity system which saturates at approximately 1 μM with a Km of approximately 0.3 μM, 2) a system with intermediate affinity for Co with a Km of approximately 3 μM and 3) a linear phase which extends to at least 500 μM.

Co influx was sensitive to the internal Co status; plants which been previously exposed to Co had lower ^{60}Co influx that plants which had not been treated with Co. Influx of Co was also sensitive to other divalent cations. The degree of inhibition of influx of ^{60}Co decreased in the order Cd>Cu>Hg>Ni>Zn>Mn>Fe>Pb. Ca and Mg were only inhibitory at high concentrations and this was interpreted in terms of direct effects of these metals on membrane surface charge. Influx was also inhibited by the sulfhydryl reagent NEM, which suggested that the uptake mechanism might involve binding of Co to –SH groups within the membrane.

Experiments with *Chara* showed that Co accumulated in both vacuole and cytoplasm. The rapid appearance of Co in the vacuole suggested that there was a high affinity Co transporter on the tonoplast or alternatively that Co transfer to the vacuole was direct (e.g. by endocytosis). ^{60}Co influx in *Chara* differed from that in mung bean in terms of pH optimum (*Chara:* 6 – 9; mung bean: 5 – 6) and a lower sensitivity to other divalent trace metals. However, influx was sensitive to NEM and to mM concentrations of Ca and Mg, as in mung beans.

Co inhibited growth of mung bean seedlings in ¼ Hoagland's solution at 5 μM but not at 0.5 μM. Co caused alterations in the concentrations of micronutrient elements and to a lesser extent macronutrient elements. The main visual symptoms of Co toxicity were similar to

those of Fe deficiency, which was consistent with the large reduction of Fe content in plants exposed to Co, even at 0.5 μM. Toxicity of Co was ameliorated by increasing Ca concentration in the range 0.1 to 1 mM. A comparison was made between the effects of Co and of other toxic metals, Hg, Zn, Cd, Pb, Cu and Ni on growth and nutrient balance of mung beans. Metals, Zn, Cn, Ni are also essential nutrients, but there are some situations (e.g high concentrations) when they can become toxic.

DECLARATION

To the best of my knowledge and belief, this thesis contains no material that has been submitted or accepted for the award of any other degree or diploma in any university, and it contains no material previously written or published by any other person, except where due reference is made in the text.

I give consent to the thesis being made available for loan and photocopying if accepted for the award of the degree.

Juhong Liu

October, 1998

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ABBREVIATIONS

A	absorbance
ACC	1-aminocyclopropane-1-carboxylic acid
AES	atomic emission spectrometry
ANOVA	analysis of variance
APW	artificial pond water
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CHES	2[N-cyclohexylamino]ethanesulfonic acid
chl	chlorophyll
CHX	cycloheximide
cpm	counts per minute
dH_2O	deionised water
ddH_20	double deionised water
DM	dry matter
EDTA	ethylene-diamine-tetra-acetic-acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]N,N,N'N'-tetraacetic acid
EPPS	N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid
FW	fresh weight
ICP	inductively coupled plasma
lsd	least significant difference
MES	2-[N-morpholino]ethanesulfonic acid
MOPSO	3-[N-morpholino]-2-hydroxypropanesulfonic acid
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
RO	reverse osmosis
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tris	tris[hydroxymethyl]aminomethane

CHAPTER 1. Introduction

1.1 Overview

1.1.1 Physical and chemical properties of cobalt

Cobalt is ranked 32nd of all the elements, and 19th of the trace elements, in terms of abundance (Peterson and Girling, 1981). Principle cobalt minerals include carrollite ($CuCo_2S_4$ or $CuS{\cdot}Co_2S_3$), linnaeite (Co_3S_4), smaltite ($CoAs_2$), safflorite ($CoAs_2$), skutterudite ($CoAs_3$), cobaltite (CoAsS), erythrite ($3CoO{\cdot}As_2O_5{\cdot}8H_2O$), sphaerocobaltite ($CoCO_3$) and asbolite ($CoO_2{\cdot}MnO_2{\cdot}4H_2O$) (Young, 1948). Cobalt is of importance due to its usage in dyes and as one of the components in alloys (Young, 1948). Major cobalt ore deposits are located in Congo, Morocco and Canada (Lide, 1991).

Georg Brandt discovered cobalt in about 1735 (Weeks, 1942; Lide, 1991), and the element is defined as follows:

Atomic weight: 58.93

Atomic number: 27

Melting point 1495^0C

Boiling point 2870^0C

Specific gravity 8.9

Valence 2 or 3

Cobalt ($4s^23d^7$) stands between iron and nickel in group VIII of the elements, and above rhodium and iridium. It is similar to iron and nickel in appearance and in ferromagnetic characteristics (Nicholls, 1974). As with iron, cobalt is a typical electropositive metal and reacts with acids to form salts. However, salts of cobalt (III) are rare and have to be prepared indirectly. These salts, such as CoF_3 and $Co_2(SO_4)_3$, are only stable in the solid state; in solution, cobalt (III) is rapidly reduced to cobalt(II) with the water being oxidised

to oxygen (Phipps, 1976). For this reason, Co (III) in simple forms is not stable and only exists as complexes in aqueous solution. In contrast, simple salts of Co (II) are the most stable form in aqueous solution and the addition of some ligands leads them to be converted into the +3 state (Nicholls, 1974). Cobalt in the cobaltous state (Co (II)) forms cobalamin or vitamin B_{12} and its derivatives, which are the only biologically active cobalt complexes found in organisms, where Co exists as Co (III) (Peterson and Girling, 1981). The mononuclear hydrolysis products of Co (II) include those between $CoOH^+$ to $Co(OH)_4^{2-}$ (Baes and Mesmer, 1976). There is also evidence for formation by polynuclear hydrolysis, of Co_2OH^{3+} and $Co_4(OH)_4^{4+}$, at relatively high concentrations of Co (II) (Baes and Mesmer, 1976). In solution, cobalt forms various complexes with ligands. Figure 1.1 shows the activity of Co complexes of 1 μM Co^{2+} as a function of pH in 1/4 modified Hoagland Solution (Johnson *et al.*, 1957) without EDTA, as computed by GEOCHEM PC (Parker *et al.*, 1995). The major Co complex is with PO_4^{3-} in various forms. Cobalt is strongly chelated by EDTA in modified Hoagland's solution (Parker *et al.*, 1995).

1.1.2 Biochemistry of Cobalt

Cobalt is an essential element for organisms requiring vitamin B_{12}, such as microorganisms and animals including humans. It should exist in the nutrients of these organisms either in the form of vitamin the B_{12} complex or in the form of ions. Ruminants require dietary cobalt for the microorganisms in their rumen to synthesize vitamin B_{12}; non-ruminants, which do not have such microorganisms, require vitamin B_{12} in their diet. Cobalt is also needed by *Rhizobia* in the root nodules of leguminous plants and some species of nitrogen-fixing blue-green algae for symbiotic nitrogen fixation (Holm-Hansen *et al.*, 1954; Lowe and Evans, 1962).

There is no evidence that cobalt is essential for other plants. However, the deficiency of Co in grass and feedstuffs leads to Co deficiency diseases in ruminants causing bone fragility, anaemia, reproductive failure, emaciation, scaly skin, rough coat, progressive loss of appetite and even death. The disease was first found in the eighteenth century in Great

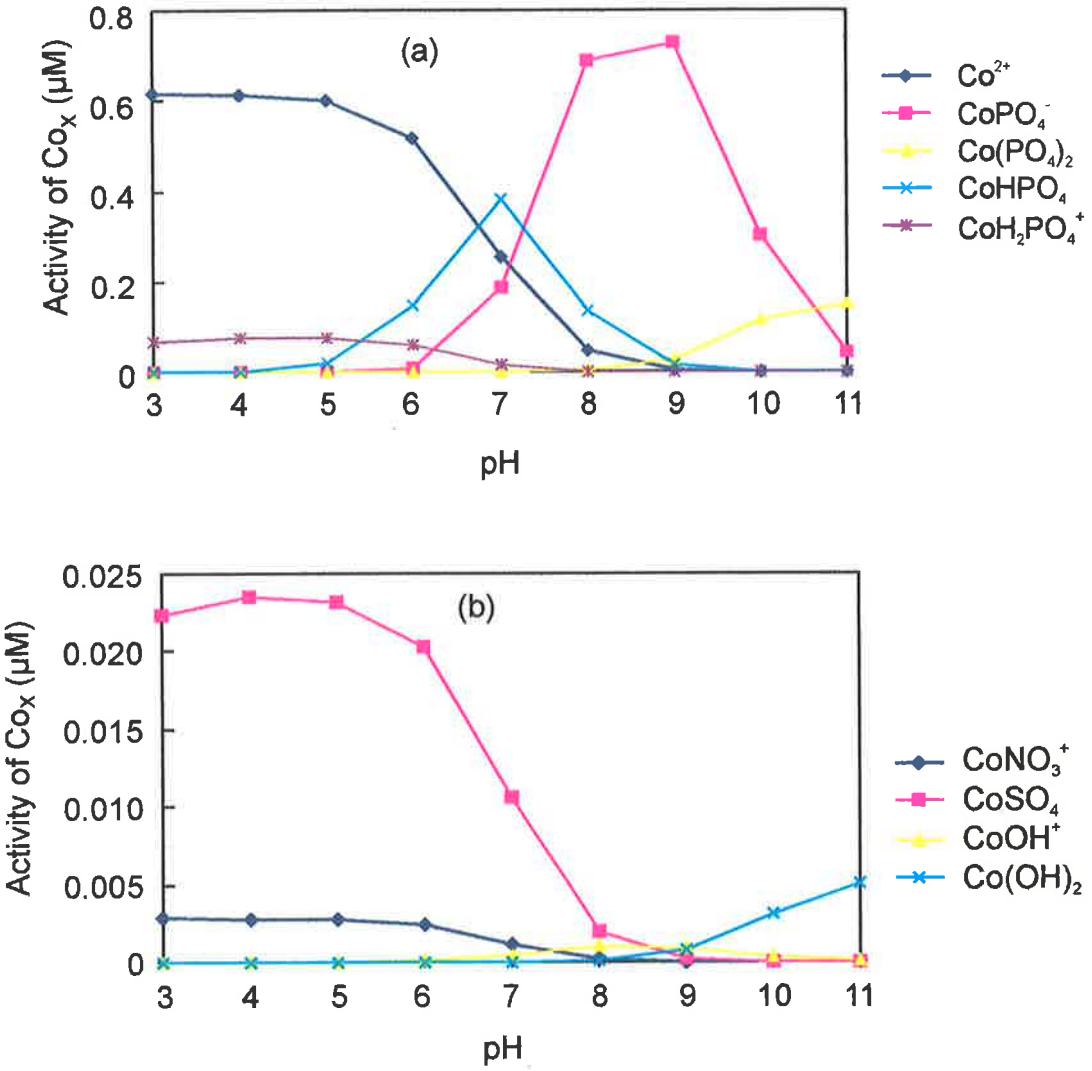


Figure 1.1 Activity of Co^{2+} and Co-complexes in ¼ Hoagland's solution containing 1 μM Co. (a) major species, (b) minor species as computed by GEOCHEM-PC.

Britain, and later in Western Australia, South Australia, New Zealand, Kenya and Florida (Lee, 1975; Sauchelli, 1969; Underwood, 1971). The real cause of the disease as Co deficiency was first revealed in Australia in 1935 (Underwood and Filmer, 1935).

1.1.3 Cobalt in plants and in the environment

The content of Co in plants varies from species to species. Generally, legumes contain more Co than grasses, and grasses contain more than grain crops (Sauchelli, 1969; Sillanpaa and Jansson, 1992). The general range of cobalt content in plants is 0.03-0.55 ppm DM (Sillanpaa and Jansson, 1992). A cobalt content in plants of 0.2 to 0.5 ppm is deemed to be sufficient, while <0.2 ppm is deemed critical and >0.5 ppm to be toxic (Bennet, 1994). However, range wheat grasses (*Agropyron spp.*) and swamp black gum (*Nyssa sylvatica*) are two exceptions in accumulating and tolerating cobalt. The former contains cobalt at up to 23 ppm, the latter at 59, or even 845 ppm, without symptoms of toxicity (Peterson and Girling, 1981). A very few hyperaccumulator species are able to grow on serpentine soils containing high concentrations of Co, and to tolerate internal Co contents of 4,000 to 10,000 ppm (Brookes and Malaisse 1989).

The content of cobalt in plants also depends on the availability of the metal in the soil. Total cobalt content in soil varies with geographical zones and with the rocks from which the soil originates. Cobalt content ranges from 0.01-300 ppm, with most soils containing 10-15 ppm (Aubert and Pinta, 1977). The availability of cobalt to plants is affected by soil pH, drainage status, and content of humus and MnO_2 (McKenzie, 1975; Palit *et al.*, 1994; Smith, 1990). Co becomes more available to plants when the pH and humus content decrease. The availability of Co decreases in well-drained soils, compared with poorly drained ones (Smith, 1990; Palit *et al.*, 1994). MnO_2 fixes Co rapidly, and the addition of MnO_2 in soil decreases the availability of the metal (McKenzie, 1975). Cobalt in most soils in the world is sufficient for plants, and toxicity to plants is rare. However, deficiency occurs in some soils, and is a major concern for some countries, such as Sierra Leone, Malta, New Zealand and Finland (Sillanpaa and Jansson, 1994).

Cobalt deficiency in South Australia is widespread. The deficiency is found most severely in coastal regions, and less severely on some types of inland soil. It affects legumes and grass, and consequently, sheep and cattle. In these areas Co supplements are needed, and the feeding of Co to sheep and cattle makes possible the raising of animals (Lee, 1951; Dewey *et al.*, 1958).

Compared to other heavy metals, Co is not regarded as very toxic to plants and other organisms. Toxicity to plants rarely occurs in natural soils (Sillanpaa and Jasson, 1994) unless they are contaminated by pollution (Ormrod *et al.*, 1980). For these reasons perhaps, attention has been paid to Co deficiency, and consequently Co has been widely used in feedstuff and fertilizers to improve animal and crop production. It has also been tested for its potential in pruning roots in container plants (Baker *et al.*, 1995) and in prolonging the shelf life or storage life of horticultural products (Abeles *et al.*, 1992).

Toxicity, when it occurs, is mainly due to human practices. For example, the application of excess Co to crops and animals can cause toxicity, not only to the crops and animals themselves, but also to their consumers through the food chain. In industry, Co is widely used in alloys; Co powder is prepared and used in manufacturing alloys and may be released during their use. The powder is also a powerful health risk for workers (Lauwerys and Lison, 1994). In the glass and ceramics industries, compounds of cobalt are used as pigment and in the oil and chemical industries, they are used as drying agents for paints, varnishes, printing, inks and catalysts (Lauwerys and Lison, 1994). In the past cobalt was also added to beer to improve the stability of the foam but was banned since the incidence of poisoning in heavy beer drinkers became clear (Mercier and Patry, 1967). The health effects of cobalt exposure include allergic contact dermatitis in skin, allergic bronchial asthma, interstitial fibrosis, lymphocytic alveolitis, lung cancer; and myocardiopathy (Sabbioni *et al.*, 1994).

In animals, Co toxicity can result from repeated feeding with Co salts over a long period of time (Young, 1979). Hill (1974) reported that Co at 100 mg/kg depressed the growth of chicks. This was partly reversed by Fe, and at 50 mg/kg Co increased the susceptibility of

chicks to *Salmonella gallinarum*. In cattle, Co toxicity is related to listlessness, rough coat, weight loss and muscular incoordination, but is extremely difficult to diagnose due to the lack of specific clinical and pathological signs (Dickson and Bond, 1974). Dickson and Bond (1974) reported that in several field cases, the death of cattle from Co feeding was related to higher Co content (20-62 ppm DM) in the liver compared to levels in healthy cattle of 0.08-0.12 ppm DM. This led the authors to highlight the potential dangers of excess Co feeding to domesticated animals. In relation to this, Huck and Clawson (1976) reported that pigs developed anorexia, growth depression, stiffness, humped back, incoordination and extreme muscular tremors when fed with 400 or 600 mg Co/kg.

In addition to the direct application of Co to plants, environmental pollution is a potential source for Co toxicity in plants. For example in the northeast of Cairo there has been a long history (10-60 years) of sewage irrigation. As a result of this, the content of Co and other metals (Fe, Zn, Mn, Cu, Ni, Pb, and Cd) in naval orange trees has increased, although no toxicity has been observed (Omran *et al.*, 1988). Similarly, it has been reported that the Co content in the saturation extracts obtained from sludges in California is consistently greater than that of California soils (Brandford *et al.*, 1975). In southern Ontario, Co severely contaminated vegetation and soil in the vicinity of a nickel refinery (Temple and Bisessar, 1981), and a reduction of 79% in shoot weight was observed on celery grown in contaminated soils containing 7500 ppm Ni, 800 ppm Cu and 100 ppm Co (Bisessar *et al.*, 1983). The contamination of soil with the trace metals was mainly due to air emissions (Linzon, 1981).

The utilization of Co may have beneficial effects for the time being, but the widespread use of Co, in combination with industrial pollution, may lead too much higher levels of Co in the environment in the future. Hence the toxicity of Co should not be ignored, either in research to better understand the mechanism of the metal on plants and other organisms, or in agricultural and industrial practices.

1.2 PHYSIOLOGICAL EFFECTS OF CO ON PLANTS

1.2.1. Beneficial Effects

Cobalt is not defined as an essential element for plants. Even in legumes, where Co is needed by *Rhizobia* for nitrogen fixation in the form of vitamin B_{12}, the plants themselves do not need it. However, the supplementation of Co to plants has been shown to be effective in improving crop yields (Young, 1979). Co can be applied to plants in experiments and in practice in three ways. The most popular way is to add cobalt sulphate, nitrate, or chloride to the soil or other media. The second method is to soak seeds or tubers in $CoSO_4$, $Co(NO_3)_2$, or $CoCl_2$ for 2-24 hours, or alternatively to dust seeds with finely powdered $CoSO_4$ or $Co(NO_3)_2$, before sowing, although the latter method is less used than the former. The third way is to spray young plants with diluted solution of $CoSO_4$ or $CoCl_2$, with a concentration of 0.005-0.2% (Young, 1979).

These methods can be combined to get the best improvement in yield. In groundnuts (*Arachis hypogaea* cv TMV_2,), application of Co by seed treatment, foliar spray, seed treatment followed by foliar spray, and seed treatment followed by Rhizobium inoculation have all resulted in significant increases in nodule development and pod yield. Among these methods, seed treatment and foliar spray obtained the highest yield (Reddy and Raj, 1975).

Crops which have been reported to benefit from the application of Co include legumes (cowpea, groundnut and soybean), cereals (barley, buckwheat, corn, oats and wheat), grass (timothy), fruit (apple and grape), vegetables (Chinese cabbage and potato), and sugar beet (Young, 1979).

The effect of Co application on leguminous plants generally includes an increase in chlorophyll content, net assimilation rate, nodule number, leghaemoglobin, growth rate, and as increase in the number of branches and leaves (Raj, 1987). It has been reported that two cycles of presowing, soaking and drying seeds with 1 ppm cobalt nitrite increases nodulation, dry matter, nitrogen content and yield in the bean *Phaseolus vulgaris* L. The

effect was more pronounced than with 2 or 5 ppm Co alone, or by combination with 1, 2, or 5 ppm sodium molybdate (Mohandas, 1985). The beneficial effects of Co application on legumes have also been reported by other workers (Riley and Dilworth, 1985; Gopal and Singh, 1995; Jana *et al.*, 1995; Viny *et al.*, 1995).

Even in legumes, the responses to the application of Co are different among species. In six legume species planted on a sandy lateritic soil of marginal Co, the addition of Co increased dry matter yield by nearly 50% in *Lupinus angustifolius*, but no effect was observed in the species *Lupinus cosentinii, Lupinus luteus, Trifolium hirtum, Trifolium subterraneum and Vicia atropurpurea* (Gladstones *et al.*, 1977).

The beneficial effects of Co to other crops include senescence retardation in lettuce (Tosh *et al.*, 1979), germination improvement in sunflower (Singh and Rao, 1993), tomato and cucumber (Helmy *et al.*, 1994), prevention of floral malformation in mango (Zora *et al.*, 1993; Zora *et al.*, 1994), prolongation of the vase life of cut flowers, maiden hair ferns (Fujino and Reid, 1983), marigolds (Chandra *et al.*, 1981), and roses (Venkotarayappa *et al.*, 1980), ozone and draught tolerance (Young, 1979; Wenzel *et al.*, 1995), and wilt disease control in guava (Dwivedi, 1991).

It is well established that Co is essential for blue-green algae and microorganisms in fixing N_2. The active form of Co in root nodules is a cobamide co-enzyme or cobalamine synthesized by nodule bacteria (Shkolnik, 1984). Cobamide co-enzymes are involved in the reactions of several enzymes (for example, methylmalony-CoA isomerase, glutamate mutase, glycerol dehydratase, diol dehydratase, ethanolamine deaminase and β-lysine mutase) for H transfer during the formation of NH_3 by *Rhizobia* (Nicholas, 1975). In non-nodulated pea plants, Fries (1962) detected traces of cobamide co-enzymes, but it was not clear if the compounds originated from microorganisms. Wilson and Nicholas (1967) reported that the growth of non-nodulated legumes and wheat grown under sterile conditions needed Co, 90% of which existed in the plants in the form of small molecular weight complexes other than the known cobamide compounds and inorganic cobalt. However, the biological

function of these complexes is not known, and neither are the functions of cobalt on non-leguminous plants. There is evidence that Co may function in respiration, photosynthesis, and protein and fatty acid synthesis in plants (Shkolnik, 1984). An improvement in photosynthesis, and the content of protein, sugar and other carbohydrates by application of Co has been reported in both legumes and non-leguminous plants (Young, 1979).

Due to the lack of the evidence for bioactive forms of Co in plants, it is not easy to understand the mechanism for the beneficial effects of Co on plants. Presumably Co exerts indirect effects on plant metabolism at low concentrations. Evidence indicates that these effects may result from the role of Co in cross-linked interactions with other elements (see below). The beneficial effects of Co on plants are also attributed to its inhibition of the production of ethylene production, which regulates such processes as germination, growth, ripening, senescence, and stress resistance. It is assumed that Co inhibits the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene through inhibiting ACC oxidase activity in the ethylene biosynthetic pathway (Yu and Yang, 1979).

The symptoms of Co deficiency in plants include chlorosis and growth retardation in wheat and non-nodulated clover (Wilson and Nicholas, 1967), and growth retardation in rubber, tomatoes and non-nodulated subterranean clover (Nicholas, 1975). Co deficiency in legumes can be assessed from the Co content of shoots. Critical deficiency occurs when shoot Co falls to between 0.04 (Ozanne *et al.*, 1963) and 0.02 mg/kg dry weight (Robson *et al.*, 1979). While a deficiency of Co in nodulated legumes may be due to a limitation on nitrogen fixation by *Rhizobia*, the symptoms of Co deficiency in other plants and non-nodulated legumes remain to be investigated.

1.2.2 Toxicity of Co in plants

The critical level of Co which causes toxicity to plants varies with species, and is generally within the range of 30-40 ppm DM (Kabata-Pendias and Pendias, 1991). The toxicity

symptoms include interveinal chlorosis of new leaves, white leaf margins and tips, leaf chlorosis and damaged root tips (Kabata-Pendias and Pendias, 1991).

1.2.2.1 Toxic effects of Co on plant yield

Toxic effects of Co have been compared to those of other trace metals. In chrysanthemum, 0.1mM Co resulted in a growth reduction of 45%, which was equal to Ni, greater than Zn (21%), but less than Cd (70%), Cu (70%) and Cr (70%) at the same concentration (Patel *et al.*, 1976). Reduction in growth was also observed in *Lolium multiflorum* grown on perlite supplemented with 100 mg Co/l (Azpiazu, 1989), and -green algae *Kirchneriella lunaris* (Issa *et al.*, 1995). In barley, the effectiveness of Co among five metals tested in decreasing DM yield of plants was in the order of Ni>Co>Zn>Mn>Cu, and, in inducing the visual symptoms of toxicity, was in the order of Ni>Co>Cu>Mn>Zn (Agarwala *et al.*, 1977). In young spring barley, the minimum content of Co in the leaves and shoots required to reduce the yield was determined at 6 ppm of DM, in comparison with other elements in the order:

Be(0.6)<V(2)<Hg(3)<Ag=Li(4)<Co(6)<Cr(10)<Mn=Cd(15)<As=Cu=Ti(20)<
Ni(26)<Se(30)<Pb(35)<Sn(63)<Mo(135)<Zn(290)<Ba(500) (Davis *et al.*, 1978).

1.2.2.2 Toxic effects of Co on seed germination and seedling growth

Several researchers have noted the toxic effects of Co on seed germination and seedling growth. For example, Co at high concentration inhibited the growth of radicles in *Vicia faba,* although the toxicity was the least among the tested elements following the order Cd > Ni > Zn = Co (Jyoti *et al.*, 1994). In other research, an inhibition by 1 ppm Co of the root growth of *Allium cepa* was associated with root twisting, abnormal nucleoli and irregularly shaped and disintegrated nuclei of root-tip cells (Liu *et al.*, 1995). Herich and Bobak (1976) reported that in the meristematic cells of *Vicia faba* L. root tips, Co treatment resulted in differentiation of the endoplasmic reticulum and dilation or destruction of membranes when the treatment was prolonged. Other researchers showed that seed soaking in Co or other metals (Cd, Ni and Zn) for 6-24 hours resulted in a decrease in germination percentage and seedling growth in soybeans, with the effects increasing as the concentration and soaking

time increased (Dubey and Dwivedi, 1987). Co has also been reported to inhibit seed germination and the elongation of the roots and hypocotyls of *Cyamoposis tetragonoloba* (Thukral and Kaur, 1987).

1.2.2.3 Toxic effects of Co on pollen germination and pollen tube growth

Co may inhibit pollen germination and pollen tube growth. The inhibition by Co of pollen germination has been observed in *Amaryllis vittata* (Bhandal and Bala, 1989). In *Lilium longiflorum,* the inhibition by Co of pollen germination and pollen tube growth has been associated with the swelling of the tip region and abnormal cell wall organization in the tube (Sawidis and Reiss, 1995).

1.2.2.4 Toxic effects of Co on photosynthesis

Cobalt and other heavy metals affect photosynthesis and the activities of related enzymes (Van Assche and Clijsters, 1990). For example, 0.4 mM $Co(NO_3)_2$ added to the root system of 10-day-old pea plants resulted in the reduction of chlorophyll content, photosynthesis rate, and fresh and dry mass, in association with the inhibition of ribulose 1,5-bisphosphate (RuBP) carboxylase activity and the stimulation of RuBP-oxygenase and phosphoenolpyruvate carboxylase activities on the fourth day following Co treatment (Angelov *et al.*, 1993). Austerfeld, (1979) reported that cobalt inhibited net photosynthesis in *Phaseolus vulgaris* at 10 μM, the inhibition being more severe in secondary leaves than in primary leaves, although more Co was accumulated in the latter. Similarly, 20 μM Co treatment was found to decrease net photosynthesis, chlorophyll content and foliar iron in the presence or absence of 20 μM iron in both iron-efficient and iron-inefficient cultivars of tomatoes and soybeans (Blaylock *et al.* 1986). Other research found that in lichen, the relative toxicity of Co on photosynthesis was in the order:

Ag=Hg>Co>Cu=Cd>Pb=Ni for short-term exposures, and

Ag=Hg>Cu≥Pb=Co>Ni for extended exposures (Puckett, 1976).

Mohanty *et al.* (1989) observed that Co inhibited 2,6-dichlorophenolindophenol-Hill activity in pea (*Pisum sativum* L. cv. Bombay) chloroplasts, possibly through modifying the secondary quinone electron acceptor (Q_B). In addition, Co affects the transport of ^{14}C-photoassimilates. Samarakoon and Rauser (1979) reported that in *Phaseolus vulgaris*, Co prevented the export of ^{14}C-photoassimilates to the young trifoliate leaves and root tips from the nearly fully expanded unifoliate leaves, where sucrose, reducing sugars and starch were accumulated but not transported to the major sink areas. Other researchers found that excess Co also resulted in the accumulation of callose on phloem sieve plates of *P. vulgaris*, but noted that there was no correlation between translocated ^{14}C-photoassimilates and the amount of callose in the petioles (Peterson and Rauser, 1979).

1.2.2.5 Tolerance of Co in plants

Plants can develop Co tolerance under conditions of high metal concentration. Two clones of *Agrostis gigantea* collected from mine waste in Ontario, Canada by Hogan and Rauser (1979) showed tolerance to cobalt, copper and nickel but not zinc, with one clone showing a higher tolerance to Co and Cu. By contrast, a commercial clone exhibited no tolerance to any of the metals. Similarly, seedlings from *Silene vulgaris* (Moench) seed collected in a heavy metal-contaminated area near a mine in Ontario, Canada have been shown to be more tolerant to elevated levels of cobalt, arsenic and nickel than those from seeds collected at an uncontaminated site (Paliouris and Hutchinson, 1991).

Cell walls appear to play some role in the protection of plants against Co toxicity. In two strains of *Chlamydomonas reinhardtii*, for example a wall-less strain was more sensitive than a walled strain to Co, Cd, Cu and Ni, among which the biggest difference between the strains was observed for Co and for Cu. Both walled and wall-less strains showed higher tolerance to Co at pH 5 than at pH 7 (Macfie *et al.*, 1994).

1.2.3. Effects of Co on enzymes in plants

Co exerts beneficial or toxic effects through modifying enzymatic reactions in plants. Much work has been done to investigate the effects of Co on enzymes in plants, including both the induction and the inhibition of enzyme activity. Generally, at phytotoxic concentrations of heavy metals, some enzymes are induced and others are inhibited, depending on their sensitivity to the metals. The induction of enzymes by metals at toxic concentrations is considered to result from the insensitivity of enzymes to the metals. It is also thought to play a significant role in stress metabolism (Van Assche and Clijsters, 1990).

1.2.3.1 Induction of enzymes

In cucumbers, Co has been shown to increase the activity of urease in the leaves, although the Co-induced urease possessed a lower specific activity than that of Ni-induced urease (Watanabe *et al.*, 1994). In *Simmondsia chinensis*, Prakash and Iyengar (1995) found that seed treatment with Co at 5 ppm for 14 hours before germination resulted in an increase in ribonuclease activity in cotyledons, and acid and alkaline phosphatase activity in both cotyledons and the embryo axis after 10 and 15 days of germination.

The induction of enzyme activity by Co has also been reported for: catalase in cotton, grape vines, meadow foxtail, potato and tomato; dehydrogenase in barley and potato; ATPase and carbohydrase in fodder beets; peroxidase in tomato, grapevines, maize and meadow foxtail; and ascorbic oxidase and polyphenoloxidase in grapevines (Young, 1979; Affa-Aly *et al.*, 1991; Myton and Fry, 1995).

1.2.3.2 Inhibition of enzymes

An enzyme may be induced at low concentration of the heavy metals, and inhibited at higher concentrations. In Japanese pear (cv. Hosui) and apple (cv. Granny Smith), for example, ACC (1-aminocyclopropane-1-carboxylic acid) oxidase activity was stimulated by Co at low concentrations (0.01mM), but inhibited at higher concentrations (>0.02mM) (Tian *et al.*, 1994). Similarly, in germinating rice seeds, Co inhibited alpha-amylase but stimulated beta-

amylase (Shaw and Ou-Lee, 1984). Bisht and Mehrotra (1989) found that Co depressed the specific activity of catalase in maize, but stimulated the activities of beta-glycerophosphatase, ribonuclease and aspartate aminotransferase while Tandon and Awasthi (1979) noted that Co inhibited peroxidase activity at the level of 0.125-2.5 mM in barley seed.

Co has a high affinity for sulfhydryl groups in proteins, peptides, and amino acids (Madsen, 1963), and the inhibitory effects of Co on enzyme activity are generally ascribed to its binding with -SH groups in enzymes. The results of an investigation into the activity of ACC oxidase by Yu and Yang (1979) are in agreement with this assumption: ACC oxidase activity in mung bean was inhibited not only by Co and Hg, but also by sulfhydryl reagents, *p*-chloromercuribenzoate, *N*-ethymaleimide and iodoacetate. Other researchers have noted that the addition of sulfhydryl compounds, cysteine and glutathione restored phenylalanine ammonia-lyase inhibited by either Co or *N*-ethymaleimide in the first internode of *Sorghum bicolor*. This indicated that one of the sites of Co action on the enzyme was the -SH group, with another being the -COOH group, since the additions of citric, malic, oxalic or tartaric acids nullified the effect of Co (Dube *et al.*, 1993).

Other heavy metals possess a similar ability to bind -SH groups and to inactivate enzymes. Experimental results on various enzymatic systems reveal that when -SH groups are involved, the order of inhibitory effect of the metals on enzyme activity is almost identical with the order of metal sulfide insolubility:

Hg, Ag>>Cu>>Pb, Cd, Zn>Co, Ni>Mn (Hewitt and Nicholas, 1963).

The inhibition by Co of enzyme activity may also be a result of its effect on *de novo* protein synthesis *in vivo*. Co forms stable complexes with cysteine (log K_I=9.3 and Log K_{II}=7.6) or the related compounds, for example, glutathione (log K=3.7) (Friendman, 1973), thus lowering the level of free amino acids in the tissue. The depletion of the amino acids would affect protein synthesis and consequently the amount of enzyme, especially for those enzymes that are short lived or which have high turnover rates. If this were the case, the

value of the total activity of the enzymes *in vivo* would be reduced even when the activity of the enzymes is unaffected. This speculation seems logical and may be supported directly or indirectly by the following research:

Evidence from animal cells has shown that protein synthesis is affected by the availability of amino acids. For example, in work by Kimball *et al.* (1996), deprivation of histidine resulted in reduced protein synthesis in both the rat liver and isolated rat hepatocytes. This inhibition was shown to be at both translational and pretranslational levels, and was reversed by the addition of histidine. Other research found that cobalt inhibited ^{14}C-glycine- incorporation into proteins, RNA, and DNA of *Azotobacter agilis*. In this case, the inhibition of protein synthesis was more than 60% at 10 mM Co (Bernlohr and Webster, 1958).

The application of cysteine and other sulfhydryl compounds reduces the toxicity of Co in various organisms. Hill (1979a, 1979b) demonstrated this in his work that found both ascorbic acid and dietary protein reduced the inhibitory effect of Co on growth in chicks. In other research on chicks, Southern and Baker (1982) noted that while excess methionine and cysteine ameliorated the toxicity of Co on the rate of gain in bodyweight, cysteine was nearly six times more effective than methionine. Such amelioration was assumed to be due to the chelation reaction of cysteine with Co (Baker and Czarnecki-Maulden, 1987). Huck and Clawson's (1976) work on pigs showed that the addition of methionine was also effective in overcoming the toxic effects of Co and restoration of serum Fe lowered by Co. Similarly, Dierickx (1996) found that the depletion of glutathione in rat hepatoma-derived Fa32 cells exacerbated the cytotoxicity of Co and other metals (Hg, Cd, Ni, Zn and Cu) 3-12 times. Other research (Singh, 1989) also demonstrated that cystine and cysteine mitigated the toxicity of excess Co (>1μM) on the growth of *Anabaena doliolum* and *Anacystis nidulans*, but methionine was ineffective in this regards.

In research by Yu and Yang (1979), the addition of Co to the incubation medium was followed by a lag period of approximately 3 hours before the onset of inhibition of ACC oxidase activity in the segments of mung bean hypocotyls. This lag was presumed to be due

to the lower level of Co accumulated, but no information on the uptake of Co in the tissue or cells was demonstrated. It could be argued that Co inhibits the synthesis of ACC oxidase and that the existing enzyme decays within three hours following Co application. This is supported by the fact that cycloheximide, a powerful protein synthesis inhibitor, also inhibits ACC oxidase activity (Chou and Kao, 1992). However, information about the uptake and transport of Co at tissue, cellular or subcellular levels is needed to obtain a clearer view.

1.3. Co Uptake, Translocation and Accumulation in Plants

The uptake of cobalt is known to be affected by several factors, such as temperature, Co concentration, pH and the light/dark cycle. The uptake of Co in barley roots has been proposed to be an active process with a Q_{10} of 2.2, and is inhibited by respiratory inhibitors, 0.5 mM difluorodinitrobenzene, 0.5 mM dinitrophenol and 0.1 mM carbony cyanide phenylhydrazone (Colclasure and Schimid, 1974). In other research into ryegrass seedlings of *Lolium perenne*, a linear relationship has been reported to exist between total uptake and the concentration of Co, ranging from 0.01 to 1.0 μM, with a similar slope to that between the rate of transport and the concentration of Co (Macklon and Sim, 1990). Research on barley, however, has shown that total uptake of Co is not linear in relation to Co concentration ranging from 1-100 μM, there being at least three inflections, indicating that a number of carrier sites exist, depending upon Co concentration (Colclasure and Schmid, 1974). In wheat, uptake has been shown to increase proportionally with time after a high initial rate (Macklon and Sim, 1990), while in barley, the slope varied with Co concentration in solutions (Colclasure and Schmid, 1974). Other research noted that uptake showed a strong dependence on pH: increasing pH from 4.5 to 6.0 increased the total uptake of Co by almost 3 times in 1.0μM Co, and more than 4 times from pH 4.5 to pH 7.8 in 0.1 μM Co (Macklon and Sim, 1990).

Uptake of Co may also be affected by light. In wheat seedlings, for example, the total uptake of Co from a solution containing 2 μM Co increased linearly during a period of more

than 60 hours of continuous illumination, while under darkness, uptake was almost totally inhibited (Macklon and Sim, 1987). However, inhibition by darkness of Co uptake was not observed in other research on ryegrass (Macklon and Sim, 1990).

Other heavy metals may also affect Co uptake in plants. For example, accumulation of either Zn or Cu has been shown to suppress Co accumulation in duckweed (Dirilgen and Inel, 1994), while in barley, it has been reported that Ni competitively inhibits Co uptake (Colclasure and Schmid, 1974). The effect of other heavy metals on Co uptake varies with species. In work by Bernal and McGrath (1995), including Zn, Ni and Cd in nutrient solutions decreased the concentration of Co in the shoots of *Alyssum murale*, a Ni accumulator, whereas in the shoots of radish, a non-accumulator of Ni, Co uptake was not affected by the other metals.

Phosphorus and iron also affect Co uptake. Vinay *et al.* (1994) reported that phosphorus increased Co uptake in cluster bean (*Cyamopsis tetragonoloba* L.) at low concentrations (<40mg P/kg soil), but decreased Co uptake at high concentrations (>40mg P/kg soil). Similarly, in the same research, Co induced P uptake at low concentration (<1mg Co/kg FW) and inhibited P uptake at high concentration(>1mg Co/kg FW) (Vinay *et al.*, 1994). In tomato, it has been reported that Co and Fe are competitive elements, and an increase in Fe concentration in nutrient solution reduced Co content in the upper leaves, thus preventing chlorosis induced by a high concentration of Co (0.5 mM) in the nutrient solution (Affa-Aly *et al.*, 1991).

Co is distributed to all parts of plants, but in most cases is mainly accumulated in roots (Young, 1979). Co, like Mn, Ni, Zn and Mo has been found to be translocated to all organs of cabbage plants, but Ti, V, Cr, Fe and Cu have been found to accumulate mainly in the roots (Hara *et al.*, 1976). While Co, V, Ti, Ag, and Cr were found to concentrate in the roots of *Phaseolus vulgaris*, Co was the most mobile metal and induced severe chlorosis at 43 or 143 μg/g dry matter of leaves (Wallace *et al.*, 1977). In the work by Patel *et al.* (1976) on chrysanthemum, Co content, like that of Cu, Cd, Ni and Cr, decreased in the

following order: roots > leaves > stems. The gradient of metals from root to shoot varied with the metals in the order Cr > Co > Cd > Ni > Cu > Zn. There was no gradient in Zn content from root to shoot in chrysanthemum. The root to stem ratios of Co content also depended on the concentration of the metal applied; the ratios were 75, 10, and 1 growth media containing 1, 10, and 100 μM Co respectively. In research on ryegrass, Macklon and Sim (1990) reported that only 11-16% Co absorbed was accumulated in the shoots, and in wheat, only 10% was transported to the shoots (Macklon and Sim, 1987). The transport of Co was not affected by water flux through the plant induced by transpiration, and the amount of Co transported to the shoots was very small in wheat or ryegrass, which led the authors to infer that, similar to Cu, Co is predominately transported in complexes with ligands, such as amino acids or larger molecules. Indeed, in sieve tubes of *Ricinus communis*, cobalt is combined with organic compounds in complexes with a negative overall charge and a molecular weight ranging from 1000-5000 (Wiersma and VanGoor, 1979). Analyzing stem exudates of tomato plants treated with 0-50 μM Co, Tiffin (1967) revealed that like Mn and Zn, Co was translocated primarily as an inorganic cation, whereas Fe was translocated as anionic complexes.

The main location of Co in the cell is unknown, although Terry (1981) found half of the Co in sugar beet leaves was detected in the chloroplast.

1.4. Interaction of Co with other metals

The interaction of Co with other metals plays a part in both its beneficial and its toxic effects on plants.

1.4.1 Calcium

1.4.1.1 Evidence for Co-Ca interaction

Cobalt and other heavy metals (Ni, Cd, Zn, and Cu) have been shown to inhibit the uptake of calcium in intact barley roots (Veltrup, 1981). In green algae, Issa *et al.* (1995) showed

that Ca partly abolished the inhibitory effects of Co on growth, and caused an increase in the ratio of photosynthesis to respiration. Other research (Wallace *et al.*, 1971) indicated that Ca overcomes the toxicity of Co to *Phaseolus vulgaris* seedlings, with the effectiveness being greater at 10 mM $CaCl_2$ than at lower concentrations (1 and 5 mM). The work of Karamushka *et al.* (1996) showed that in *Saccharomyces cerevisiae,* Co in insoluble phosphates and soluble chloride was toxic to glucose-dependent H^+ efflux mediated by H^+-ATPase on the plasma membrane. Ca alleviated this toxicity and it was proposed that this be through competitive and protective interactions at the cell surface.

1.4.1.2 Calmodulin

Calmodulin, a major intracellular calcium binding protein, regulates many key enzymes in cellular processes, for example, adenylate cyclase, guanylate cyclase, Ca-dependent phosphodiesterase, Ca-ATPase, NAD kinase, phosphorylase kinase, myosin light chain kinase, calmodulin-dependent protein kinase(s) and calmodulin-dependent protein phosphatase (Cheung, 1984). Heavy metals were found to substitute for Ca in calmodulin and the effectiveness of substitution depended on the radius of metal cation within the effective range of 100 ± 20 pm. The closer the radius is to calcium (99 pm), the more effective the cation (Chao *et al.*, 1984). The ability of Co with a radius of 72 pm in substituting for Ca in calmodulin and in preventing Ca binding to calmodulin is limited and much less than other heavy metals, Zn^{2+} (74 pm), Mn^{2+} (80 pm), Tb^{3+} (92 pm), Cd^{2+} (97 pm), Sm^{3+} (100 pm), La^{3+} (102 pm), Hg^{2+} (110 pm), Sr^{2+} (113 pm), Pb^{2+} (120 pm) (Chao *et al.*, 1984). It was speculated that heavy metals may disturb many vital cellular functions by substituting for Ca in calmodulin (Cheung, 1984). However, the hypothesis is limited in interpreting the toxicity of other heavy metals based on their ionic radii, Ag^{1+} (126 pm), Al^{3+} (51 pm), Ba^{2+} (137 pm), Be^{2+} (35 pm), Ni^{2+} (69 pm) (Lide, 1991), some of which are highly toxic.

1.4.1.3 Calcium transport

Co is known to block calcium channels in animal cells (Hagiwara and Byerly, 1981) most probably by competing with Ca^{2+} for the Ca channel binding site. The effectiveness of various metals in blocking Ca channel is in the order: La^{3+}, $UO_2^{2+} > Zn^{2+}$, Co^{2+}, $Fe^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Mg^{2+}$ in barnacle muscle (Hagiwara and Takahashi, 1967); $La^{3+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Mg^{2+}$ in egg cell of the tunicate (Okamato *et al.*, 1976); $Ni^{2+} > La^{3+} > Co^{2+} > Mg^{2+}$ for snail neurons (Akaike *et al.*, 1978). For this reason, Co is widely used as an inorganic blockade of calcium channels in animal cells.

The only data on the effect of Co on Ca channels in plant cells appears to be in the work of Piñeros (1995) who showed that wheat plasma membrane Ca channels inserted into lipid bilayers had relative permabilities to other divalent cations in the order: $Ca^{2+} > Zn^{2+} > Ni^{2+} \cong Mg^{2+} \cong Mn^{2+} >> Co^{2+} > Cu^{2+} > Cd^{2+}$.

1.4.2 Iron

The transport of iron from roots to leaves was inhibited by application of excess Co in sugar beet (Terry, 1981). For inducing Fe deficiency in bush beans (*Phaseolus vulgaris* L.), among four metals applied at the rate of 1000 ppm, Co and Ni were the most toxic elements, Cu next, and Zn the least with only mild toxicity (Wallace *et al.*, 1976). In barley, excess Co reduced Fe absorption and translocation to shoots, induced chlorosis and decreased catalase activity of young leaves (Agarwala *et al.*, 1977). The inhibition of Fe uptake by Co was also observed in tomatoes and soybeans, and during the regreening following chlorosis induced by iron-deficiency or excess cobalt, cobalt inhibited the movement of Fe from roots to leaves (Blaylock *et al.*, 1986).

When applied to the growth solution of sugar beet at 0, 17, 34, 85 and 169 μM, Co induced reductions in photosynthesis/unit leaf area, chlorosis and changes in chlorophyll-a:b ratios, carotene and xanthophyll contents, number and size of photosynthetic units and the number and average volume of chloroplasts and leaf cells. These changes were all associated with

the reduction of Fe transport, and were similar to that induced by Fe-deficiency (Terry, 1981). However, in maize, it was reported that Co excess and Fe deficiency were not synonymous, showing differences in leaf deformation, wilted appearance, the activities of peroxidase and aldolase and the pattern of recovery (Bisht and Mehrotra, 1989).

1.4.3 Other Microelements

There is limited data on the effects of Co on other microelements. Co increased the Mn content of the shoots of bush beans (Wallace *et al.*, 1976), and stimulated Cu and Zn in duckweed (Dirigen and Znel, 1994). In fodder beet and maize, seed enrichment with 0.1% Co or Zn decreased the content of the other metal in different plant organs and tissues (Anisimov and Ganicheva, 1978). The interaction was interpreted as not being a simple antagonism during the redistribution of the metals in plants, but a similar action, as observed in their effects on the aldolase and carbonic anhydrase activities and the assimilate transport. Co reduced the growth inhibition induced by Cu in duckweed (Dirilgen and Inel, 1994), and suppressed the uptake of Cd by roots, thus reducing the toxicity of Cu, Ni, Co, Zn and Cd in combination on the growth of bush bean (Wallace and Abou-Zamzam, 1989).

1.5 Summary

As a component of vitamin B12 and cobamide coenzyme, Co is regarded as an essential element for microorganisms, animals and nitrogen fixing blue-green algae. The essentiality of the metal in plants is not defined due to the lack of the evidence for its bioactive forms. While Co deficiency in plants is widespread in the world, Co toxicity rarely occurs under natural circumstances. Application of Co in agriculture and industrial pollution may lead to Co toxicity in the environment and to vegetation.

The content of Co in plants depends on the concentration of Co in soil and its chemical form in the soil, but varies with species. Although Co is translocated into all organs or parts of

the plant, it is mainly accumulated in roots. The uptake of Co is not well established at cellular and subcellular levels in plants.

The application of Co by soil addition, presowing treatment, foliar spray or the combination of the two or three methods may improve the growth and yield of crops or grasses. Legumes benefit from Co application probably because of its role in increasing nitrogen fixation by *Rhizobia*. For non-leguminous plants, the beneficial effects may result from its interaction with other trace metals. Co is also effective in promoting seed germination and seedling growth, and prolonging shelf life or vase life of horticultural products, which is associated with and attributed to its inhibition on ethylene production. It is generally accepted that Co inhibits ACC oxidase activity.

Excess Co causes toxicity to plants. Co induces leaf chlorosis and inhibits photosynthesis possibly through inhibiting Fe uptake and transport. Calcium overcomes the toxicity of cobalt in some cases. It is not clear whether Co affects calcium channels, calmodulin or Ca-regulated protein synthesis, some of which *per se* is not well defined in plants. The investigation of Co uptake and its interaction with other metals may help to understand the mechanisms of Co toxicity.

It is generally believed that the high affinity of Co with -SH groups in proteins or polypeptides is the mechanism by which it inactivates enzymes. Alternatively, it is possible that Co inhibits protein synthesis through depleting free amino acid pool by combining with cysteine or related compounds. The addition of cysteine or related compounds ameliorates Co toxicity in microorganisms, plants and animals, indicating the existence of chelation of Co by the chemicals and consequently the reality of inhibition of Co on protein synthesis *in vivo*.

In general, Co uptake, distribution within plants and its interaction with other elements is poorly understood. It is the aim of this thesis to clarify these processes.

CHAPTER 2. General Materials and Methods

2.1 PLANT MATERIAL

2.1.1 Mung bean

Mung bean seeds were bought commercially and stored at 4°C until use. Mung bean seeds were soaked in RO water for two days with aeration at 20°C for water imbibition and for germination. The germinated seeds with 1 cm radical were planted in base growth solution, 1/4 modified Hoagland's solution (Johnson et al., 1957) under illumination of 16 h (day)/8 h (night) at 28°C (day)/20°C (night) with aeration. In some experiments, FeEDTA was excluded from the growth solution because of the chelating effect of EDTA on Co. It was observed that there was no difference in growth and appearance between the seedlings supplied with and without iron in the early days of growth. The growth solution was changed every 3 days at the beginning of growth and every 2 days after 6 days growth, until harvested. Otherwise, deionised water (dH_20) was added to the original level when needed. The mung bean seedlings obtained thus were used for uptake and growth experiments. Other chemicals of various concentrations were also included in the base growth solution as specified.

2.1.2 *Chara*

Chara corallina was grown outdoors in an artificial pond on a substrate of garden soil and river sand in tap water under natural light conditions. Before experiments, individual internodal cells (40-90 mm in length and approximately 1 mm in diameter) were harvested and incubated overnight in unbuffered artificial pond water (APW) containing (in mM) 1 NaCl, 0.1 K_2SO_4, and 0.5 $CaSO_4$. Since membrane transport of ions may vary with the position of the cells in a *Chara* plant, as shown for Ca^{2+} (Reid and Smith 1992b), only

uncalcified cells from the top of the plants were obtained to ensure similarity in age and physiological conditions between cells for such experiments.

2.2 METHODS

2.2.1 Co uptake

2.2.1.1 Co uptake in mung bean

2.2.1.1.1 Long term uptake and distribution of ^{60}Co

After 3 days growth when all the parts of the seedling were established, $^{60}CoCl_2$ was added to the growth solution at final concentrations of 0.5, 5, 50, 500 and 5000 μM. Counting aliquots of solution monitored the concentration of 60Co. The plants were cultured as described above. More $^{60}CoCl_2$ was added if the concentration of Co was 15% lower than designated. The plants were harvested at 2, 5 and 8 days after application of $^{60}CoCl_2$ by removing the seedlings from the growth solution, followed by rinsing the intact roots in dH_2O for 2 seconds, but without desorption. Co content in roots, hypocotyl and epicotyl + leaves was estimated separately by liquid scintillation counter (Beckman LS 3801, Fullerton, California). Long term uptake rate (I_m) was calculated by a formula in accordance with Williams (1948):

$$I_m = (M_2 - M_1)(\ln R_2 - \ln R_1)(t_2 - t_1)^{-1}(R_2 - R_1)^{-1},$$

Where R_1 and R_2 are the root fresh weights, and M_1 and M_2 are the nutrient contents in the total plant at harvests taken at time t_1 and t_2.

2.2.1.1.2 Short term uptake and transport of ^{60}Co

Uptake. Roots of intact 10 days old seedlings were incubated in aerated uptake solution for a short period under dim light at 22°C. The base uptake solution contained various concentrations of $^{60}CoCl_2$ in ¼ modified Hoagland's solution minus FeEDTA. The

concentration of ^{60}Co in the uptake solution and the period of uptake varied in the experiments as specified. pH in the base uptake solution was 6.0, except in the experiment where the effect on ^{60}Co uptake of pH was examined. In this case, the uptake solution was buffered with 5 mM designated buffers (in brackets) to pH 4.0 (MES), pH 5.0 (MES), pH 6.0 (MES), pH 7.0 (MOPSO), pH 8.0 (EPPS), pH 9.0 (CHES), pH 10.0 (CAPS) and pH 11.0 (CAPS) adjusted with NaOH. In other experiments, specified compounds were added to the base uptake solution to examine the effect on ^{60}Co uptake of other divalent cations, NEM or cysteine.

Desorption. After the designated uptake period, the roots from the intact seedlings were rinsed in dH_2O for 2 seconds to remove surface activity, and then desorbed for 40 min in aerated desorption solution under the same conditions as uptake, with changes of solution every 10 min. The desorption solution contained 5 mM $CaCl_2$ in 1/4 modified Hoagland's solution minus FeEDTA.

Estimation of ^{60}Co content. After desorption, roots and leaves were detached and Co content therein was estimated using methods described above for long term uptake.

2.2.1.2 Co flux measurements in Chara

The base solution for influx was APW buffered with 2 mM MES and adjusted to pH 6.0 with NaOH, except in the experiment in which the effect on influx of pH was examined. In this latter case, APW was buffered with 5 mM designated buffers (in brackets) to pH 4.0 (MES), pH 5.0 (MES), pH 6.0 (MES), pH 7.0 (MOPSO), pH 8.0 (EPPS), pH 9.0 (CHES), pH 10.0 (CAPS) and pH 11.0 (CAPS).

Co uptake and influx measurements using ^{60}Co tracer were conducted as described in Reid and Smith (1992a,b) Reid *et al.* (1996a,b) for other metal ions. Individual internodal cells of *Chara* were incubated in influx solutions containing ^{60}Co in a petri dish with agitation under dim light at 22°C, followed by desorption for 30 min, or as otherwise specified. The desorption solution contained 5 mM $CaCl_2$ and 1 mM $LaCl_3$ and was changed every 5 min unless otherwise specified. To differentiate between ^{60}Co binding in the cell wall and influx

across the cell membrane, the cell was separated into cell wall, vacuole and cytoplasm using the methods described in Reid and Smith (1992a,b). Briefly, the ends of the cell were removed and the cell contents were flushed out by rapidly injecting with a syringe approximately 1 ml of deionised water through the lumen. The ^{60}Co activities in the remaining cell wall and in the flushed contents were then counted separately on a liquid scintillation counter. Where it was necessary to separate activity in the vacuole and in the cytoplasm, a modified procedure was employed. This involved gently injecting an air bubble through the cell to displace the vacuole before flushing out the cytoplasm by rapid injection of deionised water.

A significant problem encountered in early experiments was the depletion of ^{60}Co from solution as a result of the high capacity of cell walls to bind Co. To maintain constant ^{60}Co concentrations in the bathing solutions, large volumes of solution relative to the number of *Chara* cells were used (200-500 mL depending on the experiment). Additionally, the activity was monitored during uptake experiments by counting aliquots of the solution at intervals and, if necessary, adding more ^{60}Co of the same specific activity.

2.2.2 Chlorophyll content and chlorophyll fluorescence

2.2.2.1 Determining chlorophyll content

Chlorophyll was extracted and assayed according to Porra *et al.* (1989). Briefly, that is, one circular disc of primary leaves was cut with a 16 mm diameter punch (approx. 200 mm^2 surface, and 40 mg FW), and ground in a mortar and pestle in 2 ml 80% aqueous acetone buffered by 2.5 mM sodium phosphate (pH 7.8). The homogenate and three washings of the pestle and mortar (1.5 ml buffered acetone each time) were centrifuged at 3500 rpm in a bench centrifuge for 10 min. The pellet was then extracted with a further 1 ml of the buffered acetone in a Potter-Elvejhem homogeniser and the pooled supernatants were

adjusted to a final volume of 8 ml. The absorbance at 646.6 and 663.6 nm was recorded and chlorophyll concentration was calculated as follows:

Chl a $= 13.71\ A^{663.6} - 2.85\ A^{646.6}$

Chl b $= 22.39\ A^{646.6} - 5.42\ A^{663.6}$

Chl a+b $= 19.54\ A^{646.6} + 8.29\ A^{663.6}$

in nmol ml^{-1} , or

Chl a $= 12.25\ A^{663.6} - 2.55\ A^{646.6}$

Chl b $= 20.31\ A^{646.6} - 4.91\ A^{663.6}$

Chl a+b $= 17.67\ A^{646.6} + 7.34\ A^{663.6}$

in $\mu g\ ml^{-1}$ (Porra *et al.*, 1989).

Chlorophyll content was calculated either based on fresh weight ($\mu g\ g^{-1}$ or nmol g^{-1}) or based on leaf area ($\mu mol\ m^{-2}$ or $\mu g\ m^{-2}$). The correlation between the two values was analyzed, and the r = 0.98, 0.96 and 0.97 respectively for chl a, chl b and chl a+b. As a result, either of the two values could be used to compare the difference between treatments. In this thesis, the values based on fresh weight ($\mu g\ g^{-1}$ or nmol g^{-1}) were preferred since the strong inhibition by excess Co of leaf area extension resulted in a higher FW/area ratio.

2.2.2.2 Determining chlorophyll fluorescence

Chlorophyll fluorescence was estimated using an OS-100 Modulated Fluorometer (OPTI - Sciences Inc.), and the yield of energy conversion (Y = (Fms-Fs)/Fms) was recorded using the light adapted test according to the manufacturer's manual.

2.2.3 Growth parameters and nutrient uptake

2.2.3.1 Growth parameters

After a designated period of growth, 12 seedlings were harvested randomly to measure the growth parameters of individual plants, including the number of side roots at least 2 mm in

length, the length of main root, hypocotyl and epicotyl, and fresh weight and dry matter of roots, primary leaves and stems (hypocotyl + epicotyl). The dry matter of leaves, stems and roots was the average of three replicates consisting of 12 plants after drying at 85°C overnight.

2.2.3.2 Nutrient uptake

The dried samples obtained above were analysed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) after digestion by nitric acid (Zarcinas *et al.*, 1987) for the content of elements in the plant, including B, Ca, Co, Cu, Fe, K, Mg, Mn, Na, P, S and Zn.

2.2.4 Data Analysis

Data were calculated and analyzed statistically using the analytical tools of Microsoft Excel 5.0a, ANOVA (single factor or two factor with replication) and Correlation. Multiple comparison between treatments was carried out using least significant difference ($lsd._{05}$ and $lsd._{01}$) or the Newman-Keuls multiple comparison test.

CHAPTER 3. Cobalt Uptake in Mung Beans

3.1 INTRODUCTION

Plants absorb a wide variety of trace metals, but little is known about the actual mechanisms by which the metals are transported across plant cell membranes. Many metals, for which data were available, show uptake isotherms that suggest multiple transport systems. If these transporters are relatively specific for each metal, there are likely to be very large numbers of different membrane transport proteins. This is relevant to those seeking to modify, by molecular means, the characteristics of transporters. It allows a high degree of selectivity for individual trace metal systems, but, at the same time, increases the number of genes and proteins that must be targeted. An alternative proposition is that there are a relatively small number of transporters with affinities and selectivity, which are controlled by internal factors, such as nutrient status or growth rate, and by external factors, such as pH and competition for uptake between metal cations.

Co uptake has been investigated in a number of studies and has been shown to be affected by several factors, such as temperature, pH and light/dark cycle (Colclasure and Schimid, 1974; Macklon and Sim, 1987; Macklon and Sim, 1990). An active uptake mechanism is supported by the sensitivity of uptake to metabolic inhibitors (Colclasure and Schimid, 1974). Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} also inhibit uptake (Colclasure and Schimid, 1974; Affa-Aly et al., 1991; Dirilgen and Inel, 1994; Bernal and McGrath, 1994). However, in these studies no attempt was made to distinguish between total absorption (i.e. including cell walls) and membrane transport itself. This chapter deals with the characteristics of membrane transport of Co in mung bean, and the effects on Co uptake of a range of environmental variables, such as pH, sulfhydryl agent (cysteine) or reagent (NEM), as well as divalent macronutrient metals (Ca and Mg) and various trace metals (Cd^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, Mn^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+}).

3.2 METHODS

Uptake of Co was measured in two ways. For short-term uptake (0 – 24 h), the radioisotopic tracer ^{60}Co was used. This allowed examination of the processes that affect Co uptake into the root and distribution to the shoot. To follow the accumulation of Co during growth, chemical analysis by ICP was employed. The specific methods used are given in Chapter 2.

3.3 RESULTS

3.3.1 Validity of ^{60}Co uptake measurements

The cell wall has a high capacity for binding metal cations and it is therefore important to be able to distinguish between binding in the cell wall and actual membrane uptake. Reid and Smith (1992a, 1992b) have shown that in giant algal cells it is difficult to remove divalent cations bound to the cell walls and they developed a method for separating the cell wall from the cell contents. Co uptake in *Chara* using this technique is examined in Chapter 4. This technique, however, is not practical with roots because of their more complex structure. Nevertheless, in attempting to measure membrane fluxes in roots, it is necessary to determine the extent to which influx or net uptake is overestimated by binding in the cell wall. In the current research, preliminary experiments were conducted to examine the uptake of ^{60}Co and the desorption of extracellular Co.

In the experiment whose results are shown in Fig. 3.1, roots of intact seedlings were exposed to 1 µM ^{60}Co for 4.75 hours, after which the roots were excised, rinsed briefly in dH_20 then desorbed in 5 mM $CaCl_2$ in ¼ strength Hoagland's solution (200mls/ approx.0.8g roots, solution changed at each point on the graph). Between 0 and 5 minutes of rinsing, only 16% of the ^{60}Co activity associated with the root was removed. After this time, the amount of ^{60}Co in the tissue remained steady or decreased only slowly. At 20 minutes, the

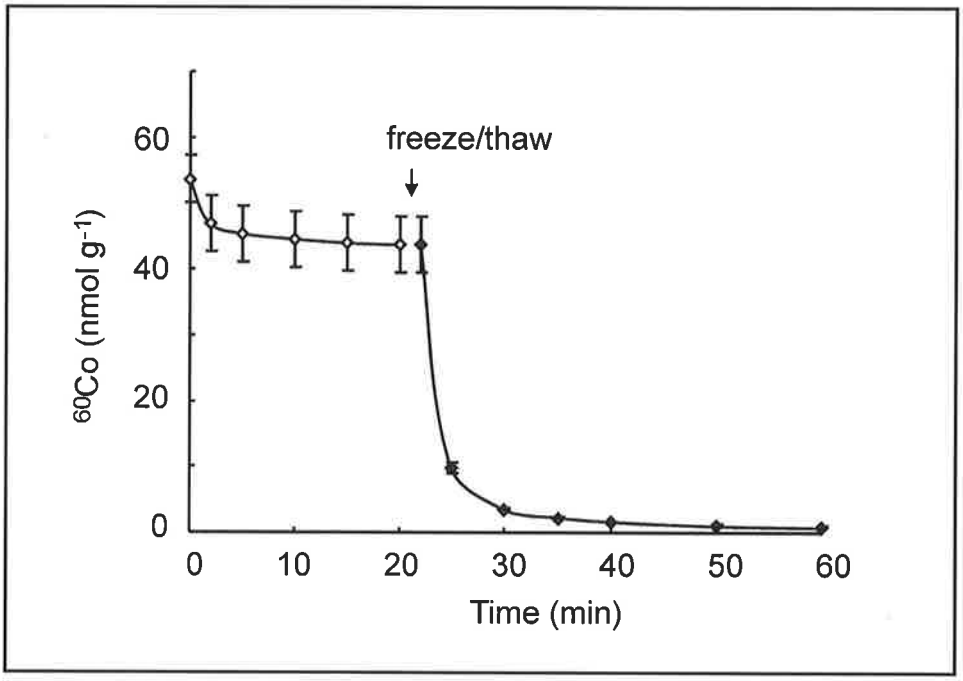


Figure 3.1. Desorption of mung bean roots following 4.75 h incubation in 1 μM ^{60}Co in 1/4 Hoaglands solution. Desorption solution contained 5 mM $CaCl_2$ in 1/4 Hoagland's solution and was changed at each sampling time. After 20 mins the roots were rapidly frozen in liquid nitrogen and thawed after 2 mins by addition of desorption solution. Each point is the mean of 4 replicates containing the roots of 2 plants excised after the ^{60}Co uptake period.

roots were drained and rapidly frozen in liquid nitrogen. The roots were thawed after 2 minutes by placing them back in the desorption solution, and desorption continued. It can be seen from Fig. 3.1 that freezing/thawing released approximately 80% of the total ^{60}Co (97% of the remaining ^{60}Co activity). This suggests strongly that the ^{60}Co came from inside the cells.

A similar experiment conducted with roots loaded with ^{45}Ca showed a very different pattern. Fig 3.2 shows the desorption curve for mung bean roots incubated for 4.75 h in 1.25 mM ^{45}Ca. In this case, the initial loss represented a larger proportion of the total ^{45}Ca activity and required a longer period (20 min) to stabilize. After freezing/thawing, the loss of activity was gradual rather than sudden, as with ^{60}Co. For ^{45}Ca, therefore, it was difficult to be confident whether what was lost after freezing/thawing was mostly intracellular ^{45}Ca or mostly a slowly exchanging fraction of ^{45}Ca within the cell walls. Similar uptake and desorption experiments with lower concentrations of ^{45}Ca (10 μM) and ^{65}Zn (1 μM) also showed that there was a component of metal activity that was not released by freezing and thawing (data not shown).

3.3.2 Uptake in excised roots

Many previous studies of nutrient uptake have used excised roots for convenience. Before a detailed analysis was made of Co uptake in mung beans, a comparison was made between uptake in intact and in excised roots. Excised roots were found to absorb ^{60}Co at almost twice the rate of intact roots (Fig. 3.3). This difference might be due to the easy access of ^{60}Co to cells inside the endodermis or to loss of control signals from the shoot. From this point of view, the uptake rate obtained in excised roots would be higher than that which exists in natural conditions in intact roots. For this reason, in order to obtain true values of ^{60}Co uptake rate, intact roots were employed in all further experiments.

The results of the experiments described in 3.3.1 suggested that desorption of mung bean roots was quantitatively effective at removing extracellular ^{60}Co. Uptake experiments with

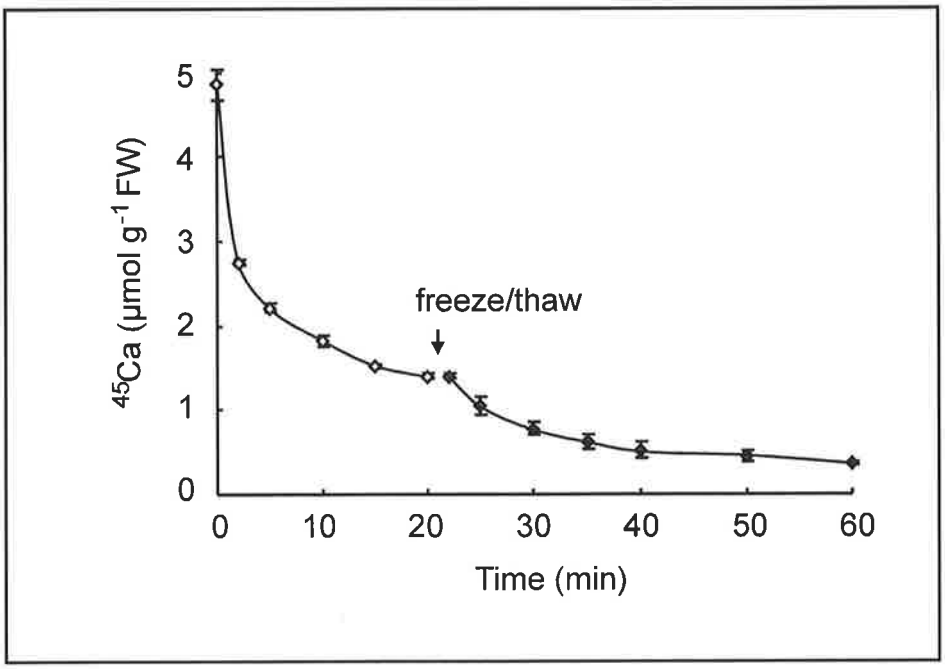


Figure 3.2. Desorption of mung bean roots following 4.75 h incubation in 1.25 mM ^{45}Ca in 1/4 Hoaglands solution. Desorption solution contained 5 mM $CaCl_2$ in 1/4 Hoagland's solution and was changed at each sampling time. After 20 mins the roots were rapidly frozen in liquid nitrogen and thawed after 2 mins by addition of desorption solution. Each point is the mean of 4 replicates containing the roots of 2 plants excised after the ^{45}Ca uptake period.

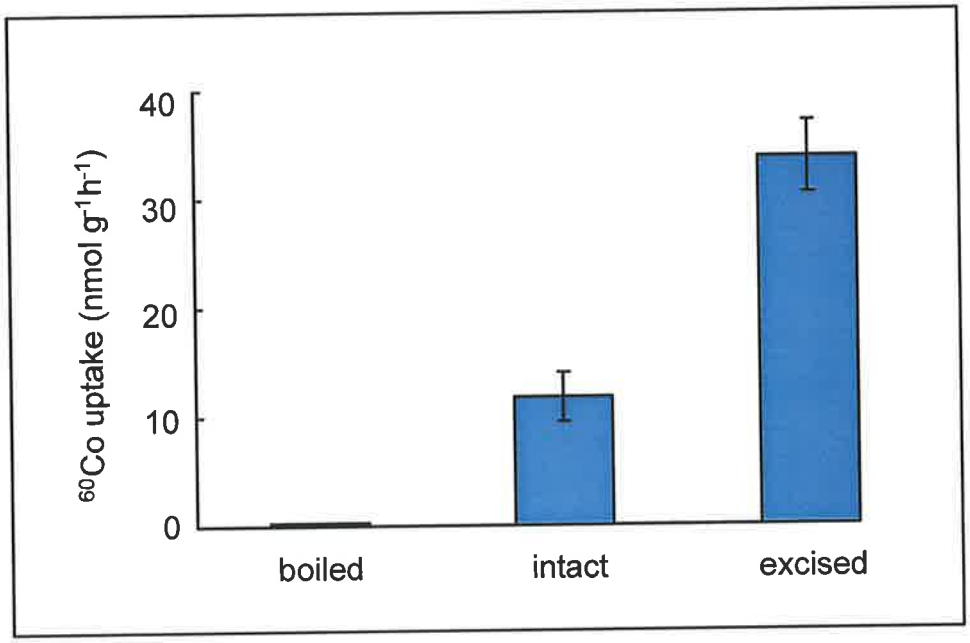


Figure 3.3. Comparison of uptake of ^{60}Co in roots which were boiled before uptake, with roots which were excised or roots which remained attached to the seedlings. Uptake period = 4 h. ^{60}Co = 1 μM in 1/4 Hoagland's solution. Uptake for the boiled roots is expressed in terms of the fresh weight before boiling. n = 6 plants.

roots, which were boiled prior to incubation in 60Co, seemed to confirm that exchange of Co in mung bean cell walls was rapid, with little residual bound Co after desorption (Fig. 3.3).

3.3.3 Time course of uptake

^{60}Co uptake showed two phases; an initial rapid phase which lasted approximately 4h, followed by a linear phase lasting for at least 72 h (Fig 3.4). The desorption data presented above (Fig 3.1) make it seem unlikely that the rapid phase is largely or solely due to undesorbed ^{60}Co on the cell wall. The linear phase after 4 h may result from the saturation of a small, rapidly exchanging component, such as the cytoplasm. In the work by Macklon and Sim (1990) rapid uptake was also noted but was attributed to cell wall binding.

3.3.4 Concentration dependence of uptake

^{60}Co uptake measured over 4 h showed multiphasic kinetics (Fig. 3.5) with respect to concentration. There were possibly 3 distinguishable systems as follows: 1) a high affinity system which saturates at approximately 1 μM with a K_m of approximately 0.3 μM, 2) a system with intermediate affinity for Co with a K_m of approximately 3 μM, and 3) a linear phase which extends up to at least 500μM (Figs 3.5 a & b). At higher concentrations, Co might become toxic even over this short period of uptake and the kinetics would therefore be unreliable.

3.3.5 Effect of Co pretreatment on uptake

The uptake of many nutrients is regulated by the internal nutrient status. For example, high P status leads to lower P uptake (Mimura 1995, Schachtman *et al.* 1998). To determine if Co uptake was sensitive to internal Co, uptake experiments with ^{60}Co were conducted with seedlings that had been grown with or without Co for 2 days. At the lower concentration (1 μM), Co pretreatment did not have any effect on ^{60}Co uptake, while at the higher concentration (50 μM), uptake was greatly reduced by Co treatment (Table 3.1). It is not

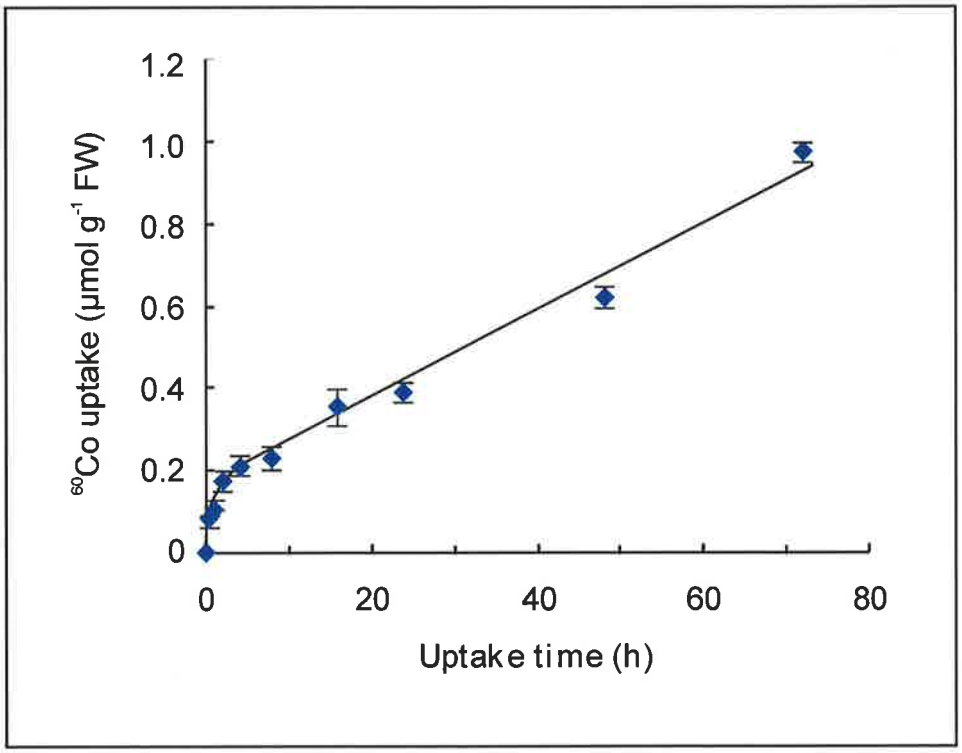


Figure 3.4. Time course of uptake of Co by mung beans from a solution containing 1 µM ^{60}Co in 1/4 Hoagland's solution. Roots were desorbed in 5 mM $CaCl_2$ after the uptake period.

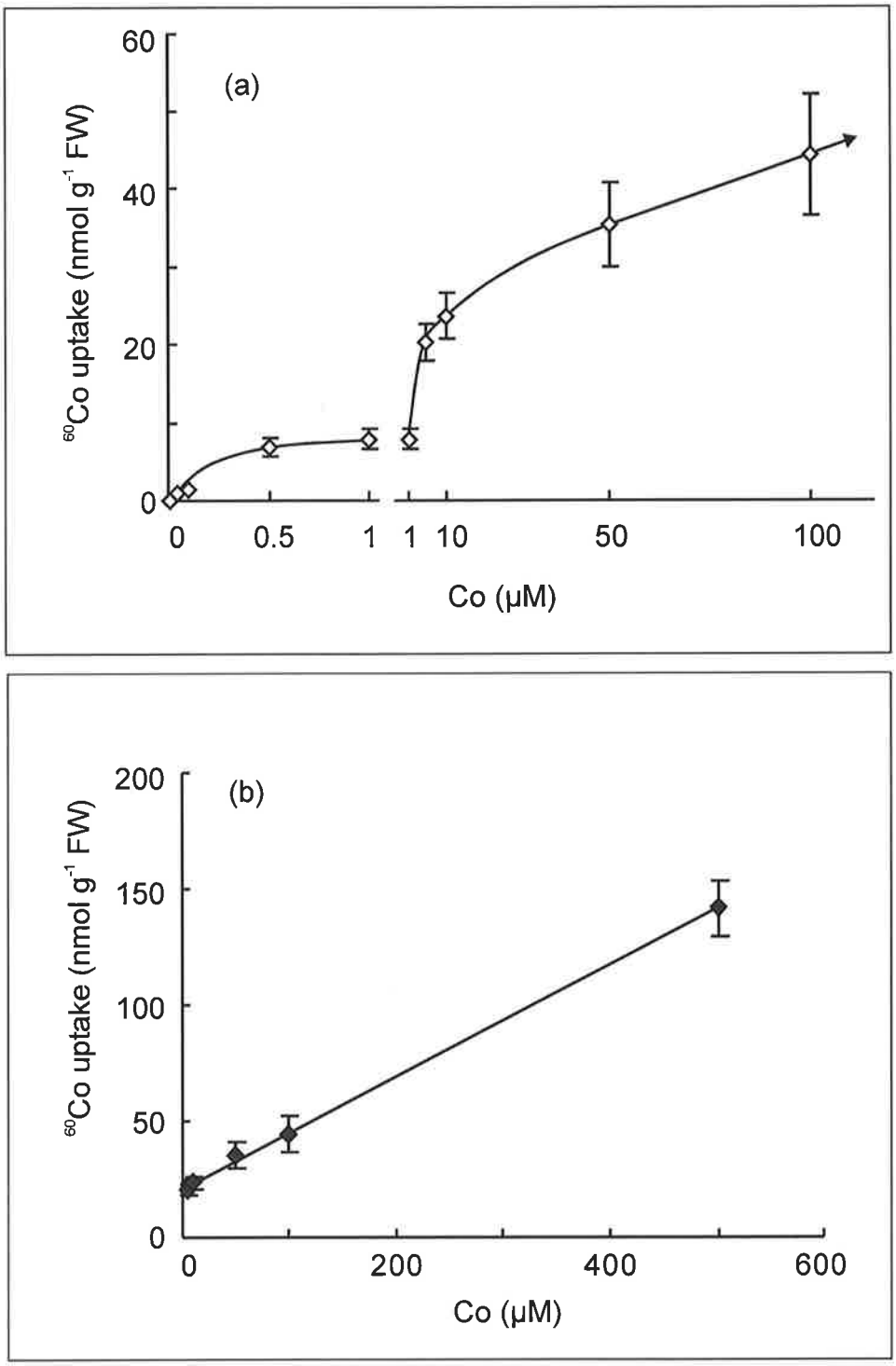


Figure 3.5. Concentration dependence of Co uptake in mung beans. (a) low and intermediate concentration range, (b) higher concentration range. Uptake t = 4 h followed by 40 min desorption. n = 6 seedlings.

known whether inhibition by Co at the higher concentration resulted from regulation by the internal nutrient status as observed for other nutrients, or from the toxicity of excess Co. Visible symptoms of toxicity did not appear after 2 days pretreatment although in longer-term experiments, Co at 50 μM strongly inhibited the growth and development of mung bean (see Chapter 5). Macklon and Sim (1990) also found little difference of pretreatment in Co (0.1 and 1 μM) for ^{58}Co uptake in ryegrass seedlings.

Table 3.1 Effect of Co pretreatment on ^{60}Co uptake. Mung bean seedlings grown for 2 days ± Co. Uptake measured using 1 μM ^{60}Co over 4h with desorption. Mean ± s.e. of 6 seedlings.

Pretreatment	^{60}Co uptake (nmol g^{-1} h^{-1})
0 Co	9.7 ± 1.1
1μM Co	9.7 ± 0.6
50μM Co	2.1 ± 0.2

3.3.6 Uptake rate as a function of growth

When mung bean seedlings were grown in solutions containing varying concentrations of ^{60}Co, the ^{60}Co content of the roots reached a concentration of approximately 4.5 μmol g^{-1} FW. This concentration was independent of the concentration of Co between 5 and 500 μM in the external solution (Fig 3.6a). At lower external Co concentrations, it took longer to reach this level. The content in the leaves continued to increase over the growth period and after 8 days the content reached nearly 2 μmol g^{-1} FW at the highest concentration (Fig 3.6b). Although the experiment did not extend past 8 days, it is possible that the leaves would also have reached a plateau of concentration, as was observed in the roots.

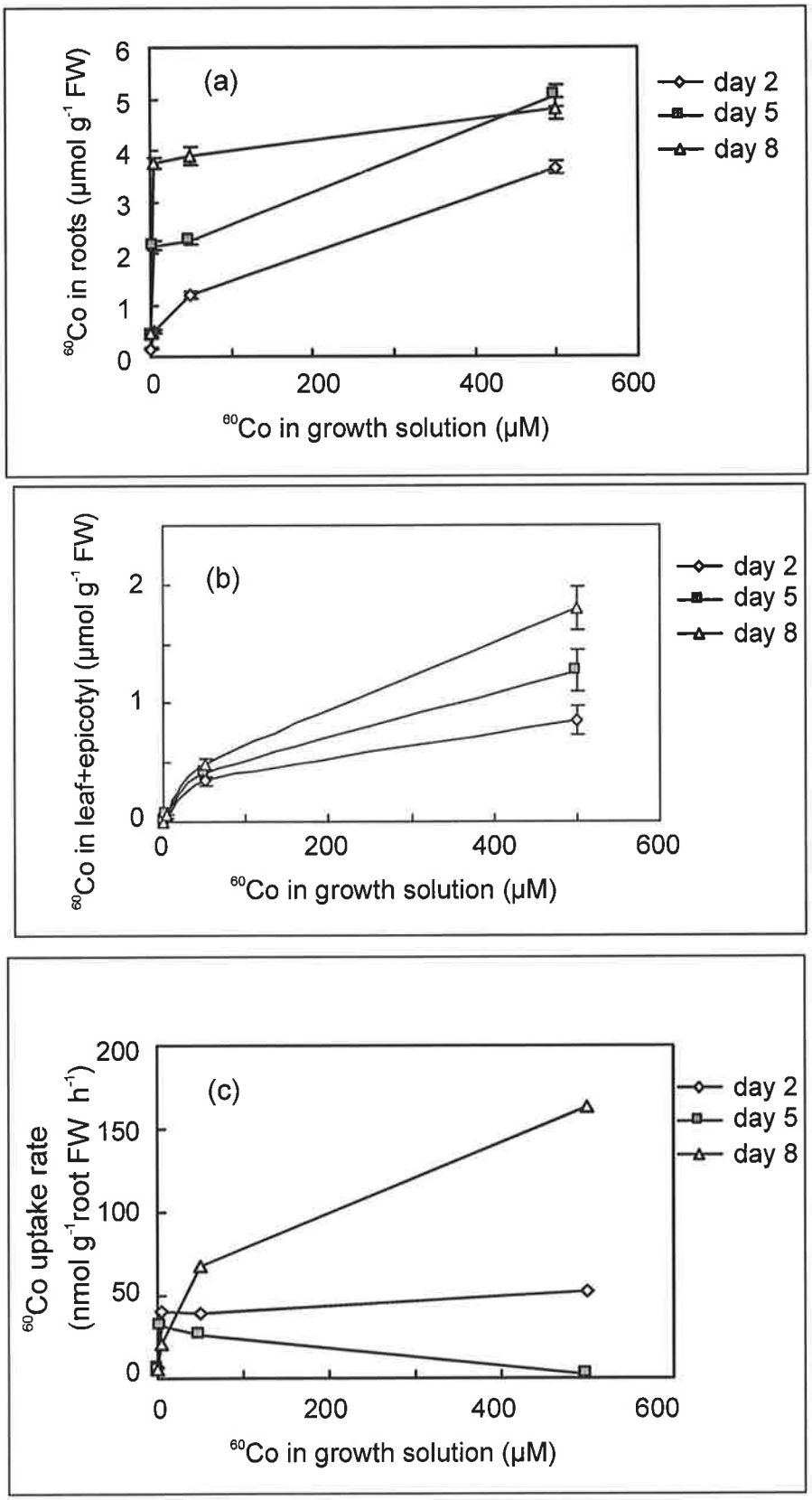


Figure 3.6. Distribution of ^{60}Co in mung bean seedlings grown in varying concentrations of ^{60}Co. (a) root content, (b) leaf+epicotyl content. (c) whole plant ^{60}Co uptake rate based on the root fresh weight. Roots were desorbed. n = 6 plants

The whole plant ^{60}Co uptake rate, expressed on a root fresh weight basis, decreased as the internal concentration approached the plateau level (Fig 3.6c).

3.3.7 ^{60}Co distribution with growth

Figure 3.7 shows the pattern of distribution of ^{60}Co in mung bean seedlings at different stages of growth. The plants were germinated and then transferred to ^{60}Co and grown for 5 days. After this time, the distribution of ^{60}Co between roots, hypocotyl and epicotyl+leaves depended on the concentration of ^{60}Co in the growth solution. At the lowest concentration (0.5 μM), almost all of the Co remained in the roots, while at the highest concentration, the roots retained only approximately 50% of the total plant ^{60}Co. Correlation analysis showed that Co content in the roots inversely correlated with Co allocation % in the roots (r = -0.78), while it positively correlated with the allocation in the shoots (r = 0.71). Similarly, Co concentration in the solution inversely correlated with Co allocation % in the roots (r = -0.80) and positively correlated with the allocation % in the shoots (r = 0.70). The results suggest that transport to the shoot depend on root content. It appears that the transport of Co to the shoots might be governed by its availability and the uptake capacity of the root cells.

3.3.8 Effect of pH on Co uptake

^{60}Co uptake in mung bean seedlings generally decreased as the pH of the growth solution increased. This may partly be due to the effect of pH in decreasing the activity of the Co^{2+} ion in solution. Fig. 3.8a shows that in ¼ Hoagland's solution (Fe omitted), the activity of Co^{2+}, as calculated by GEOCHEM-PC (Parker *et al*, 1995) was strongly influenced by pH. ^{60}Co uptake was closely correlated with the activity of Co^{2+} up to pH 7 but at higher pH the computed activity was low, suggesting either that other species of Co are taken up at high pH or that possibly the pH at the membrane surface is lower than in solution. When the experiment was performed in simple solution (buffered APW) there was a peak in uptake between pH 5 and pH 6 and the correlation between Co^{2+} activity and uptake was poor.

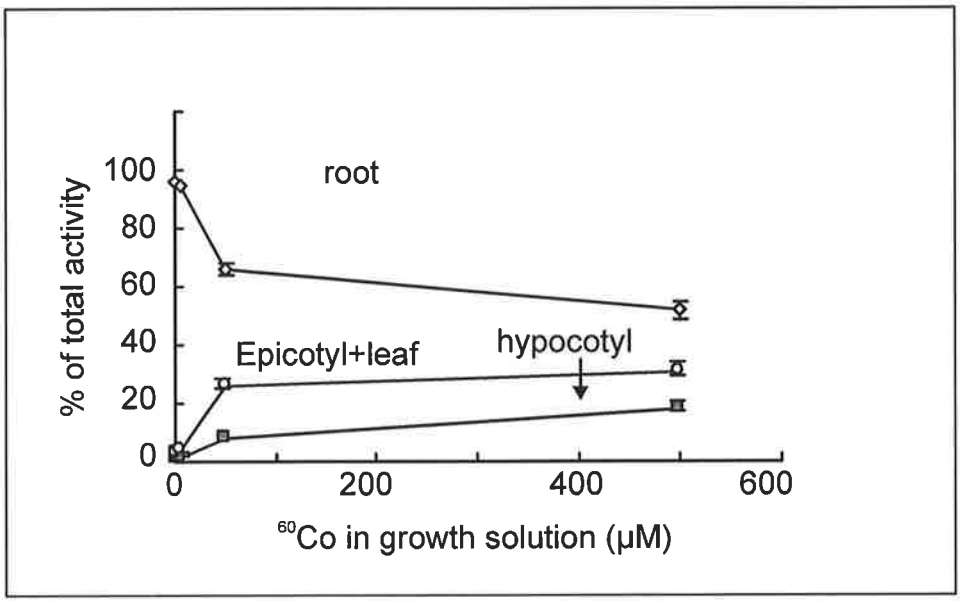


Figure 3.7 Distribution of ^{60}Co in mung bean plants after 5 days growth on 1/4 Hoagland's solution containing various concentrations of ^{60}Co. n = 6 plants

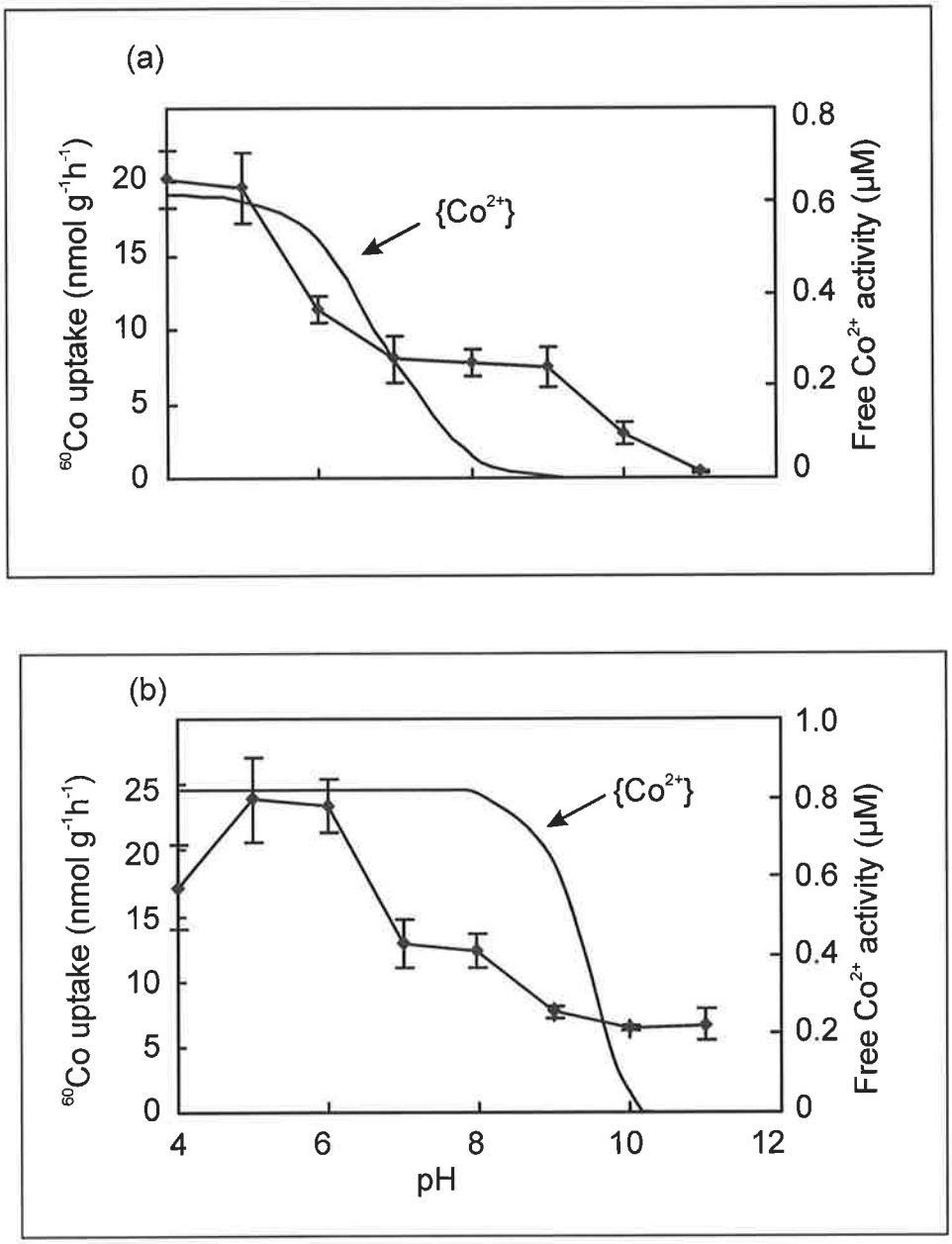


Figure 3.8 pH dependence of ^{60}Co uptake in (a) 1/4 Hoagland's solution and (b) simple solution (APW: 0.4 mM NaCl, 0.1 mM KCl, 0.5 mM $CaCl_2$. Solutions were buffered (see methods) and contained 1 μM ^{60}Co. Uptake t = 4 h, n = 6 seedlings.

Macklon and Sim (1990) found that in ryegrass Co uptake increased with increasing pH in the range 4 – 6 and then remained fairly constant up to pH 8.

3.3.9 Competition with other cations

3.3.9.1 Trace metals

3.3.9.1.1 Uptake experiments in simple solution.

The effect of other trace metals on Co uptake was determined by measuring Co uptake from solutions containing 1 μM ^{60}Co in the presence of 5 μM of a single trace metal (Fig 3.9a). For these experiments a simple solution (APW, pH 6) was used to avoid problems caused by having more than one competing trace metal present (see further experiments with complete nutrient media below). All of the trace metal cations tested were inhibitory to Co uptake. Cd was strongly inhibitory while Pb and Fe(III) were only mildly inhibitory. The relative inhibition of ^{60}Co decreased in the order:

$Cd > Co > Cu >= Hg >= Ni = Zn >= Mn > Fe^{2+} > Pb, Fe^{3+}$

Over the short uptake period of this experiment, only a small fraction of the total uptake appeared in the shoot.

3.3.9.1.2 Uptake experiments in nutrient solution.

The effect on Co uptake of trace metals added to nutrient solution was investigated by measuring uptake of Co in ¼ Hoagland's solution with varying concentrations of divalent metals. Cu, Mn or Zn were either deleted from the nutrient solution or were added at their normal concentrations or at 10x their normal concentration in ¼ Hoagland's solution.

Excess Cu, Mn or Zn inhibited Co uptake compared to those without the metals (nil) at 5% significance level (Fig. 3.10). The excess was not significantly different from the control (i.e. concentrations in ¼ Hoagland's solution), except for Cu. Absence of Cu, Mn and Zn (nil) was also not significantly different from the control. This might be due to the presence of the other two metals in the solutions. The three trace metals were then treated as a single

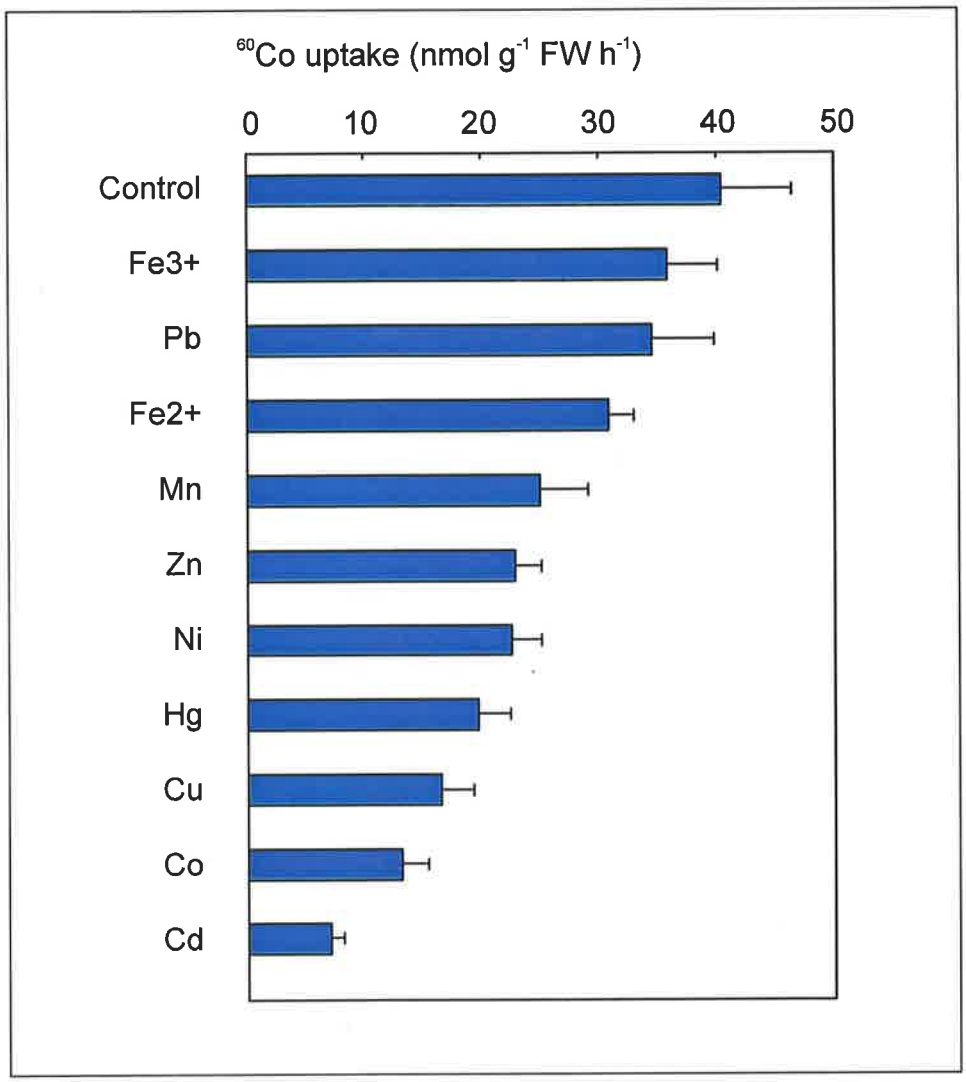


Figure 3.9a. Effects of trace metals on uptake of Co in mung bean roots. Uptake was measured in a solution containing 1 μM ^{60}Co in APW pH6. Other trace metals were present at a concentration of 5 μM. Uptake t = 4 h; n = 6 plants.

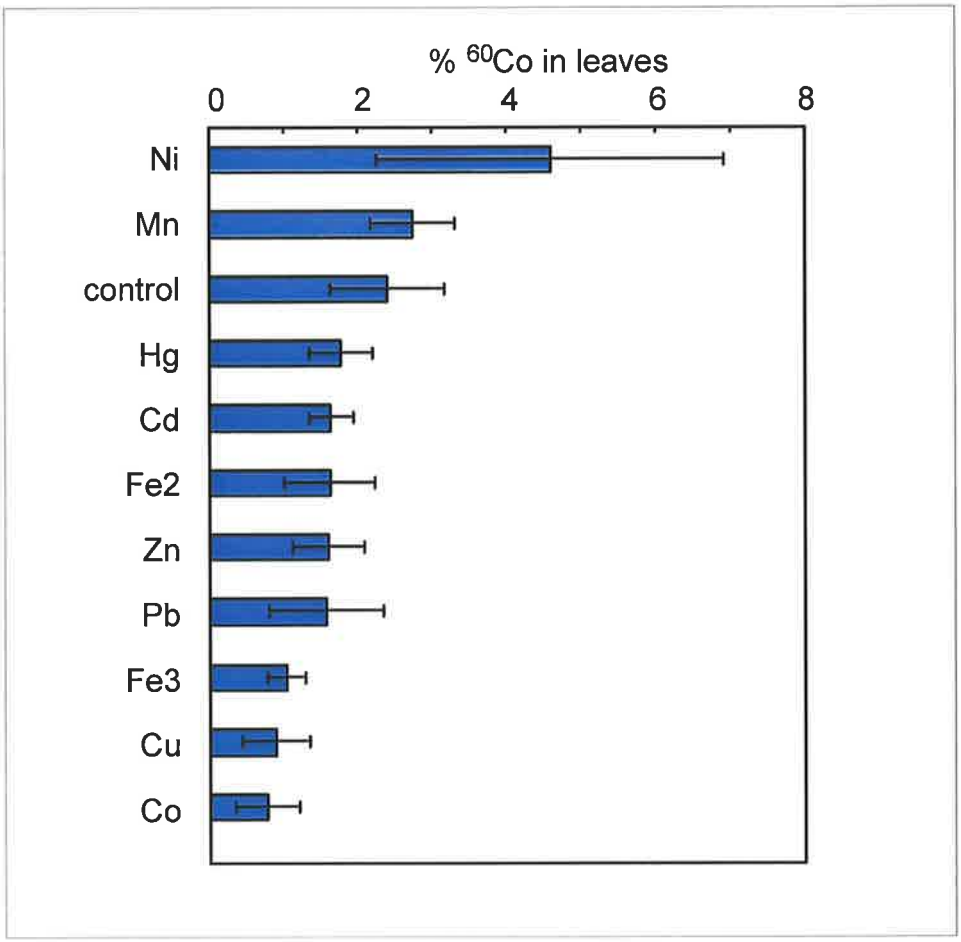


Figure 3.9b. Effect of trace metals on Co translocation from roots to shoots. Translocation is expressed as a percentage of the root Co content to compensate for effects of trace metals on uptake of Co. Conditions as in Fig. 3.9a.

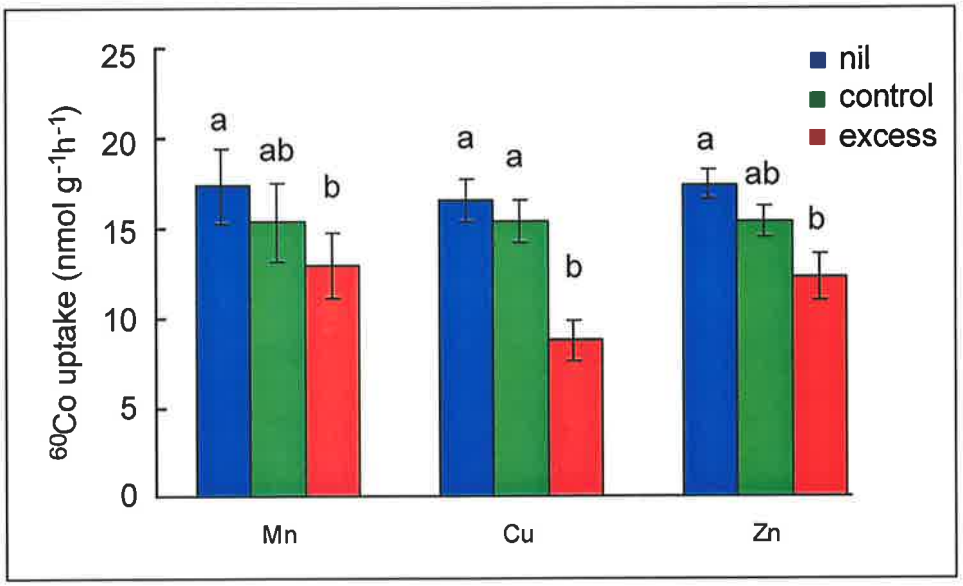


Figure 3.10. Effect of micronutrient cations on uptake of Co in mung bean roots. Treatment solutions contained 1 μM Co in 1/4 Hoagland's solution. In the 'nil' solutions the specified micronutrient was absent, in the 'control solutions the micronutrients were present at their normal concentrations (0.5 μM Mn and Zn, 0.125 μM Cu) and in the 'excess' solutions at 10x these concentrations.
Uptake t = 4 h, n = 6 plants. Uptake to the whole plant is expressed on the basis of root fresh weight. Treatments with different letter are significantly different ($p < 0.05$).

factor and added to the solution at various concentrations (Table 3.2). ^{60}Co uptake was increased significantly by deleting all the three metals (nil), and decreased by increasing the concentration ten-fold (excess) of those in ¼ Hoagland's solution (control), indicating that these three metals have a cumulative effect or possess a similarity in inhibiting ^{60}Co uptake.

Table 3.2 Effects of micronutrient metals in ¼ Hoagland's solution on uptake of ^{60}Co in mung beans. Co = 1 μM. Uptake time = 4 h. n = 6 plants

treatment	Concentration in ¼ Hoagland's solution	Uptake (nmol g^{-1} root FW h^{-1})
nil	0	20.9 ± 1.7
control	0.5 μM Zn, 0.125 μM Cu, 0.5 μM Mn	15.3 ± 1.2
excess	5 μM Zn, 1.25 μM Cu, 5 μM Mn	4.5 ± 0.8

When added to the base growth solution, Ni and Cd also inhibited ^{60}Co uptake, with the inhibition increasing as the concentration increased from 1 μM to 5 μM (Table 3.3). As in the experiments in simplified solution, Ni appeared to stimulate the transport of Co to the shoot.

Table 3.3 Effect of Ni and Cd on uptake of ^{60}Co in mung beans. The base solution contained 1 μM Co in ¼ Hoagland's solution. Uptake t = 4h. n = 6 plants

Metal	concentration	^{60}Co uptake (nmol g^{-1} root FW h^{-1})	^{60}Co translocation (nmol g^{-1} root FW h^{-1})
control		24.4 ± 2.3	0.46 ± 0.09
Ni	1 μM	21.8 ± 1.5	0.74 ± 0.19
	5 μM	19.5 ± 1.9	1.16 ± 0.25
Cd	1 μM	8.9 ± 1.2	0.1 ± 0.02
	5 μM	2.6 ± 0.5	0.02 ± 0.01

3.3.9.2 Ca and Mg

The effect of the macronutrient divalent metal ions, Ca and Mg, on ^{60}Co uptake was investigated by adding 0.9, and 9.9 mM Ca and Mg to a simple base solution (0.4 mM NaCl, 0.1 mM KCl, 0.1 mM $CaCl_2$) containing 1 μM ^{60}Co. Both Ca and Mg inhibited ^{60}Co uptake to a similar extent, with the greatest sensitivity being observed between 0.1 and 1 mM (Fig 3.11).

3.3.10 Effect of cysteine and NEM on Co uptake

^{60}Co uptake was strongly inhibited by NEM, a sulfhydryl reagent (Fig 3.12a), which binds to –SH groups in proteins. This suggests that the uptake may involve binding to a site on a membrane cation transporter that is closely associated with amino acids containing –SH residues (e.g. cysteine). NEM also inhibited the translocation of ^{60}Co to the leaves (Fig 3.12b), possibly due to the inhibited uptake. Cysteine added to the solution (10 μM) did not significantly affect the uptake, but did inhibit translocation (Fig 3.12 a, b). The absence of an effect of cysteine on Co uptake may reflect the low binding of Co to cysteine at pH 6 (only 5% of 1 μM Co would by bound in a solution containing 10 μM cysteine) while the inhibition of transport of Co to the shoot may be due to the greater affinity of cysteine for Co at the pH prevailing in the cytoplasm (approximately 50 % of 1 μM Co would be bound in a solution containing 10 μM cysteine at pH 7.8).

3.4 Discussion

Co uptake appears to be a complex function determined by concentration, pH and the presence or absence of other trace metals. There is also some evidence that uptake can be controlled or regulated by plant Co status. The general characteristics of Co uptake are

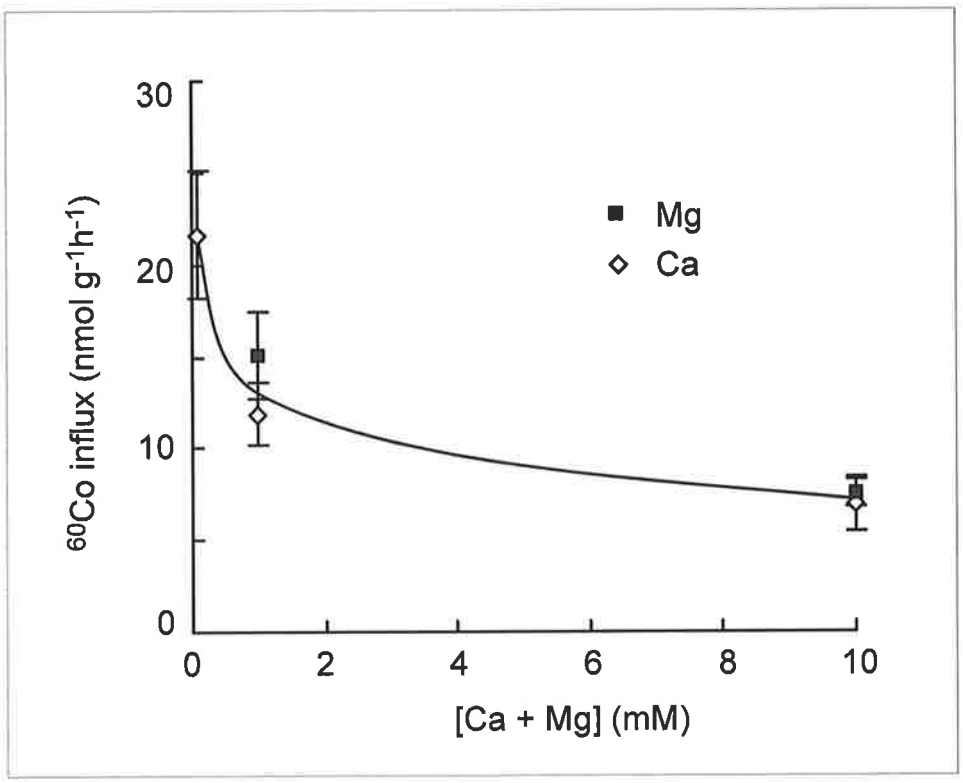


Figure 3.11 Effects of Ca and Mg on Co uptake in mung bean roots. Base solution contained 1 μM Co in 0.4 mM NaCl, 0.1mM KCl + 0.1 mM $CaCl_2$. Ca or Mg were added to the concentrations indicated.
Uptake t = 4 h; n = 6 plants

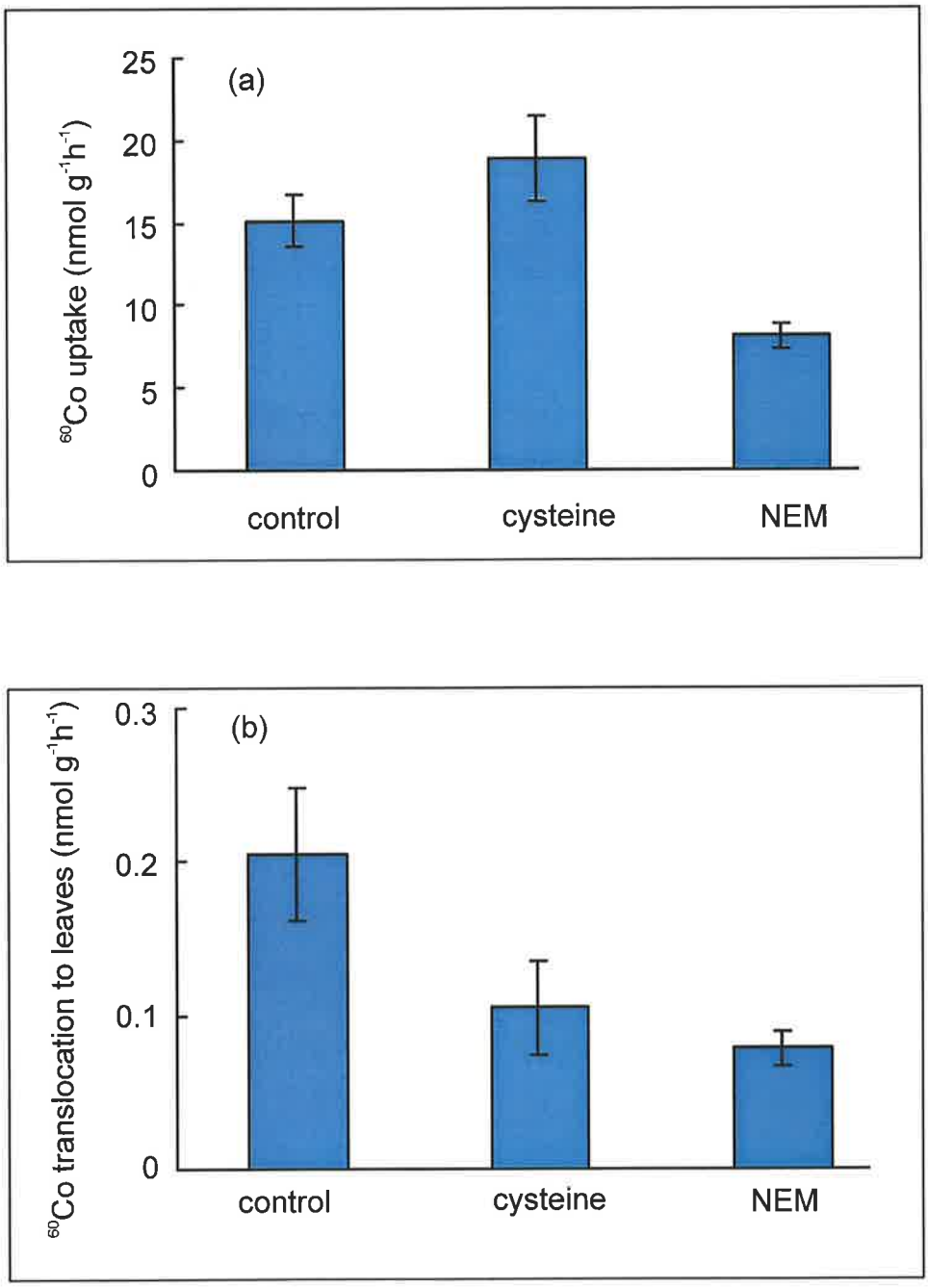


Figure 3.12 Effects of 10 μM cysteine and NEM on (a) ^{60}Co uptake in roots and (b) translocation of ^{60}Co to leaves (based on root fresh weight). Uptake t = 4 h, n = 6 plants.

worth close examination in order to try to deduce a possible mechanism for membrane transport of Co.

3.4.1 Cell wall binding

This thesis was not primarily concerned with binding of Co to cell walls. It was necessary to consider this process only in terms of its effect on the accuracy of measurements of Co fluxes across membranes. The initial desorption experiments indicated that at least at low concentrations, Co is not tightly bound in the root cell walls of mung bean.

The difference between Ca and Co in response to freezing/thawing during desorption is hard to interpret, but there are two possible explanations. First, the concentrations of Co and Ca were very different both in the cell and in the uptake solution. At low concentrations, the rate of uptake may be high relative to the binding in the cell wall, especially if plants were previously grown without the metal, and thus internal concentrations were low. Second, there may be specific binding sites for Ca and Co in the cell wall. For Ca, these may include -COOH in the galacturonic acid of pectins and in the glucuronic acid of xylans located on the cell wall (Brett and Waldron, 1996). For Co, besides -COOH, there may also be -SH groups in proteins in the cell wall. Co bound to -COOH would be exchanged by Ca, but that bound with -SH groups would not be exchanged by Ca because of the low affinity of Ca for –SH groups in accordance to the review by Nieboer and Richardson (1980) and Woolhouse (1983). It is not known if freezing/thawing releases the protein from the cell wall. However, since the -SH containing protein is rarely present in cell wall (Brett and Waldron, 1996), this part of Co would account for only a small fraction of the total remaining after desorption treatment. From these considerations, the overall conclusion is that, after desorption; most of the ^{60}Co is from intracellular compartments.

3.4.2 Concentration dependence of Co uptake

The concentration dependence of Co uptake raises the question of why a plant that does not need Co would have 3 uptake systems. It is known that multiple kinetics (more than one

transporter) or multiphasic kinetics (a single transporter with concentration-dependent phase changes) exist in plants for macro- and micronutrients (for review, see Reid, 1998). These allow plants to survive in low nutrient conditions by using high affinity systems, and to utilize less energy intensive low affinity systems when nutrients are abundant. The presence of the three possible systems for Co uptake (Fig 3.5) is therefore not easy to interpret. The competition between Co and essential metals, such as Cu, Mn, Zn, Fe, and Ni as shown in 3.3.9 suggests that Co may be taken up through transporters for essential metals, and that there may be 3 uptake systems for different concentration ranges. However, it can not be ruled out that plants have developed a high affinity system specifically for Co, or, at least, a system that can be shared by Co. Although Co may not be essential for plant growth, there is some evidence that at low concentrations it is beneficial (see also Chapter 5) and may be able to substitute for other trace metals when they are deficient.

The apparent linear dependence of Co uptake on concentration in the high range may be a potential risk for mung bean plants growing in the presence of high Co (e.g. in serpentine soils), since this dependence will not efficiently prevent the entry of excess Co, which is harmful for growth and development.

3.4.3 pH dependence of Co uptake

Macklon and Sim (1990) reported that in ryegrass seedlings, Co uptake increased almost three-fold between pH 4.5 and pH 6.0 in experiments with 1 μM Co, and more than four-fold between pH 4.5 and pH 7.8 in 0.1 μM Co. This is different to the results obtained with mung bean, where Co uptake increased by only about 40% between pH 4 and 5 and then decreased with increasing pH. This suggests that the uptake processes for Co in mung bean are fundamentally different from those in ryegrass. It is difficult to examine pH dependence in complex media such as Hoagland's solution because of the effects of pH on the free activity of Co^{2+} and on the activities of other metals that may be competing with Co for uptake. In ¼ Hoagland's solution (minus FeEDTA), ^{60}Co uptake decreased with increasing pH (Fig. 3.8a) and was correlated with the free activity of Co in solution (less well at pH >

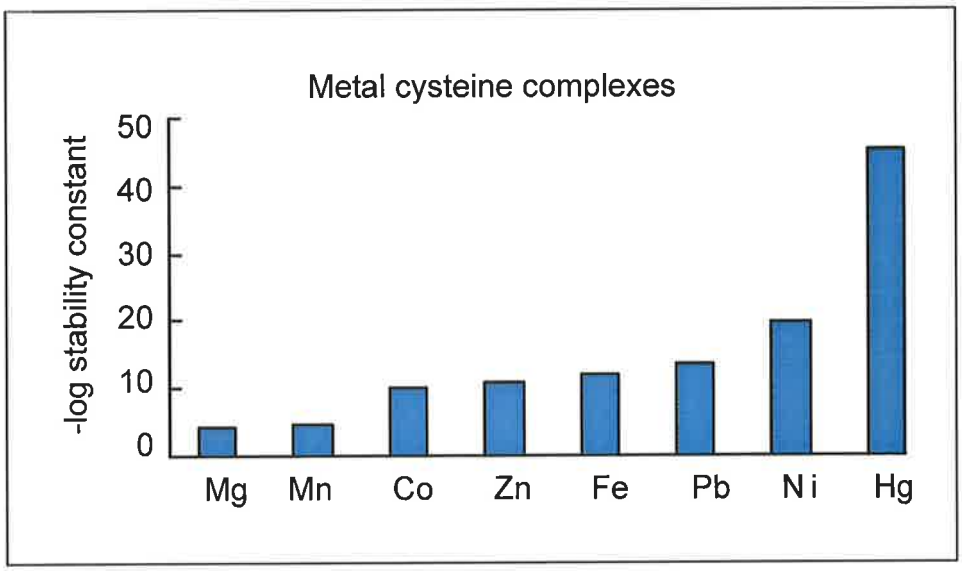
7.5). In the simplified medium (APW), ^{60}Co uptake was poorly correlated with activity (Fig. 3.8b), which suggests that the pH effects are due to responses of the transporter. Another possibility is that the buffers used at higher pH complexed Co. Stability constants for buffer-Co complexes are not available in the GEOCHEM database.

3.4.4 Specific mechanisms?

As discussed above (3.4.2), while Co uptake may use 3 systems for uptake, it is not known if there is a system specifically for Co uptake, or if Co is transported by the systems for essential metals. It would seem more likely that a specific system would exist for high affinity uptake. The inhibition of Co uptake in the high affinity range by other trace metals is consistent with competition between metals for a common uptake system. The inhibition of Co uptake by the sulfhydryl reagent NEM may indicate that the selectivity of the transporter is governed by the affinity of trace metals to -SH groups in the transport protein. This affinity is shown in Fig 3.13, as calculated by GEOCHEM-PC (Parker *et al.*, 1995). Except for Hg and Pb, the inhibitory effect of trace metals correlated with their affinity for -SH groups. After Cd, Co was the most powerful inhibitor of Co uptake (Fig 3.9a), indicating the presence of some specificity in Co uptake.

3.4.5 Cobalt uptake and membrane surface charge

Ca and Mg were much less inhibitory to Co uptake than the trace metals and this is consistent with the proposal that binding to the uptake site involves –SH groups, since Ca and Mg have low affinities for these groups. High concentrations of Ca and Mg decreased Co uptake, which may be due to competition for uptake. However, this effect might also be due to the effect of high cation concentrations on the membrane surface charge. According to Kinraide (1994) the membrane possesses a significant charge due to the ionisation of groups on membrane proteins and phospholipids. These ionised groups generate an electrical potential difference between the membrane surface and the bulk solution, which results in the electrostatic attraction of ions of the opposite charge. The net result is that the



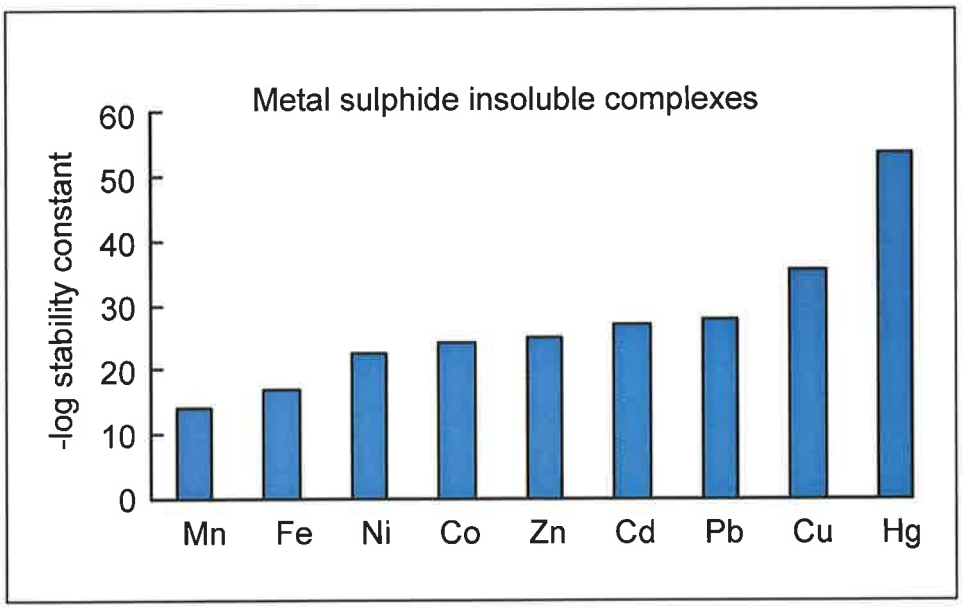


Figure 3.13 Stability constants for complexation of various divalent metals with cysteine and for the formation of insoluble sulphide complexes.

concentration of ions at the membrane surface may be very different to that in the external solution. The membrane surface potential is normally negative and is suppressed by high concentrations of cations in the solution, particularly divalent and trivalent cations. Kinraide (1994) has devised a computer program to enable the calculation of membrane surface charge in solutions of different composition. This program was kindly provided by Dr Tom Kinraide and was used to estimate the effects of Ca and Mg on the concentrations of Co at the membrane surface. Figure 3.14 shows ^{60}Co uptake as a function of the computed surface concentration (data from Fig. 3.11). The points can be fitted with a Michaelis-Menten equation with a Km of 4 μM. Recalculation of the data from Fig. 3.5a in the range 0 – 1 μM Co in terms of their computed surface activities gives uptake values which fall remarkably close to the curve fitted for the conditions in which surface activity varied with changes in the concentrations of Ca and Mg (Fig 3.14).

3.4.6 The energetics of Co uptake

The concentration inside the root of 0.46 μmol g^{-1} after 5 days growth in 0.5 μM Co (Fig. 3.6a) represents a concentration based on tissue water content of more than 0.46 mM. This exceeds the external concentration by a factor of more than 1000. In terms of passive distribution, this concentration could be achieved if the inside of the cell was more negative than the external solution by 90mV (Nernst equation). Measured values of membrane potential difference are commonly more negative than –90mV (Findlay and Hope, 1976). In theory, at least, Co uptake could be passive. However, Macklon and Sim (1987) proposed an active uptake based on the stimulation of Co uptake by light. However, this effect may also be due to other light-affected factors, such as growth.

3.4.7 Co translocation in plants

The distribution of Co in plants has been the subject of several previous studies (Hara *et al.*, 1976; Patel *et al.*, 1976; Wallace *et al.*, 1977; Macklon and Sim, 1987; Macklon and Sim, 1990). Co was found to be distributed to all parts of the plant, but in most cases, it was

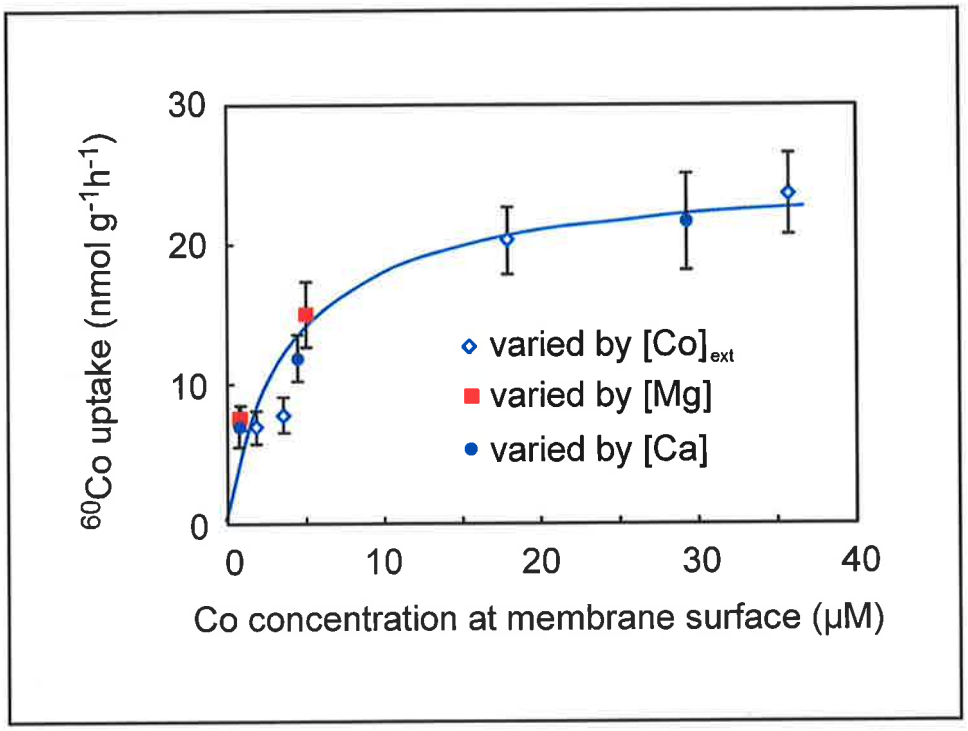


Figure 3.14 Co uptake as a function of the concentration of Co at the membrane surface, calculated according to the Gouy-Chapman-Stern model for membrane surface charge (Kinraide 1994) with the following parameter values:
density of negatively charged ligands = 0.145 μmol m^{-2}
density of neutral ligands = 2.4 μmol m^{-2}
pH of solution = 5

Open symbols - Co concentration at the membrane surface for 0.5, 1 and 5 μM Co in 1/4 Hoagland's solution. Closed symbols - Co concentration at the membrane varied by addition of Ca or Mg to suppress the surface charge. External solution = 1 μM Co in 0.4 mM NaCl, 0.1 mM KCl, 0.5 mM $CaCl_2$.

The line was fitted to a Michaelis-Menten equation with Km = 4 μM and Vmax = 25 nmol g^{-1}h^{-1}.

mainly accumulated in roots (Hara *et al.*, 1976; Wallace *et al.*, 1977; Young, 1979). At least some of the Co would have been bound in the cell wall rather than in intracellular phases. In the study by Wallace et al. (1977) of metal uptake and distribution in *Phaseolus vulgaris,* Co was more mobile than V, Ti, Ag, and Cr, although Co was also concentrated in the roots. In mung bean, it was found that distribution was dependent on the concentration of Co in the roots, which in turn was a function of the Co concentration in the growth medium (Fig. 3.7). When the root concentration was low, a greater proportion of the plant Co was retained in the roots. As the root concentration increased, more was exported to the shoots, possibly to avoid toxicity in the roots. Similar results were found in chrysanthemums, where the root to stem ratios of Co content depended on the concentration of the metal applied, with the ratios of 75, 10, and 1 in the growth medium containing 1, 10, and 100 μM Co, respectively (Patel *et al.*, 1976). At low concentrations, most of the Co may be bound to –SH group in cytoplasmic proteins, and therefore may be unavailable for transport. The inhibition of translocation, but not of uptake, by cysteine might possibly be explained by complexation of Co by absorbed cysteine in the cytoplasm. This result also argues against translocation of Co as a complex with cysteine. Most trace metals inhibited both uptake and translocation of Co and it is therefore difficult to isolate the specific effects of these metals on translocation.

CHAPTER 4 ^{60}Co Uptake and Distribution in *Chara*

4.1 INTRODUCTION

Previous tracer uptake experiments by Reid and Smith (1992a, 1992b and unpublished experiments) using higher plants indicated that in the case of Ca and Zn, there was a significant fraction of metal remaining after desorption which was not released by the disruption of cells by freezeing/thawing or by methanol treatment. Because of their inability to confidently attribute this residual activity to either intra- or extracellular compartments, Reid and Smith turned to giant algal cells where the extent of cell wall binding could easily be assessed. The large size of the individual internodal cells of the algae permitted easy separation of the cell wall from the intracellular components; the proportion of the undesorbed fraction that was intracellular was therefore measured directly. Membrane fluxes could thus be measured accurately, even over short uptake times. These methods have been used successfully to describe uptake of Ca^{2+} (Reid and Smith 1992a,b), Zn^{2+} (Reid *et al.* 1996a), and Al^{3+}, Ga^{3+} and Sc^{3+} (Reid *et al.* 1996b).

It was originally intended in the current work to use the charophyte system to characterise Co influx and net Co uptake, and then to compare these characteristics with net uptake in mung bean measured over longer periods where cell wall binding would be relatively small compared to overall uptake. It was only after much of the *Chara* work had been completed that the desorption experiments with mung bean were conducted. The aim here was to find the shortest uptake period that would allow a reasonable distinction between actual cellular uptake and residual cell wall binding after desorption. It was at this stage that it was discovered that, unlike ^{65}Zn and ^{45}Ca, very little ^{60}Co remained after freezing/thawing and, therefore, that desorption of Co was relatively effective, at least for uptake periods of more than about 1h. A more detailed investigation of Co uptake in mung bean was therefore

justified, the results of which have been presented in the previous chapter. The data collected for *Chara* are nevertheless interesting for several reasons. Firstly, they allow comparison of the characteristics of Co uptake in two very different types of plants, one a higher plant that normally grows in soil, the other a freshwater alga. Secondly, it is pos with *Chara* to separate the cytoplasm from the vacuole and to obtain some insight into the transfer of Co across the tonoplast as well as across the plasma membrane. This would be extremely difficult to carry out with the small cells of mung bean roots.

4.2 MATERIALS AND METHODS

Details of the methods for culturing *Chara corallina* and for the measurement of ^{60}Co uptake are described in Chapter 2. Because of the large number of cells needed for the experiments, it was not possible to source all material from the same cultures and to use cells of precisely the same developmental stage. Consequently, there were often differences between similar treatments in different experiments. Effects of treatments are therefore related to the control for each experiment. ^{60}Co uptake is expressed on a surface area basis.

4.3 RESULTS

4.3.1 ^{60}Co uptake into whole cells

Figure 4.1 shows the time-course of accumulation of ^{60}Co at 0.5 µM by whole *Chara* cells without desorption (cells rinsed approximately 2 sec in deionised water to remove surface activity only). Uptake was initially rapid but plateaued after 2 h, presumably due to saturation of cell wall binding sites. The data presented below indicate that the much greater binding to the cell wall obscures influx of ^{60}Co.

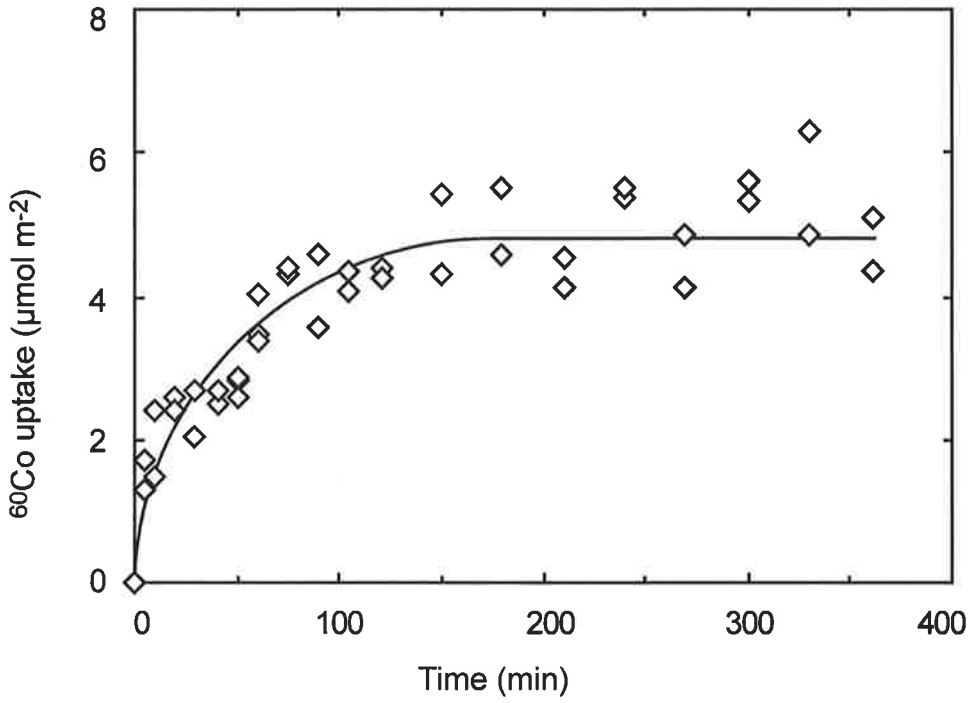


Figure 4.1 Time course of ^{60}Co uptake into *Chara* without desorption. Internodal cells were incubated in APW pH 6 containing 0.5 µM $^{60}CoCl_2$. Each point represents a single cell.

4.3.2 Desorption of ^{60}Co

Rinsing cells to desorb apoplasmic Co assessed the extent to which cell wall binding contributes to overall uptake. Cells were first incubated in 1 μM ^{60}Co for 4 h then desorbed in one of 3 desorption solutions: (1) unbuffered APW, (2) 5 mM $CaCl_2$, and (3) 5 mM $CaCl_2$ + 1 mM $LaCl_3$. Desorption solutions were changed at intervals over 75 min and their ^{60}Co activity determined. The time-course of desorption is shown in Fig. 4.2. Approximately 75% of the total cellular ^{60}Co activity was removed within 20 min using either Ca or Ca+La solutions, while APW alone was much less effective, removing less than 50% over the same period. After 75 min the ^{60}Co activity was only 17 - 20% of the original activity for the 3 desorption solutions.

4.3.3 ^{60}Co distribution within cells

The shape of the desorption curve suggested that most of the ^{60}Co activity in the cell wall was removed in the first 20 min and that the slowly-exchanging fraction which remained after 75 min represented intracellular ^{60}Co activity (Fig. 4.2). However, fractionation of desorbed cells revealed that most of the remaining ^{60}Co activity was, in fact, in the cell wall. Table 4.1 shows the distribution of ^{60}Co in the cell wall, cytoplasm and vacuole. Only 8.2% of the remaining cell-associated activity was found in the intracellular compartments. The significance of this result is that the estimate of Co influx in whole desorbed cells would have been an overestimation of the true influx by a factor of more than 10 because of the strong residual binding in the cell wall.

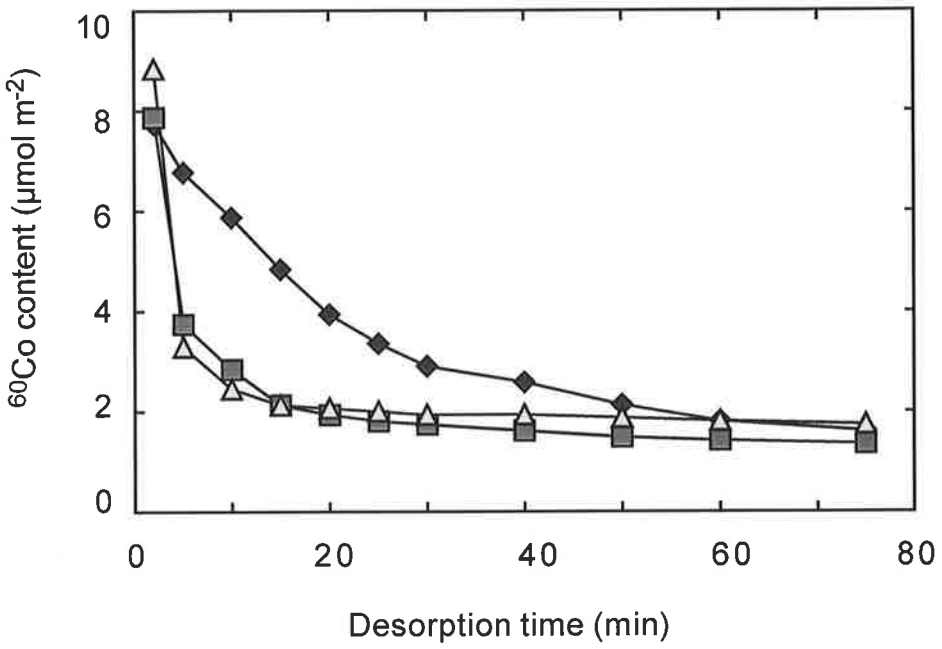


Figure 4.2 Desorption of ^{60}Co from *Chara* using unbuffered APW (diamonds), 5 mM $CaCl_2$ (triangles) or 5 mM $CaCl_2$ + 1 mM $LaCl_3$ (squares). Influx t = 4 h from APW containing 1 µM ^{60}Co. Each point is the average of 7 pooled cells.

Table 4.1. Distribution of ^{60}Co in *Chara*. Cells were incubated in APW (pH 6.0) and 1 μM $^{60}CoCl_2$ for 4 h, followed by desorption for 75 min in 5 mM $CaCl_2$ + 1 mM $LaCl_3$ (mean ± s.e., n=9).

Cell compartment	^{60}Co (nmol m^{-2})	Equivalent influx (pmol m^{-2} s^{-1})	% of total cell activity
Cytoplasm	55 ± 7	3.8 ± 0.6	3.2 ± 0.5
Vacuole	84 ± 10	5.8 ± 0.8	4.9± 0.8
Intracellular content	139 ± 13	9.6 ± 1.1	8.1 ± 1.1
Wall	1588 ± 72	110.3 ± 6.0	91.8 ±1.1
Whole cell	1727 ± 72	119.9 ± 6.0	[100]

4.3.4 Time course of ^{60}Co uptake and distribution

The desorption and fractionation experiments indicated that it would be difficult to quantitatively remove ^{60}Co from the cell wall in order to measure membrane fluxes. Therefore, in subsequent experiments, ^{60}Co influx was measured in cells in which activity in the cell walls and in the cell contents was separated. The desorption time was reduced to 30 min to minimise the error caused by any efflux during the desorption period.

The time-course of uptake from solutions containing 1 μM ^{60}Co is shown in Fig. 4.3. Accumulation within the cell occurred after a lag period of approximately 30 min, in contrast to uptake to the whole cell (predominantly cell wall binding) which was most rapid in the first few minutes of exposure to ^{60}Co (Fig. 4.1). The lag is most likely due to the depletion of ^{60}Co in the apoplast caused by initial rapid binding to the cell wall.

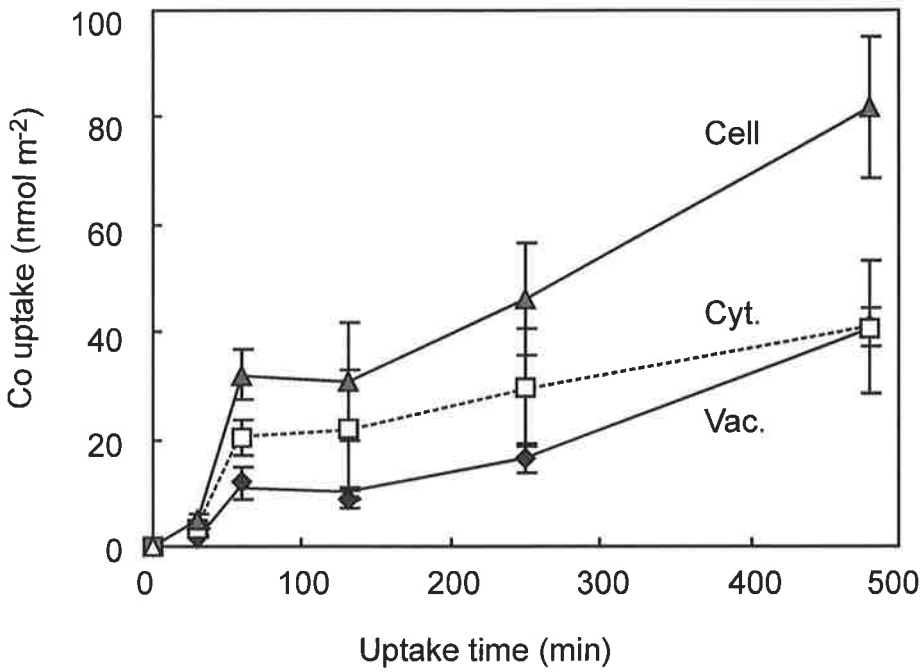


Figure 4.3 Time course of ^{60}Co uptake and distribution in *Chara*. Cells were incubated in APW (pH 6) containing 0.1 μM ^{60}Co then desorbed for 30 min before fractionation into cytoplasmic (Cyt) and vacuolar (Vac) fractions. Cell content is the sum of Vac + Cyt fractions. Each point is the mean ± SE of 6 cells.

An interesting feature of the cellular influx was the rapid appearance of ^{60}Co in the vacuole. The absence of any detectable delay that would indicate a dependence of vacuolar uptake on cytoplasmic filling suggests either that the tonoplast pump has a very high affinity for Co, or perhaps that Co is transferred directly to the vacuole (e.g. by endocytosis).

4.3.5 Effect of pH on ^{60}Co influx

^{60}Co influx was strongly affected by the external pH, with a broad pH optimum between pH 7 and 9 (Fig. 4.4a). Influx was much lower in the range pH 4 - 6. The fall off at pH 9 is probably due to the pH-dependent change in the chemical speciation of Co in solution (Fig. 4.4b). At pH less than about 8, Co is almost entirely present as the free divalent cation. At a pH above 8 there is a progressive conversion to hydroxy species. The significance of this result is that it points to the divalent cation as the transported species, rather than the monovalent or neutral hydroxy species.

4.3.6 Effect of sulfhydryl reagents on ^{60}Co influx

Co^{2+} has a high affinity for sulfhydryl groups in proteins and other molecules and this may be important in the mechanism of membrane transport. To test whether this was true, N-ethylmaleimide (NEM) was applied to bind sulfhydryl groups and thereby mask them from Co. ^{60}Co influx into the cytoplasm and vacuole was significantly inhibited by 10 μM NEM (Fig. 4.5). Cysteine also has sulfhydryl groups which complex Co and it might therefore either enhance Co uptake if the complexed form was membrane-permeable, or reduce Co uptake if it was not. Addition of 10 μM cysteine strongly inhibited Co uptake (Fig. 4.5), which supports the latter proposition, but it also demonstrates the ability of sulfhydryl reagents to bind Co.

4.3.7 Effect of other divalent cations on ^{60}Co influx

The question of whether there are specific transporters in *Chara* for Co was examined by analysing the effects on ^{60}Co influx of the presence in the uptake solution of other divalent

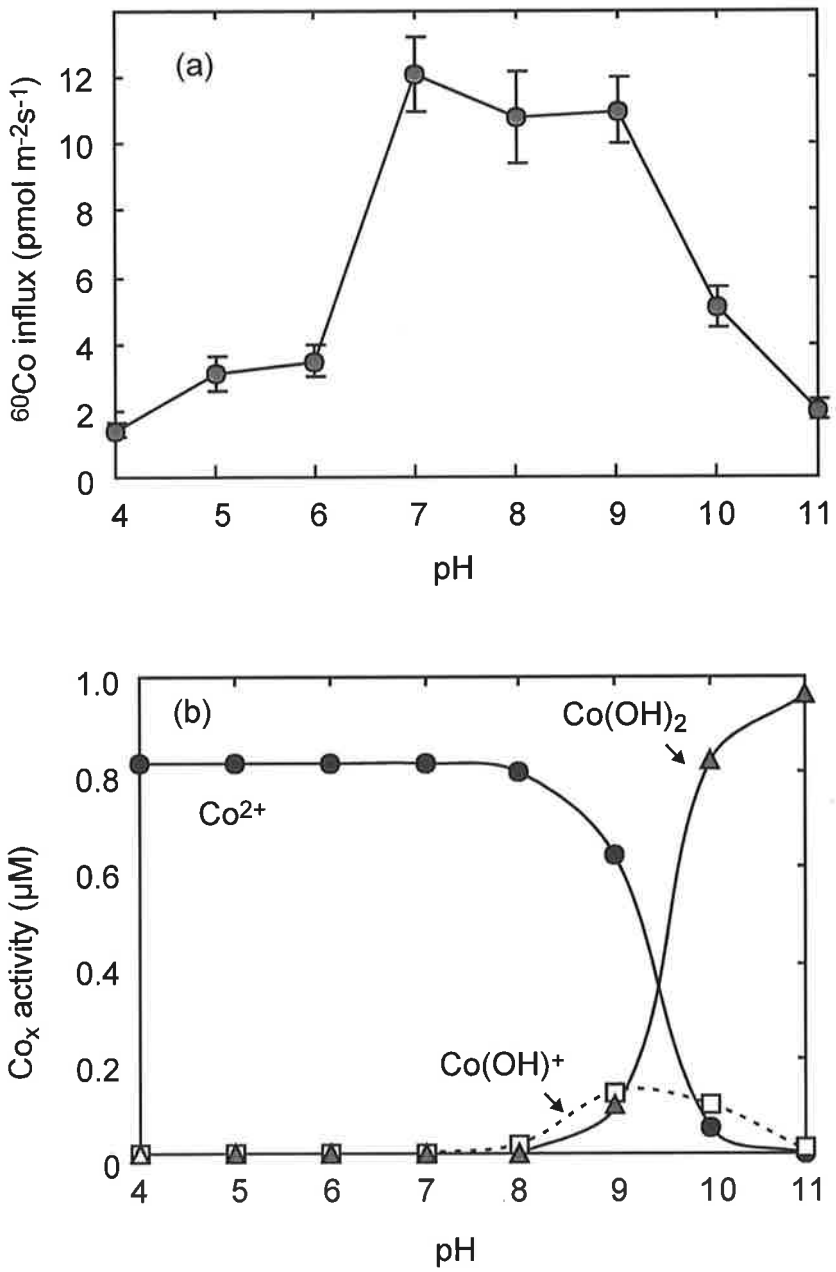


Figure 4.4 (a) ^{60}Co influx into *Chara* as a function of pH in the medium. Influx t = 4 h from APW containing 1 μM ^{60}Co; desorption t = 0.5 h. (b) speciation of Co in APW as a function of pH, calculated by GEOCHEM-PC (Parker et al. 1995). n = 6 cells.

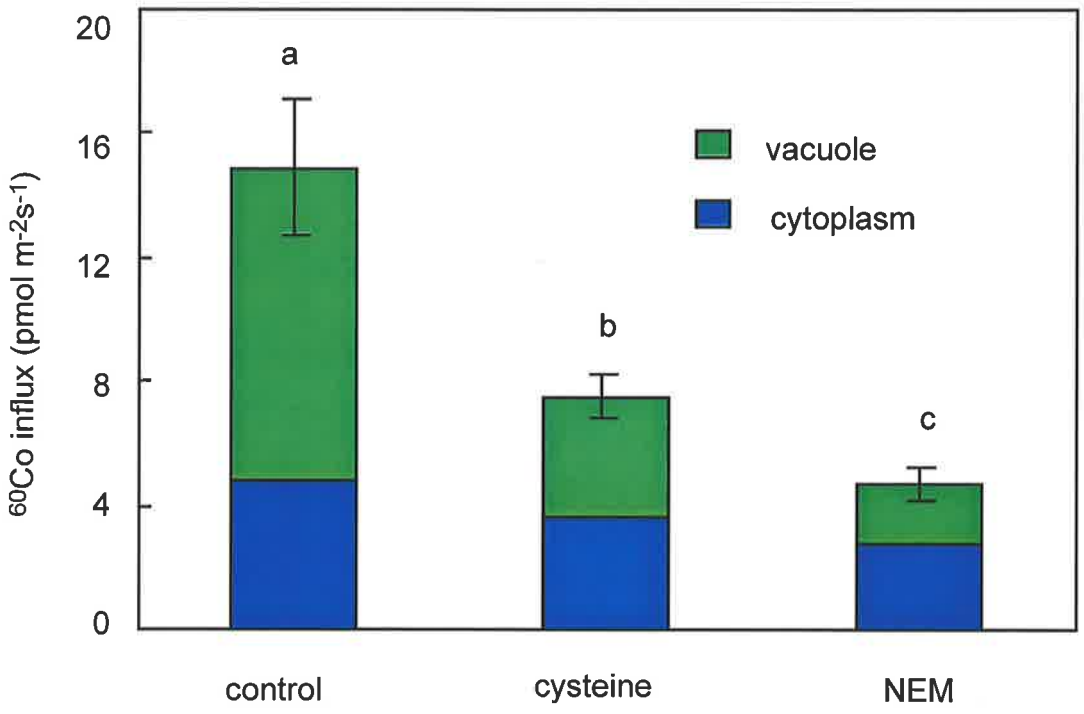


Figure 4.5 Effect of 10 μM NEM and 10 μM cysteine in APW on ^{60}Co influx and compartmentation in *Chara*. Influx t = 4 h from APW containing 1 μM ^{60}Co; desorption t = 0.5 h. n = 10 cells. The treatments with different letters were different at 0.05 significance level by Newman-Keuls multiple comparisons test.

cations. Cells were incubated for 4 h in uptake solutions containing 1 μM ^{60}Co plus 5 μM Cd^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+} as sulphates or chlorides. The effects of the divalent metals on ^{60}Co influx varied considerably (Fig. 4.6). Cd^{2+}, Cu^{2+} and Zn^{2+} inhibited ^{60}Co influx, but Ni^{2+} and Mn^{2+} had no significant effects.

At much higher concentrations, both Ca^{2+} (5 mM) and Mg^{2+} (4.5 mM) had significant effects on the uptake of ^{60}Co compared to the control (APW) (Fig. 4.7).

4.4 Discussion

4.4.1 pH dependence of ^{60}Co influx in *Chara*

^{60}Co influx showed a distinct pH profile which can be attributed to specific pH effects on the transport process and to pH effects on the speciation of Co in solution. Fig. 4.4b shows that as the pH increases above 9 there is a progressive conversion of the divalent Co^{2+} to monovalent $CoOH^+$ and then to the neutral $Co(OH)_2$. While it might reasonably be expected that the plasma membrane would be more permeable to neutral species than to a divalent cation, influx at a pH greater than about 7 correlated well with Co^{2+} concentration (Fig. 4.4a,b) and not with the concentration of $Co(OH)_2$. This suggests that Co^{2+} is the species that permeates and that the higher rates reflect a catalysed process. At a pH less than 7 there is little effect on Co speciation, so the lower influx observed may be due either to direct kinetic effects of pH on the transport process, or, perhaps, to reduced electrostatic attraction to membrane surface charges because of a lesser degree of ionisation of acidic side groups at low pH.

The pH dependence of Co uptake in *Chara* differs from that in mung bean in that the pH optimum lies within the range 7-9, whereas in mung bean the highest uptake was observed in the range pH 5 – 6 in APW solution (Fig 3.8b). This may simply reflect differences in preferred environmental pH.

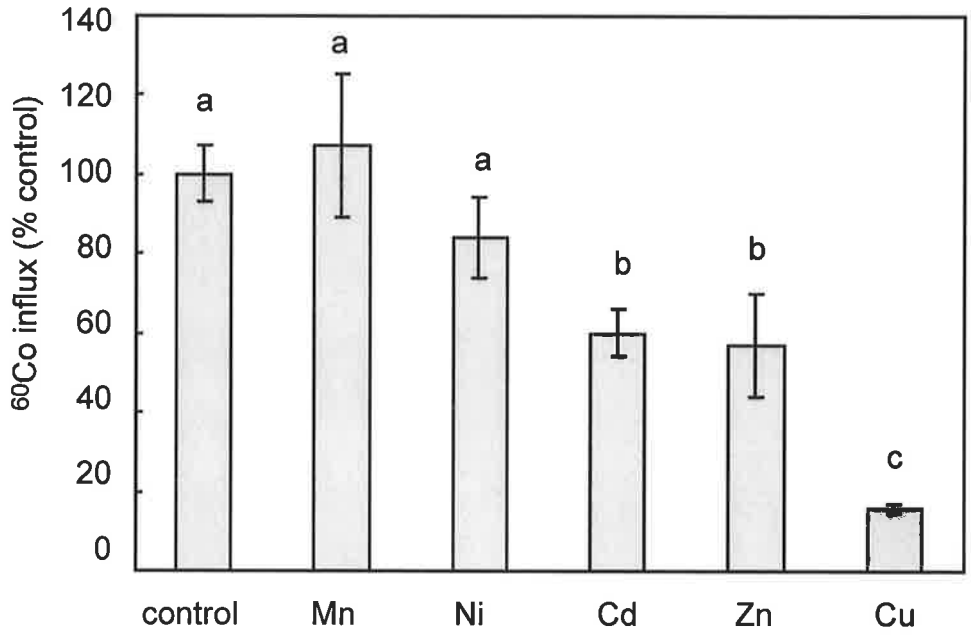


Figure 4.6 Effects of trace metals on ^{60}Co influx in *Chara*. Co was present at 1μM in APW and the trace metals were added at 10 μM as chloride (Ni, Cd) or sulphate salts (Mn, Zn, Cu). Influx t = 4 h; desorption t = 0.5 h. n = 10 cells. The treatments with different letters were different at 0.05 significance level by Newman-Keuls multiple comparisons test.

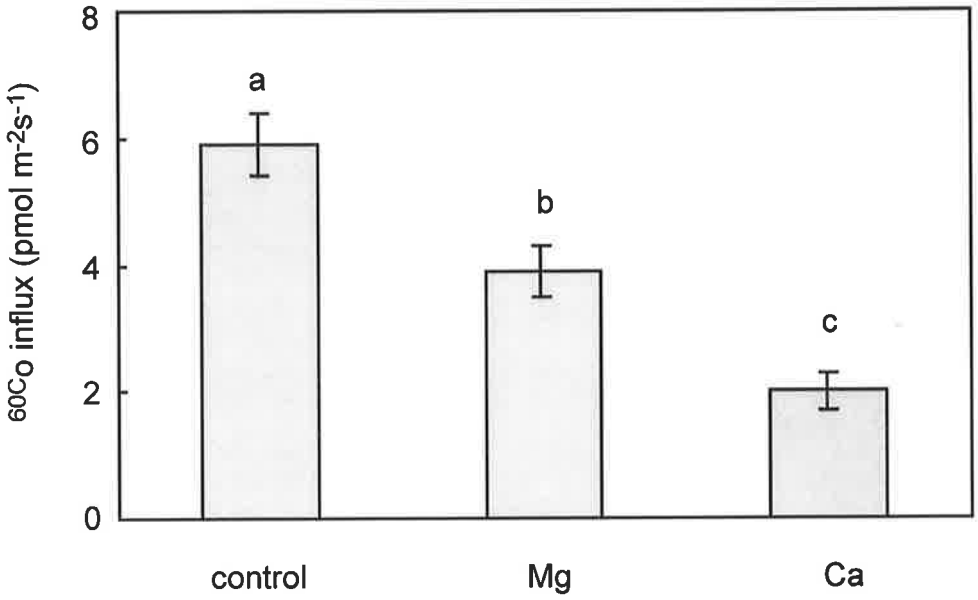


Figure 4.7 Effects of Ca and Mg on ^{60}Co influx in *Chara*. Co was present at 1 μM in APW (contains 0.5 mM Ca). Extra Ca and Mg were added at 4.5 mM. Influx t = 4 h; desorption t = 0.5 h. n = 10 cells. The treatments with different letters were different at 0.05 significance level by Newman-Keuls multiple comparisons test.

4.4.2 Inhibition of Co influx by other metal cations

The question of whether there are specific transporters for trace metals such as Co, or whether Co uptake occurs via a relatively non-specific divalent metal transporter, remains unanswered. It is shown here that some metal cations inhibit Co uptake while others have no effect, and the same results were obtained with mung bean, as shown in Chapter 3. This seems to argue in favour of the existence of transporters with a degree of selectivity, but it would be necessary to demonstrate that the inhibitory metals are actually being transported, and not simply blocking or allosterically inhibiting the activity of the transport protein.

Longer term studies have also shown inhibition of Co uptake by other divalent metals (Colclasure and Schmid, 1974; Affa-Aly *et al.*, 1991; Bernal and McGrath, 1994, Dirilgen and Inel, 1994), but these are less easy to interpret because of the possible complications caused by selective efflux, compartmentation and intracellular detoxification mechanisms which occur over longer uptake periods.

The order of potency in reducing Co uptake of $Cu^{2+} >> Cd^{2+}$, $Zn^{2+} > Ni^{2+}$ = Control = Mn^{2+} is similar, but not identical, to that for mung bean (see Chapter 3). If the inhibition of Co uptake by other metals is due to competition (Fig. 4.6), this would indicate that the proteins involved in the metal uptake may not be specific for Co^{2+} but may also be involved in the uptake of other trace metals. It also indicates that if the speculation concerning the possible existence of ion channels for micronutrient metals (Kochian *et al.*, 1991; Welch *et al.*, 1993; Reid *et al.*, 1996a) is correct, then the selectivity is most likely conferred by -SH groups of protein sites within the channel which are enriched with cysteine. This would distinguish these channels from those of macro-metals, such as Ca^{2+} and Mg^{2+}, that have low affinity for -SH. Indeed, macro-metals, Ca^{2+} and Mg^{2+} were only effective in inhibiting ^{60}Co uptake at very high concentrations compared to trace metals (Fig. 4.7).

4.4.3 Distribution of Co between cytoplasm and vacuole

The rapid appearance of Co in the vacuole points to a mechanism in *Chara* for pumping Co, and maybe other excess metals, across the tonoplast. The vacuole of *Chara* has been reported to be slightly more positive electrically than the cytoplasm, which means that Co would only enter the vacuole passively, if there was a large concentration gradient from the cytoplasm. Since the charophyte cells used in these experiments were not previously exposed to Co (except that existing naturally in pond water), the Co concentration of the cytoplasm would not be expected to be high and therefore the specific activity of ^{60}Co in the cytoplasm might be similar to that in the external solution, and the ^{60}Co activity in the cytoplasm and vacuole would permit estimation of relative concentrations. Since the vacuolar volume in *Chara* is roughly 20x greater than that of the cytoplasm, the activities shown in Fig. 4.3 would correspond to concentration gradients across the tonoplast of between about 40:1, at earlier times, and 20:1, after 500 min. This may be sufficient to counter the opposing electrical gradient and permit passive accumulation in the vacuole.

CHAPTER 5 Physiological Effects of Co in Mung Bean in Relation to Nutrient Balance

5.1 INTRODUCTION

Cobalt affords both beneficial effects and toxicity to plants. It has long been applied to plants either to raise crop yields (Young, 1979) or to increase Co content in forage in order to prevent Co deficiency diseases in animals in certain areas of the world, such as Britain, Western and South Australia, New Zealand, Kenya and the U.S.A. (Underwood 1971). Unlike in animals and micro-organisms, where Co is known as an essential element in the form of vitamin B_{12}, the essentiality of Co in plants is not recognized, although it is needed by *Rhizobia* in root nodules of leguminous plants and by some species of nitrogen-fixing blue green algae for symbiotic nitrogen fixation (Holm-Hansen *et al.*, 1954; Lowe and Evans, 1962). It is not only legume crops (cowpea, groundnut and soybean) which are reported to benefit, but also non-leguminous plants, such as cereals (barley, buckwheat, corn, oats and wheat), grass (timothy), fruit (apple and grape), vegetables (Chinese cabbage and potato), and sugar beet (Young, 1979).

Excess Co induces toxicity in plants, the critical level varying with species, but being generally within the range of 30-40 ppm dry matter (Kabata-Pendias and Pendias, 1991). In most cases, excess Co results from the direct application to plants in agricultural practices or from environmental pollution (Linzon, 1981; Brandford *et al.*, 1975; Temple and Bisessar, 1981; Bisessar *et al.*, 1983). Toxicity symptoms include interveinal chlorosis of new leaves, white leaf margins and tips, leaf chlorosis and damaged root tips (Kabata-Pendias and Pendias, 1991). Co inhibits many processes in plants, such as photosynthesis (Van Assche and Cliijsters, 1990; Mohanty *et al.*, 1989), seed germination and seedling growth (Dubey and Dwivedi, 1987; Thukral and Kaur, 1987), and growth and yield (Patel *et al.*, 1976).

The mechanisms by which Co affects plants are not yet clearly known. It has been proposed that Co interacts with the uptake of other macro- and microelements (Palit *et al.*, 1994). Co

would appear to be toxic when the uptake and/or action of essential elements such as Ca and Fe is inhibited (Terry, 1981; Agarwala *et al*, 1977; Wallace *et al.*, 1971; Veltrup, 1981). However, it would appear to be beneficial when the uptake and action of toxic elements such as Cd, Cu, Ni and Zn is inhibited (Dirilgen and Inel, 1994; Wallace and Abou-Zamzam, 1989). There is still a lack of evidence for the confirmation of the role of Co interaction with other elements in both a beneficial and toxic manner in a diversity of plants. In this study, the effect of Co was investigated in relation to other elements, mainly Ca and Fe, on the parameters of growth, photosynthesis and nutrient uptake in mung bean in order to elucidate the mechanisms of Co effects (mainly toxicity) in plants.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Mung bean seedlings were grown as described in Chapter 2. Co was applied in the growth solution one week after germination. 25 μM $FeCl_3$ was used as specified. To evaluate the effect of Ca on Co toxicity, the concentration of Ca ranged from 0.2 to 5 mM as specified.

5.2.2 Methods

Analytical methods for chlorophyll content, radiotracer methods and determination of nutrient and Co concentrations by ICP-AES were carried out as described in Chapter 2.

5.3 RESULTS

5.3.1 Comparative effects of Co and Fe deficiency on the growth of mung bean

In order to compare the effect of Co and Fe deficiency on the growth of mung bean, seedlings were grown in ¼ Hoagland's solution containing Fe plus 0 (control), 0.5 and 5 μM $CoCl_2$, or not containing Fe (Fe-deficient). $FeCl_3$ (25 μM) was added to the growth solution instead of FeEDTA to avoid Co chelation by EDTA (Parker *et al.*, 1995).

Excess Co (5 μM $CoCl_2$ + 25 μM $FeCl_3$) and Fe deficiency inhibited the growth of mung beans and caused similar degrees of chlorosis on the primary and trifoliate leaves. Figure 5.1 shows that the treatment of 5 μM Co + 25 μM Fe and Fe deficiency treatment both significantly depressed the FW of whole plants compared to the control, while 0.5 μM Co + Fe had no significant effect. The decrease in FW was more or less the same for the roots, stems and leaves (data not shown).

The difference between excess Co + Fe and Fe deficiency appeared in the roots. While both significantly depressed the elongation of the main root compared to the control, Fe deficiency (73% control) had a more inhibitory effect than 5μM Co + 25 μM $FeCl_3$ (89% control) (Fig 5.2a). Additionally, 5 μM Co + 25 μM $FeCl_3$ significantly reduced the number of side roots (82% control), but Fe deficiency (102% control) had no significant effect (Fig 5.2b).

The Fe content of seedlings was strongly affected by Co (Fig 5.3). In the seedlings treated with 5 μM Co + 25 μM Fe, Fe content in the leaves was reduced by 80%, and even at the non-toxic concentration of 0.5 μM Co + 25 μM Fe, the Fe content was reduced by 51% (Fig 5.3a). By contrast, the Fe content of the roots was not significantly affected (Fig 5.3b).

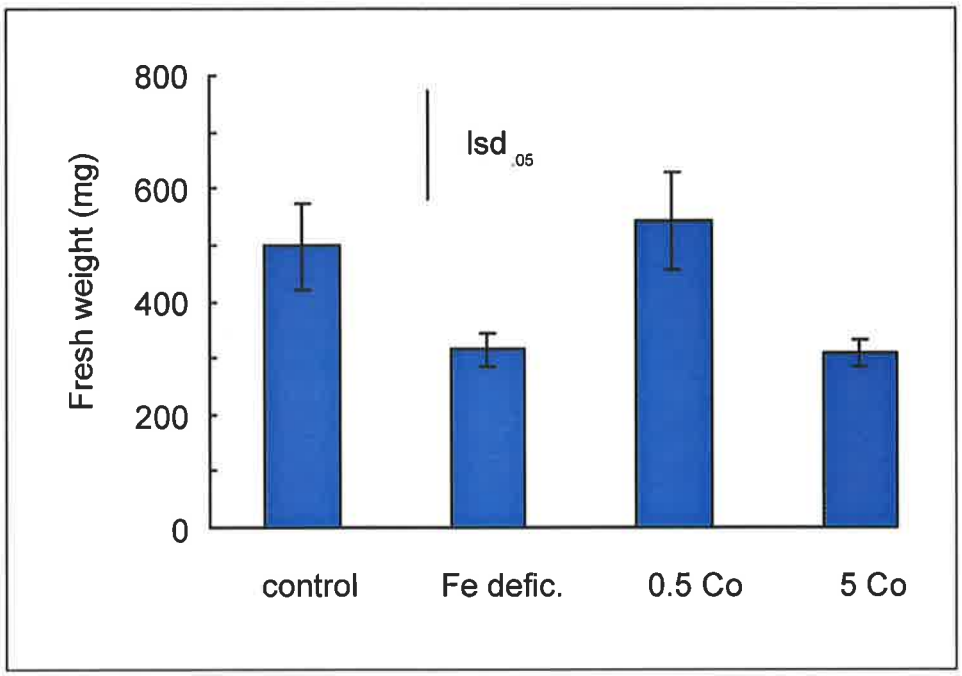
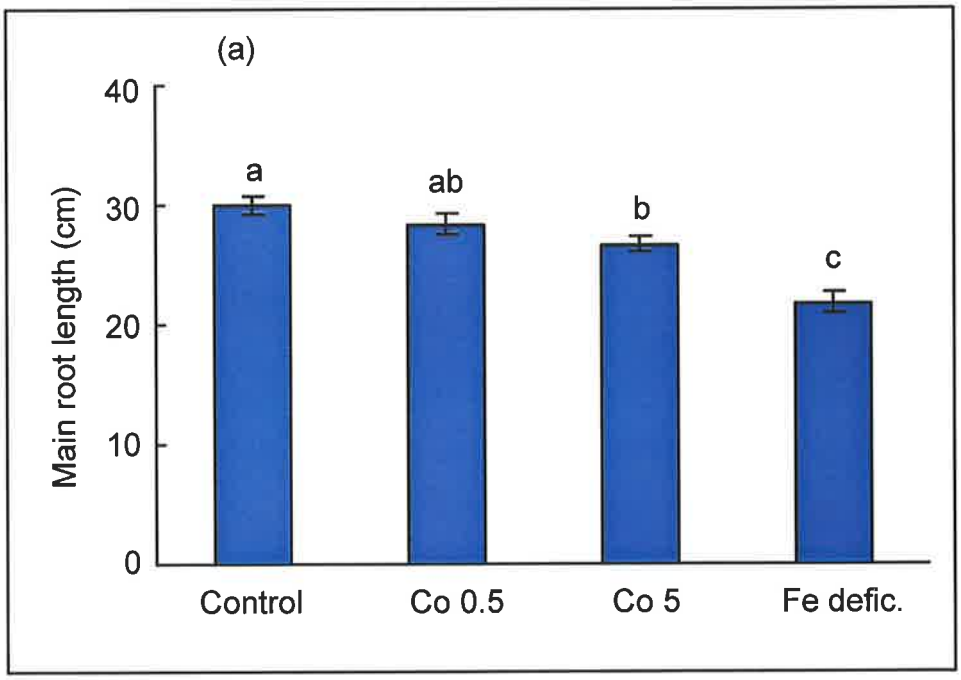


Figure 5.1 Effects of Fe and Co on the growth of mung beans. All treatments except Fe deficient contained 25 μM $FeCl_3$. n = 3 replicates consisting of 12 plants each.



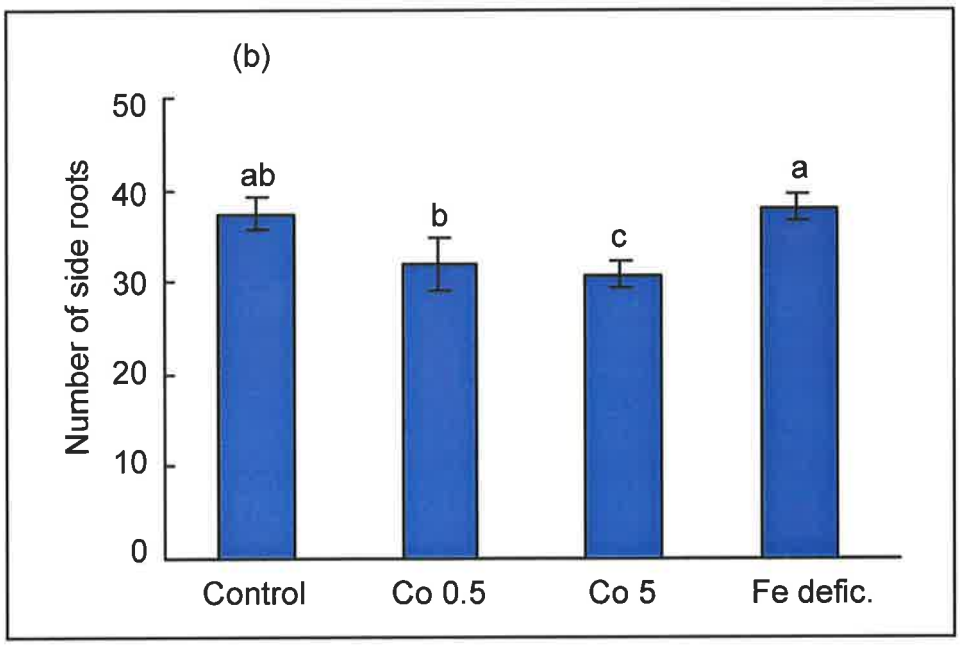
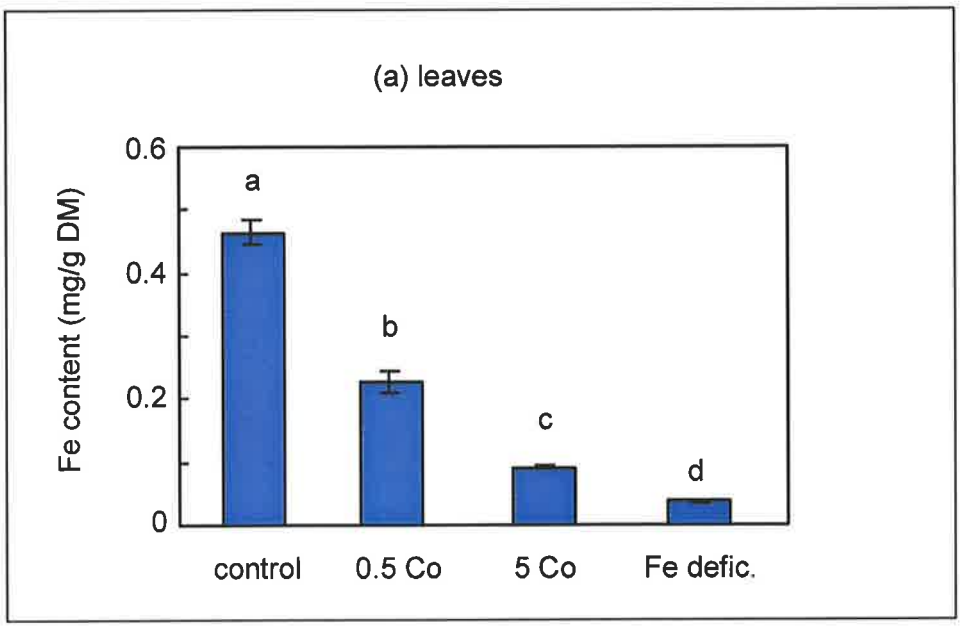


Figure 5.2 Effects of Fe deficiency and Co on (a) root length and (b) number of side roots of mung bean seedlings. Treatments with different letters are significantly different. All treatments except Fe deficient contained 25 μM $FeCl_3$. n = 3 replicates consisting of 12 plants each.



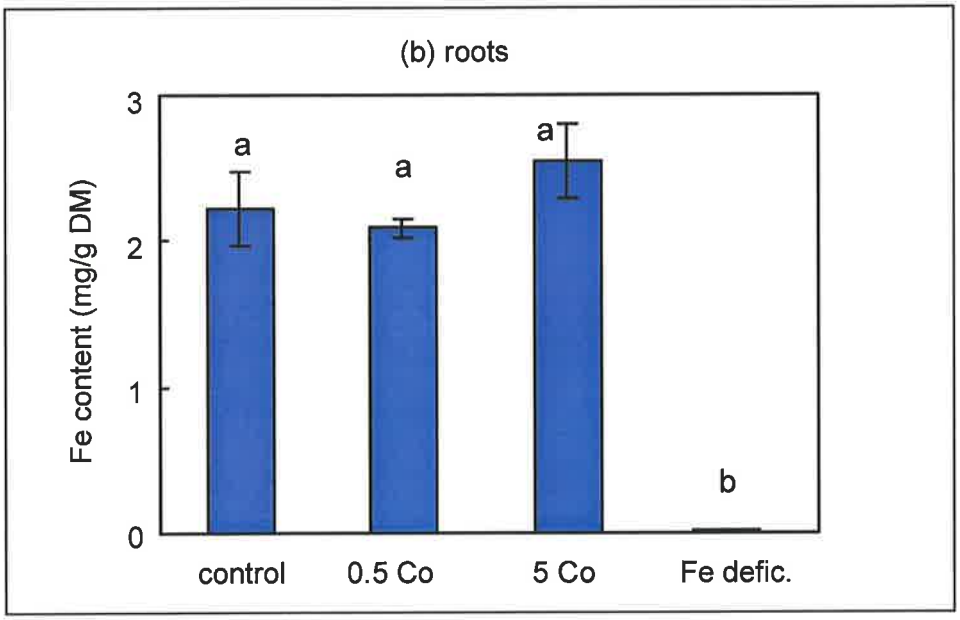


Figure 5.3 Effect of Fe deficiency and Co on Fe content of (a) leaves and (b) roots of mung bean seedlings after 10 d of growth. All treatments except Fe deficient contained 25 μM $FeCl_3$. n = 3 replicates consisting of 12 plants each.

5.3.2 Effect of Co on growth of mung bean

Since Co and Fe deficiency share some similarities in the inhibition of growth of plants, to examine other mechanisms of Co on plant growth (rather than on Fe uptake), seedlings were raised in a growth solution in the absence of Fe but containing various concentrations of Co from 0.5-500 μM. Co at 5 μM or higher concentrations inhibited the growth of mung bean, and this inhibitory effect increased with the duration of the Co treatment (Fig 5.4). After 5 d growth with Co at 5, 50 and 500 μM, growth was depressed by 10%, 25% and 68%, respectively, whereas after 8 d, the depression was 29%, 53% and 77%, respectively. The depression of growth of whole seedlings was associated with the decrease in both the fresh weight of roots and of the upper parts (leaves plus hypocotyls) (data not shown). Excess Co induced chlorosis and necrosis in primary leaves. The chlorophyll content of primary leaves was strongly inhibited by Co at 5 μM and higher concentrations. Chl a+b was reduced by 4%, 33%, 49% and 48% at 0.5, 5, 50 and 500 μM Co, respectively, as a result of decreases in both chl a and chl b content (Fig 5.5). However, although both chl a and chl b were reduced by Co, chl b content was less sensitive to Co concentrations over 5 μM. The decrease of chl a was 4%, 31%, 50% and 53% at 0.5, 5, 50 and 500 μM Co, respectively, compared to the control, whereas the decrease of chl b was 3%, 38%, 45% and 34%, respectively.

The visual appearance of seedlings exposed to Co is shown in Fig 5.6.

5.3.3 The role of Ca in ameliorating Co toxicity

5.3.3.1 Growth

In the absence of added Co, increasing Ca from 0.2 to 5 mM had little effect on seedling FW up to 5 d of growth (Fig 5.7a), possibly due to adequate Ca in the cotyledons up to this stage. However, between 5 and 8 d, FW was dependent on the Ca supply, increasing in a linear fashion with increasing Ca concentration in the medium (Fig. 5.7b). In plants to which 50 μM Co was supplied, growth was severely inhibited at low Ca concentrations, but

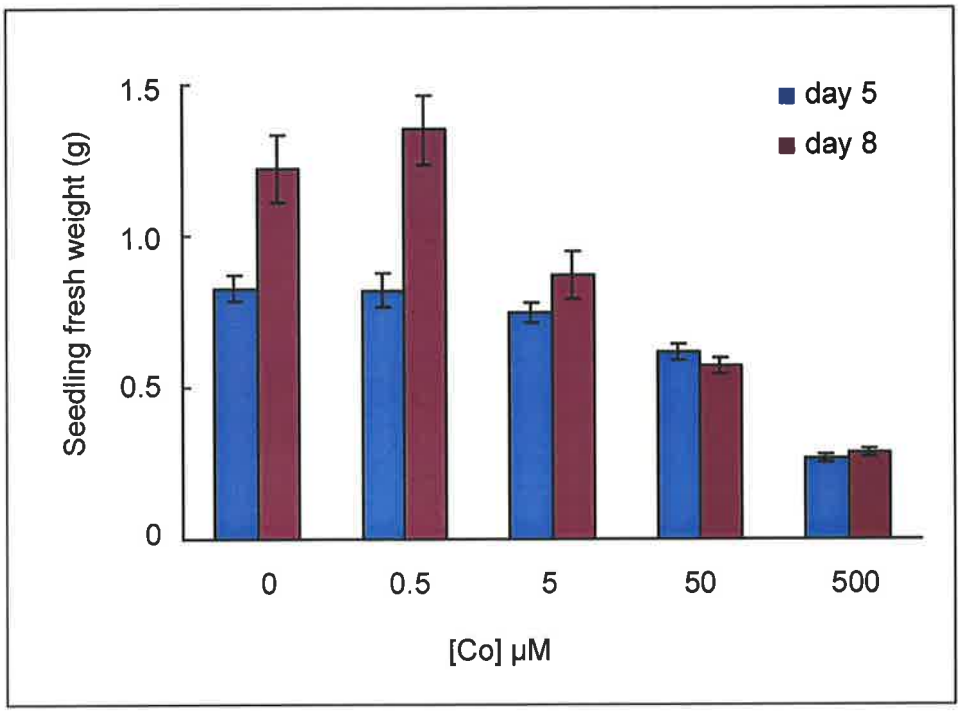


Figure 5.4 Effect of Co on growth of mung bean seedlings in the absence of Fe.. Plants were grown in 1/4 Hoagland's solution minus Fe for 10d. The solutions were changed every 3 d. n = 8 seedlings.

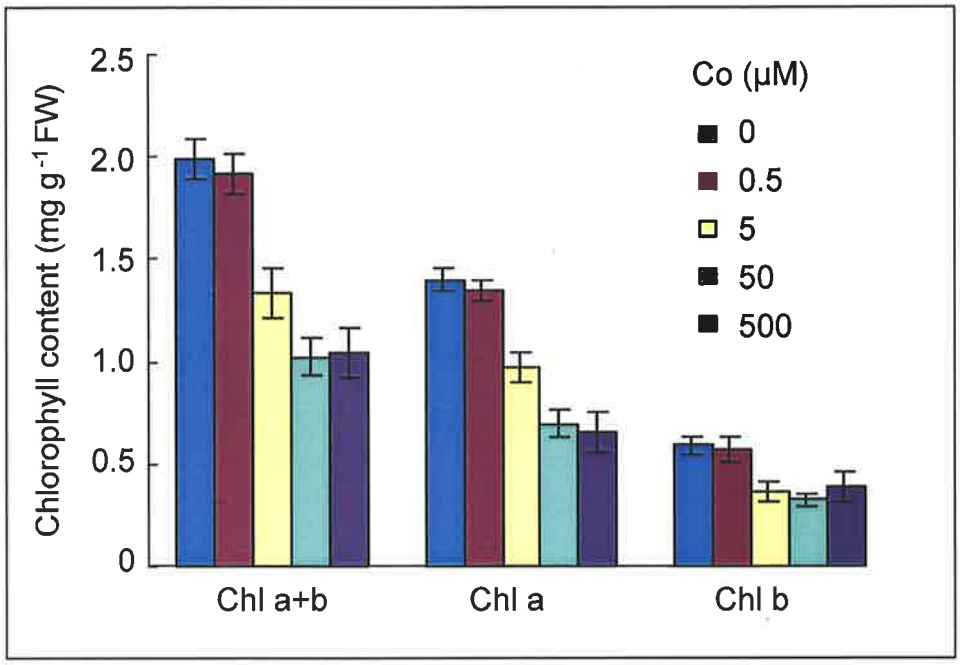
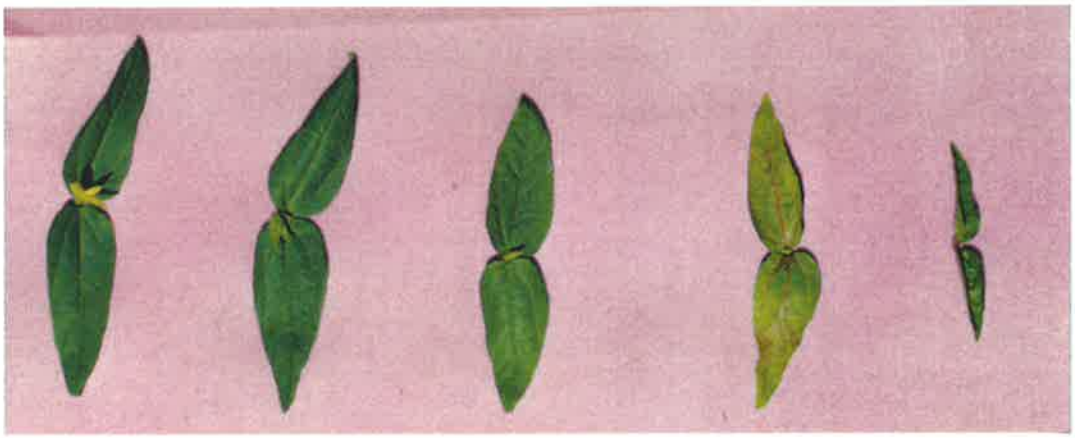


Figure 5.5 Effect of Co on the chlorophyll content of the primary leaves of mung bean. Seedlings were transferred to Co solutions after day 3 and chlorophyll was measured after day 9. n = 4 seedlings.



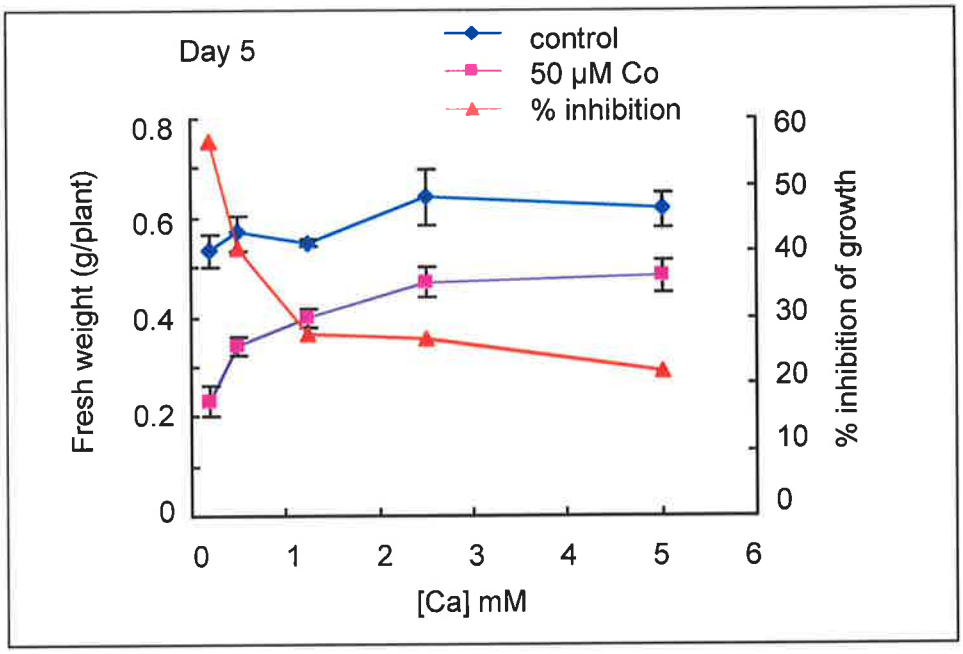
0 0.5 5 50 500

Cobalt concentration (μM)

0 0.5 5 50 500

Cobalt concentration (μM)

Figure 5.6. Effect of Co on growth and morphology of mung bean plants.
Top - primary leaves showing progressively more chlorosis and necrosis with increasing Co concentrations in the growth medium.
Bottom - whole seedlings showing overall inhibition of growth and reduction in lateral roots. Note also browning of roots.



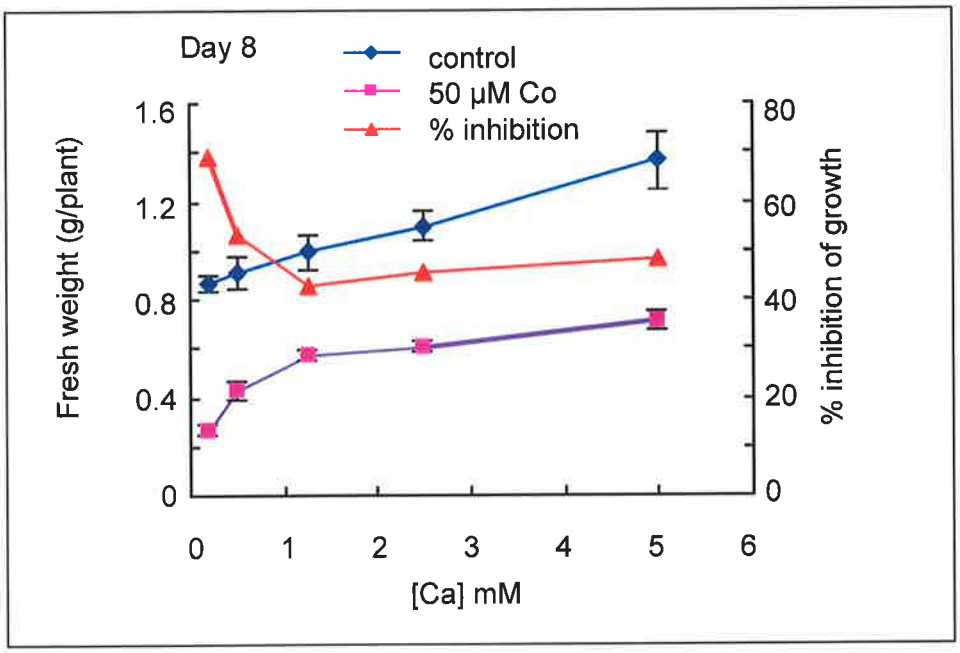


Figure 5.7 Effect of Ca on the inhibitory effects of 50 µM Co on growth of mung bean seedlings after 5 d and 8 d. n = 8-10 seedlings.

improved as the Ca concentration increased to 1.25 mM (the concentration in ¼ Hoagland's solution). Thereafter, the increase in growth was similar to that in the plants without Co. However, even at relatively high Ca concentrations, there remained a significant inhibition of growth by Co (Fig 5.7). Thus, there appear to be both Ca-sensitive and Ca-insensitive components of Co toxicity, and Ca deficiency may therefore intensify the toxicity of Co.

5.3.3.2 Photosynthetic efficiency

Photosynthetic capacity can be assessed by chlorophyll fluorescence, which is related to the absorbed light energy for photosynthesis and other heat dissipating processes (Krause and Weis, 1991). In the current study, the photochemical yield (Y= (Fsm-Fs)/Fsm, as defined in Chapter 2) of photosynthesis system II (PSII) reaction centres in light was measured using light-adapted methods. Co lowered the yield of energy conversion in photosynthesis, and the inhibition was dependent on Ca concentration in the growth solution (Fig 5.8).

5.3.3.3 Cobalt uptake

The effect of Ca in reducing the toxicity of Co may be due to the inhibition on Co uptake. Fig 5.8 shows that short term uptake of Co in mung bean roots was dependent on the concentration of Ca in the medium. Three days after germination, mung bean seedlings were transferred to growth solutions containing various concentrations of Ca, with or without 50 μM Co. After 7 days growth in these solutions, the seedlings were incubated in solutions containing the same concentrations of Ca as for growth plus 50 μM ^{60}Co for both Co pretreated and control (without Co during growth).

In plants pretreated with Co, uptake of ^{60}Co was considerably lower than in plants that had not previously been exposed to Co, in agreement with the results for shorter term pre-treatments, shown in Table 3.1. Increasing Ca concentrations in both the pretreated and non-pretreated plants inhibited uptake of ^{60}Co. Some of the reduction in Co uptake could be attributed to the lowering of the activity of free Co^{2+} by Ca (Fig. 5.9). As with the effects on growth, there appeared to be Ca-sensitive and Ca-insensitive components of Co uptake. The role of Ca in preventing Co uptake is nevertheless limited in the presence of 50 μM Co.

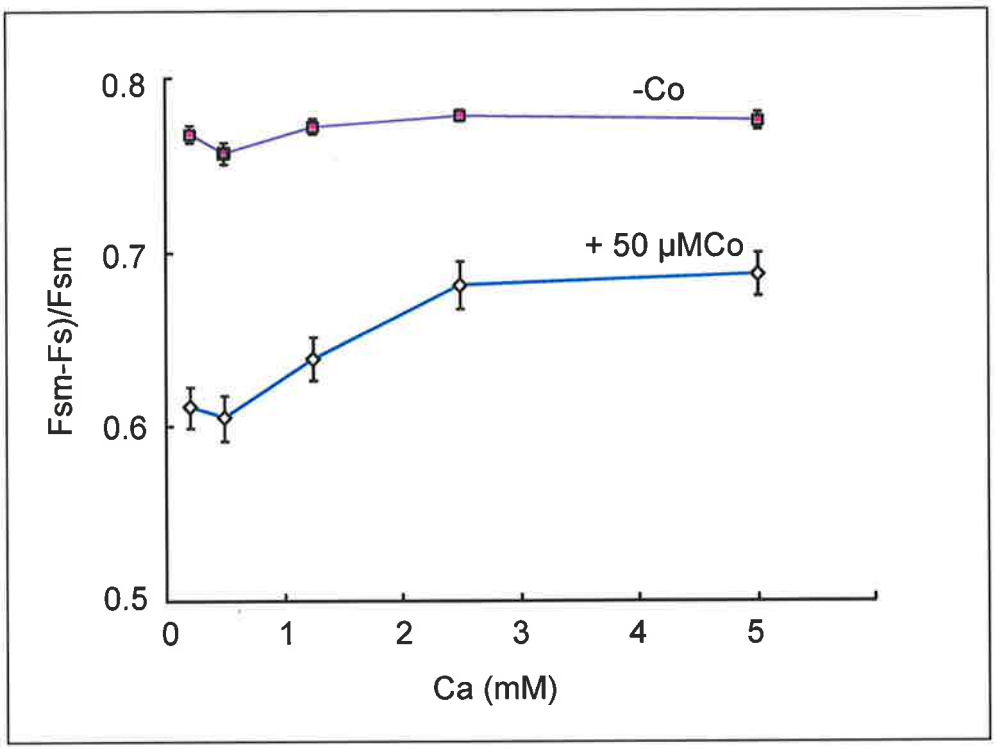


Figure 5.8. Effect of Co on chlorophyll fluorescence of mung bean leaves as a function of Ca in the growth medium. Three days after germination, seedlings were transferred to growth medium containing various concentrations of Ca, with or without 50 µM Co. Fluorescence was measured after 9 d. n = 2 seedlings.

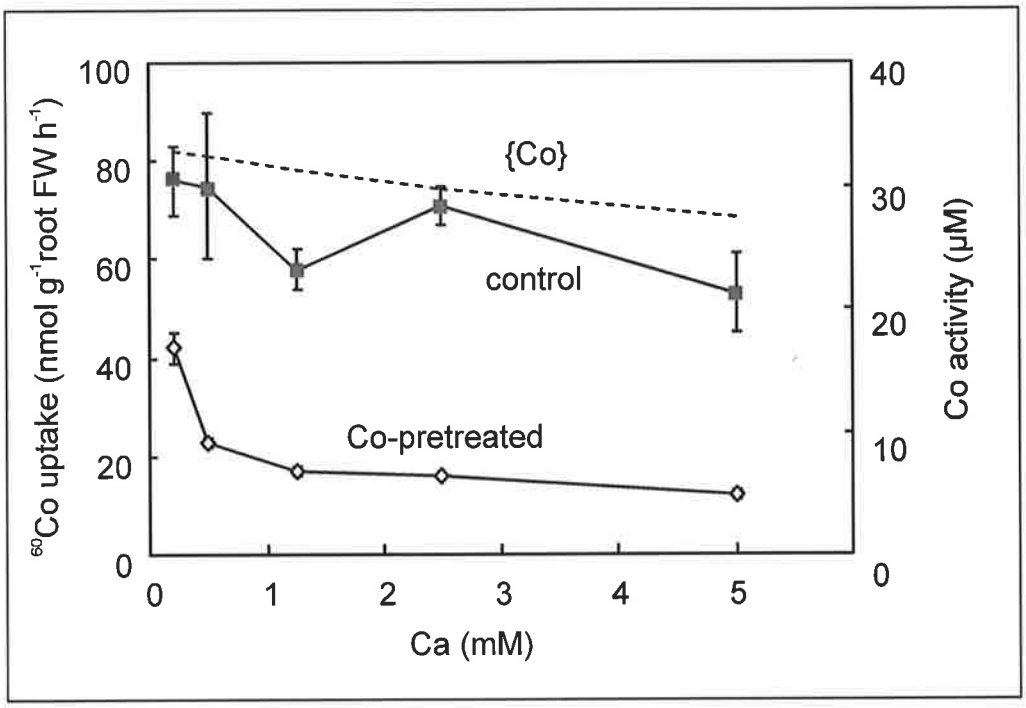


Figure 5.9 Effect of Co pretreatment and Ca on ^{60}Co uptake in mung beans. Three days after germination, seedlings were transferred to growth solution containing various concentrations of Ca with or without 50 µM Co. The uptake experiment was performed after 7 days at the same Ca concentration and with 50 µM ^{60}Co. Uptake t = 4 h; n = 6 seedlings.

5.3.4 Effect of Co on nutrient content of seedlings

The effect of Co on nutrient balance was examined by growing mung beans in ¼ Hoagland's solution containing various concentrations of Co for 2 weeks. Two Co concentrations were tested: 0.5 μM (non-toxic) and 5 μM (moderately toxic). Fe deficient plants were also exposed to 50 μM Co (strongly toxic). The variation in the level of nutrients as a result of Co treatment varied with the particular nutrient, with the plant part, and with Fe status.

5.3.4.1 Co content

Long term accumulation of Co as a function of Co concentration in the growth solution is shown in Fig 5.10. The Co content of leaves and stem (hypocotyl + epicotyl) increased linearly with the concentration of Co (Fig 5.10a), but in the roots (Fig 5.10b) it seemed to plateau at 5 μM, which is consistent with the ^{60}Co uptake experiments described in Chapter 3.

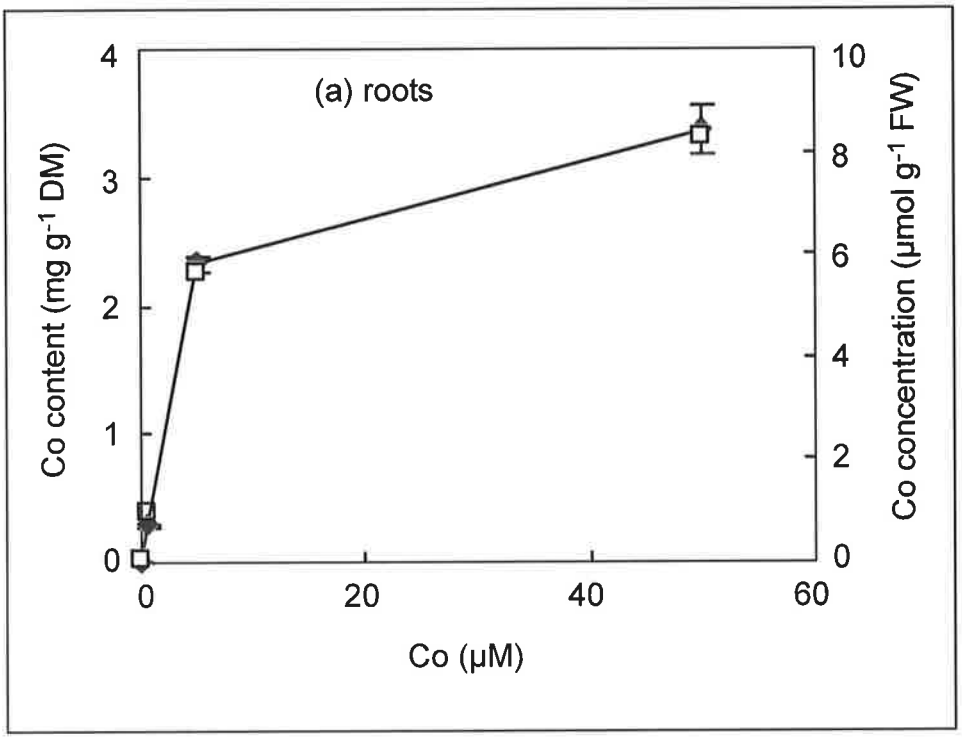
5.3.4.2 Micronutrient content (+Fe plants)

In mung beans seedlings grown in ¼ modified Hoagland's solution with adequate Fe, the content of Mn, Zn and Cu of leaves and roots was not significantly altered by 0.5 μM Co (Fig 5.12). At 5 μM Co, the Mn content of roots was reduced by more than 45% while leaf content was higher by 27%. The Zn content of leaves was also reduced (-25%).

5.3.4.3 Micro-nutrient content (-Fe plants)

The interaction of Co and Fe was investigated further by comparing micronutrient content of plants grown without Fe with those grown with Fe (see 5.3.4.2 and Fig 5.11).

Mn. Co treatment reduced the Mn content of the roots. At 0.5, 5, and 50 μM Co, the content of Mn decreased 44, 35, and 79%, respectively (Fig 5.12a). On the other hand, Mn content of the leaves increased by 48% at 0.5 μM Co, but decreased 21 and 71%, respectively, at 5 and 50 μM, compared to the control (Fig 5.12a). There was a significant negative correlation between Co content and Mn content in the roots (r= -0.77) and in the leaves (r = -0.83), which suggests that the two metals are antagonistic for uptake and/or



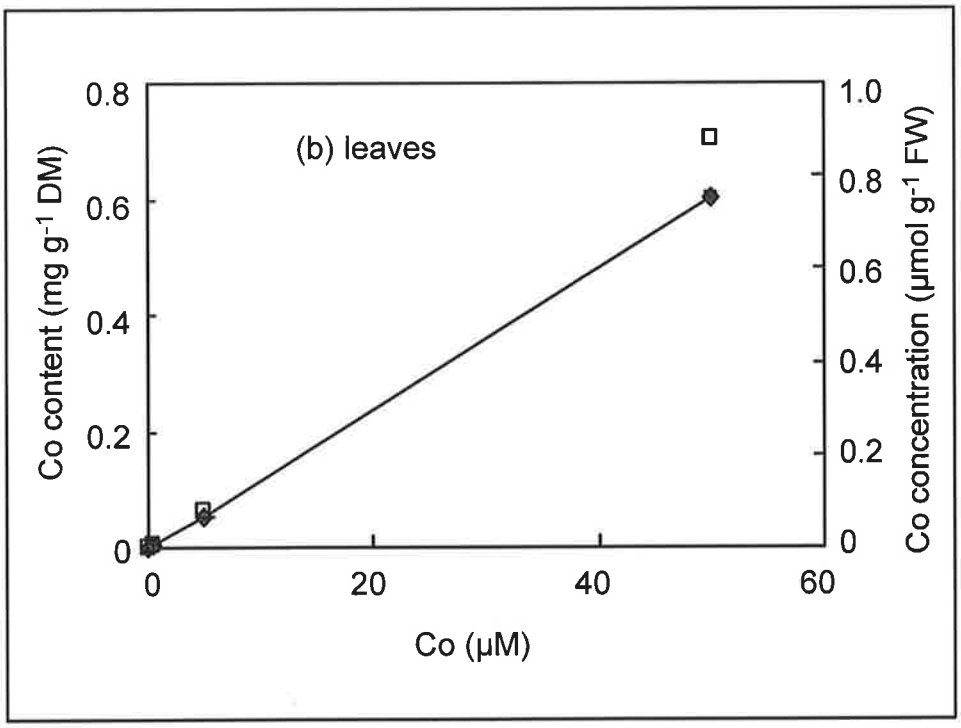


Figure 5.10 Co content of (a) roots and (b) leaves of mung beans as a function of the concentration of Co in the external medium. Content is expressed on a dry weight basis (closed symbols) and converted to a fresh weight basis for comparison with ^{60}Co experiments in Chapter 3. n= 3 seedlings.

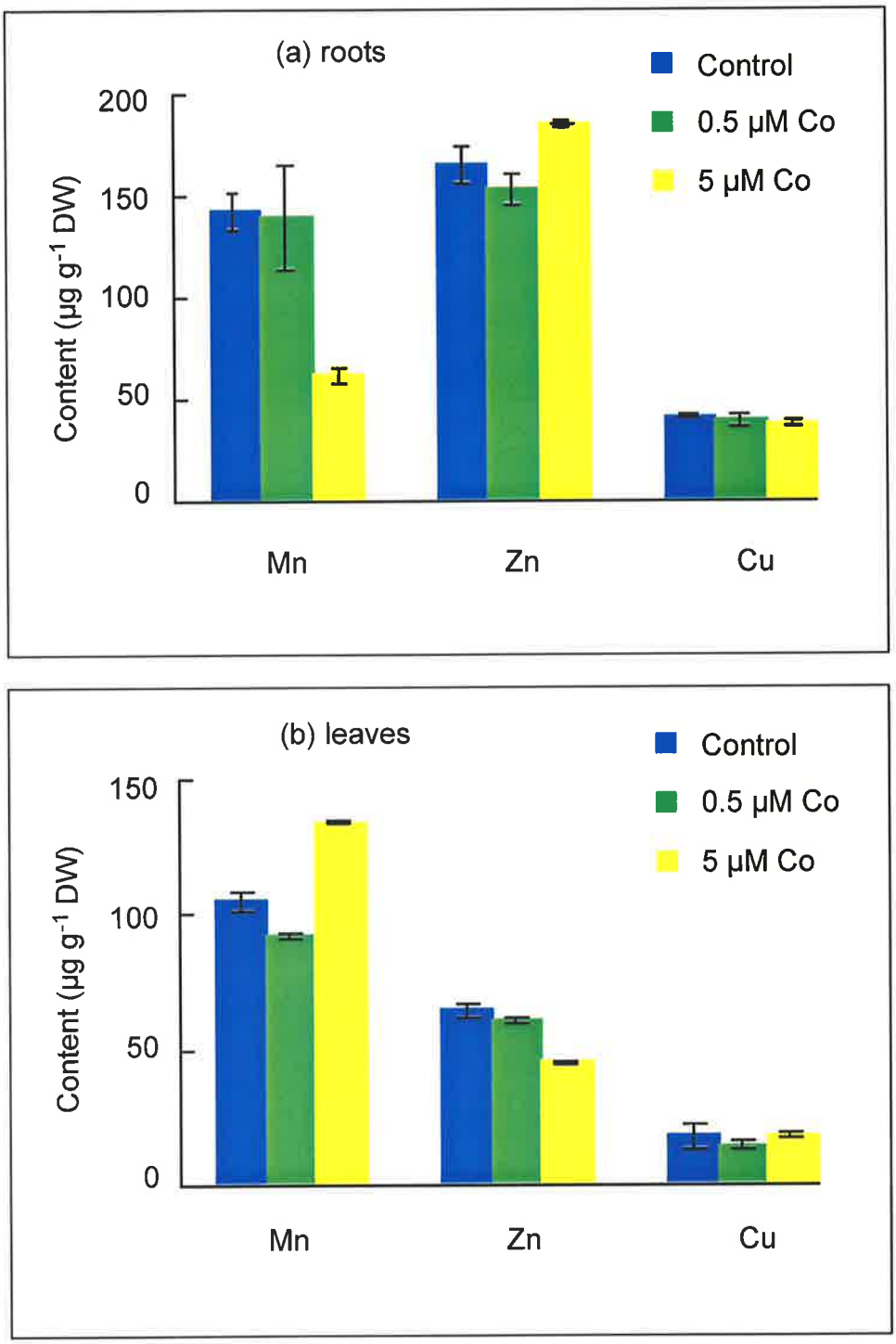


Figure 5.11 Effect of Co on the content of Mn, Zn and Cu in(a) roots and (b) roots of mung beans in 1/4 Hoaglands solution containing 25 μM Fe. n = 3 seedings.

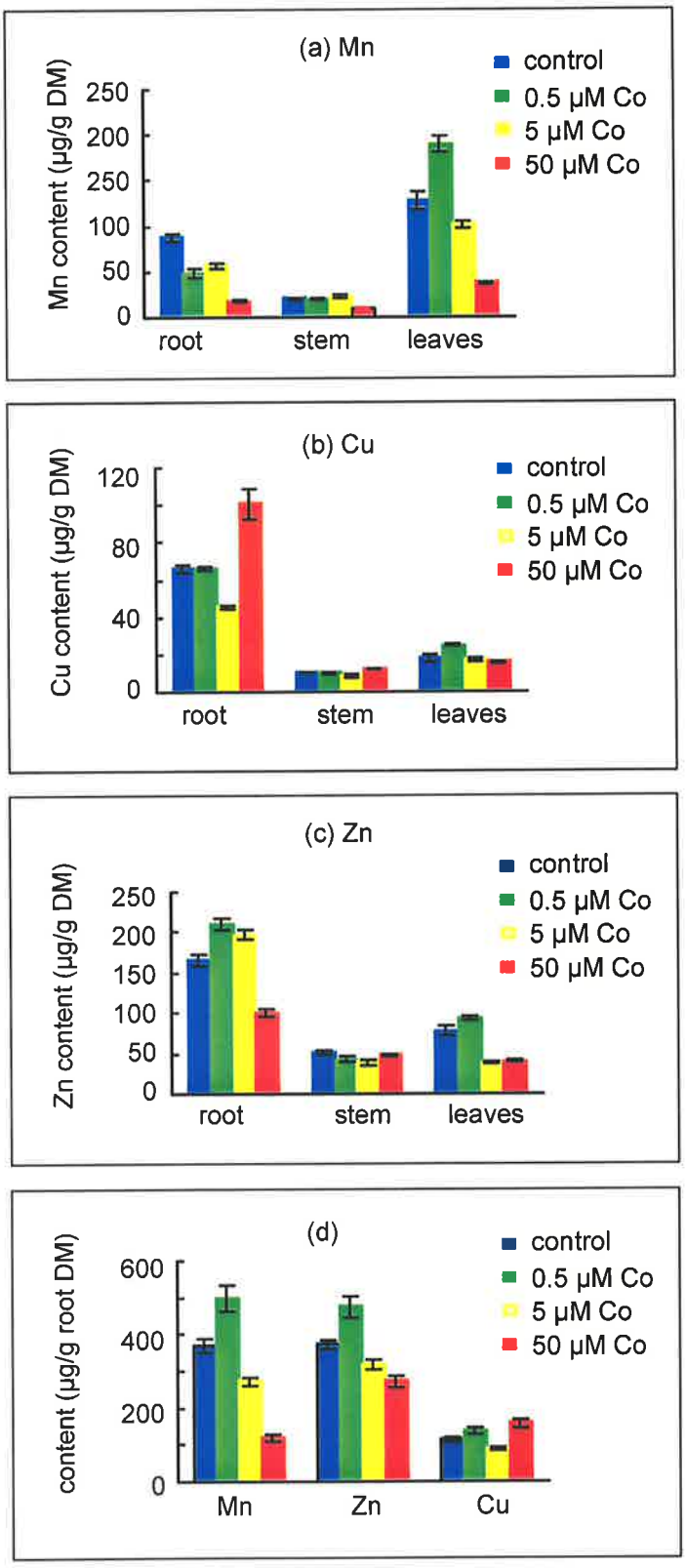


Figure 5.12 Effects of Co on the (a) Mn, (b) Cu and (c) Zn content of roots, stems and leaves and (d) whole plant content based on root DM, in mung beans grown for 2 weeks in 1/4 Hoagland's solution without Fe. n = 3 seedlings.

transport. The increased Mn transport to leaves by Co might be attributable to the saturation of common intracellular metal binding sites and sequestering mechanisms in the roots.

Cu. The Cu content of roots was unaffected by 0.5 μM Co, reduced by 5 μM Co and increased substantially by 50 μM Co (Fig. 5.12b), possibly as a result of toxicity and breakdown of membrane permeability or selectivity. Cu content of the stem and leaves was not strongly affected by Co, although 0.5 μM Co increased leaf Cu content.

Zn. Zn content of roots was increased by 0.5 and 5 μM Co, but inhibited by 50 μM Co. Leaf Zn content was greatly reduced by 5 and 50 μM Co (Fig. 5.12c). There were significant negative correlation's between Zn content and Co content in the roots ($r = -0.61$) and in the leaves ($r = -0.58$).

Whole plant content. To obtain an approximate measure of the effect of Co on the uptake of Mn, Cu and Zn, the whole plant contents of these metals were calculated on the basis of root DM at harvest (Figure 5.12d). It is clear from this data that at the non-toxic concentration of 0.5 μM Co; Co enhanced 'uptake' of all 3 nutrients. For Mn and Zn, 'uptake' was inhibited by higher Co concentrations, while for Cu the pattern was more complex.

The relative effects of different Co concentrations of Cu, Mn and Zn contents of roots and leaves of mung bean are summarised in Table 5.1.

Table. 5.1 Effect of Co on the content of Cu, Mn and Zn in leaves and roots of mung bean and on 'uptake' measured as the whole plant content divided by the root weight. The data are presented as increase (+) or decrease (-) compared to the controls.

	treatment	effect on content
root accumulation	0.5μM Co	Mn (-44%) > Cu (-1%) > Zn (+26%)
	5μM Co	Mn (-35% > Cu (-32%) > Zn (+18%)
	50μM Co	Mn (-79% > Zn (-40%) > Cu (+52%)
leaf accumulation	0.5μM Co	Zn (+21%) > Cu (+38%) > Mn (+48%)
	5μM Co	Zn (-51%) > Mn (-21%) > Cu (-4%)
	50μM Co	Mn (-71%) > Zn (-48%) > Cu (-13)
root uptake	0.5μM Co	Cu (+20%) > Zn (+27%) > Mn (+68%)
	5μM Co	Mn (-26%) > Cu (-22%) > Zn (-15%)
	50μM Co	Mn (-61%) > Zn (-27%) > Cu (+39%)

5.3.4.4 Macro-nutrients

The effects of Co on macronutrient content were qualitatively similar in –Fe and + Fe plants. The data set for –Fe plants were more complete and so to avoid repetition the following analysis is given for these results.

Ca. Co at 0.5 μM reduced Ca content by 31% both in roots and stems, but increased the transport to leaves by 63%, compared to the control (Fig 5.13a). At 5 and 50 μM, Co reduced slightly the Ca content of the roots and leaves, and strongly in the stems. There was a significant negative correlation between Co content and Ca content in the stems ($r = -0.70$), but not in the leaves or roots.

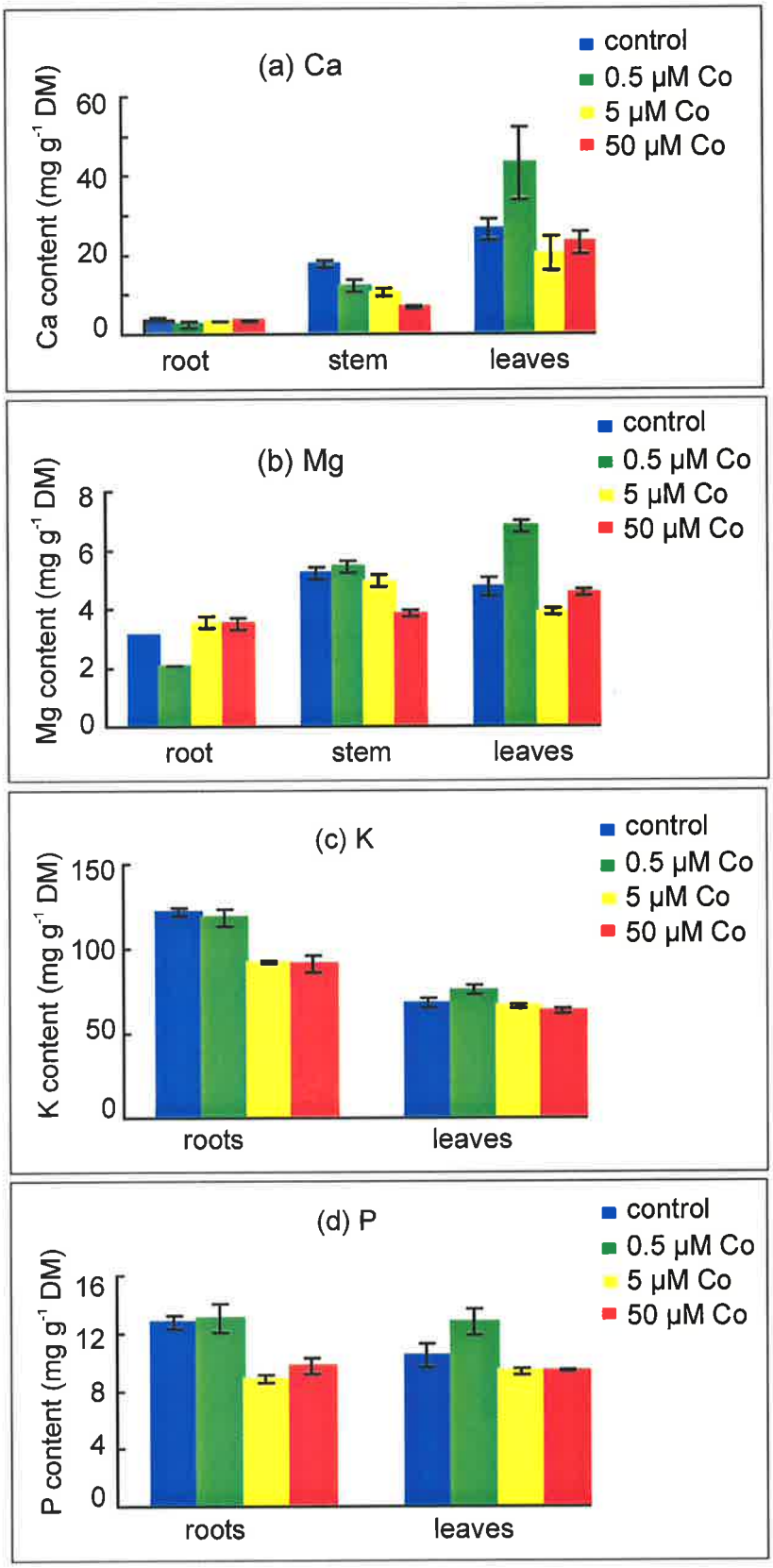


Figure 5.13 Effect of Co on the uptake and transport of macro nutrients in 2 week old mung bean. (a) Ca, (b) Mg, (c) K and (d) P.

Mg. As with Ca, 0.5 μM Co reduced the Mg content of the roots (-33%), but increased it in the leaves (+43%) (Fig 5.13b). In the roots, Co content and Mg content were positively correlated ($r = 0.68$).

K. The K content of roots was reduced at higher Co concentrations (5 and 50 μM), but there was little effect of Co on leaf K content (Fig 5.13c). There was a significant negative correlation between Co content and K content in roots ($r = -0.89$), in stems ($r = -0.65$) and in the leaves ($r = -0.56$).

P. The P content of roots was reduced at the higher Co concentrations (5 and 50 μM) while in the leaves, P content increased by 22% at 0.5 μM Co but was decreased slightly by 5 and 50 μM Co (Fig 5.13d). P content and Co content in the roots were negatively correlated ($r = -0.79$).

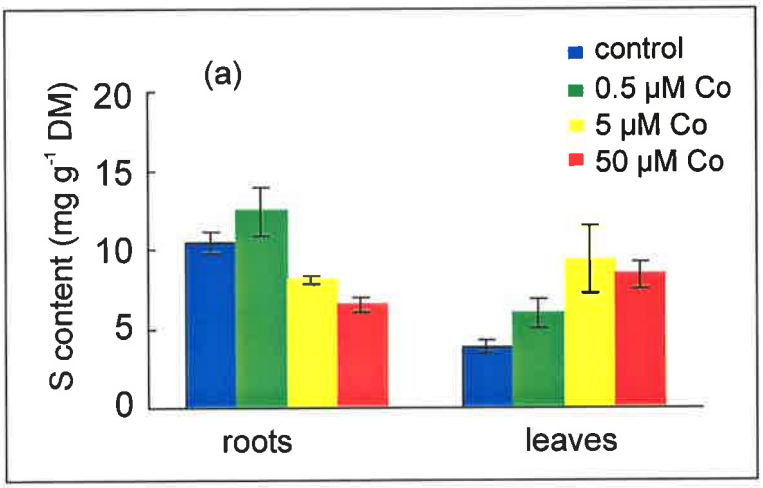
S. Co had a much greater effect on the uptake and distribution of S than on that of the other macronutrients. The leaf S content was greatly increased by Co treatment while the root content was higher at 0.5 μM Co but lower at 5 and 50 μM (Fig 5.14a). The overall tendency was to increase the ratio of S in the leaves/roots. The ratio of S in leaves/roots was 0.37, 0.48, 1.15 and 1.29 at 0, 0.5, 5 and 50 μM Co, respectively. Co content and S content were significantly correlated in the roots ($r = -0.80$), but not in the leaves ($r = 0.37$).

Overall uptake of S increased with increasing Co in the solution (Fig 5.14b). This contrasted with the other macronutrients where root uptake was increased at 0.5 μM Co but decreased at 5 μM or 50 μM Co (data not shown).

5.4 Discussion

5.4.1 Mechanisms of Co effects in plants

There is no evidence for the involvement of Co in the bioactive molecules in plants and the mechanisms of any beneficial or toxic effects of Co are not known. Co has been reported to



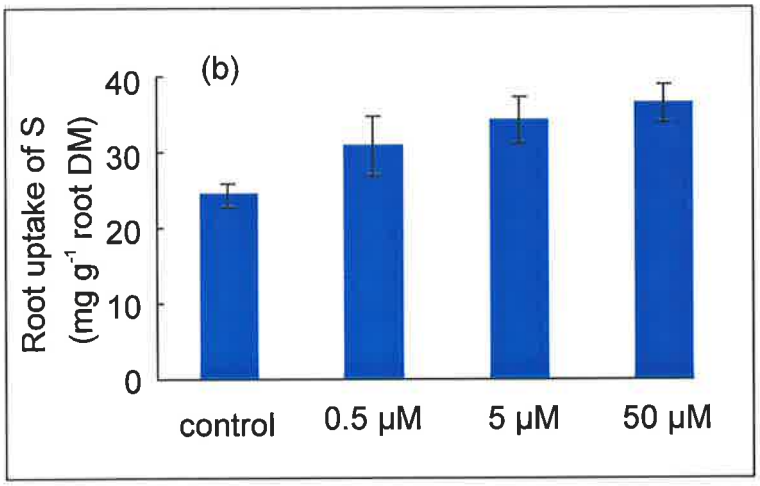


Figure 5.14. Effect on Co on (a) S content of roots and leaves of mung bean, and (b) total plant uptake of S based on root DM. n = 3 seedlings.

interact with other elements for uptake and action in plants (Wallace *et al.*, 1971; Agarwala *et al*, 1977; Terry, 1981;Veltrup, 1981; Wallace and Abou-Zamzam, 1989; Dirilgen and Inel, 1994; Palit *et al.*, 1994), but information on the specific modes of action of Co is scarce. In this chapter, attention has been paid to the physiological effects of Co in relation to nutrient status in mung bean plants. The results have shown that Co influences the nutrient composition of plants and therefore may exert toxic effects by altering the balance of essential nutrients within the plant through an interaction with nutrient uptake. An alternative mode of toxicity might be via the direct intervention of Co in internal plant processes (e.g. enzymatic reactions, calmodulin), but it was not possible within the scope of this work to investigate this possibility in any detail. However, there does appear to be a broad correlation between Co uptake and toxicity, which would support the possibility of direct toxicity. The amelioration of Co toxicity by Ca is consistent with the reduction in Co influx caused by increasing Ca concentration (Table 3.1, Fig 5.9). Thus, an internal mode of action of Co is supported. The effects of Co on nutrient uptake suggest an external mode of action. These issues will be discussed in detail.

5.4.1.1 Effect of Co on Fe uptake, transport and function.

The interaction of Co with uptake and transport of Fe in plants has been emphasised in several previous studies (Agarwala *et al.*, 1977; Terry, 1981; Blaylock *et al.*, 1986) and it was therefore important to examine this area closely. The results of the experiments in this chapter show that all of the concentrations of Co reduced the Fe content of leaves, but not of roots (Fig 5.3). The inhibition of growth therefore cannot be attributed to Fe deficiency in the roots. Additionally, total plant Fe content was not significantly affected by Co (data not shown but note the high Fe content of roots compared to leaves in Fig 5.3) which argues against inhibition of Fe uptake into the roots by Co. The main effect of Co on Fe appeared to be on the transport of Fe from root to shoot. This raises the question of whether the inhibition of growth is simply a consequence of the inhibition of supply of Fe to the leaves. Growth was inhibited to a similar extent by Fe deficiency and by 5 μM Co (Fig. 5.1) but the

Fe content of leaves in the Co treatment was significantly higher (Fig. 5.3a). This indicates that Co either intensifies the effects of low leaf Fe, or that it has independent effects on growth. The differing root morphologies under Fe deficiency and Co treatments (Fig. 5.2) would suggest that the two treatments are not equivalent. This is in agreement with the observation of Bisht and Mehrotra (1989) on maize regarding leaf deformation, wilted appearance, and the activities of peroxidase and aldolase and the pattern of recovery. From this point of view, it is prudent not to overestimate the assumption that the effect of Co is completely due to its interaction with Fe. An interesting comparison is that of the % inhibition of growth caused by Co in plants grown with and without Fe (Table 5.2). While there were differences in root/shoot ratios between the +Fe and –Fe treatments, the effects of Co on both whole plant FW and leaf FW was almost exactly the same. This further supports the notion that Co exerts some effects on growth independently of its effects on plant Fe status.

Table 5.2 Comparison of Co effects in mung bean in the presence and absence of Fe.

		FW of control (mg)	FW of 5μM Co (mg)	Co/control (%)
Expt 1 Fe- sufficient				
	whole plant	1137 ± 80	760 ± 10	67
	leaves	393 ± 61	288 ± 11	73
Expt 2 Fe-deficient				
	whole plant	1190 ± 108	849 ± 77	71
	leaves	619 ± 45	446 ± 42	72

5.4.2 Mechanisms of Co interaction with other elements in uptake and transport

The interaction of Co with other nutrients was complicated. In general larger effects were seen on micronutrient content than on macronutrient content.

5.4.2.1 Micronutrient metals (Cu, Mn and Zn)

It seems unlikely, based on the small effects of Co on the content of micronutrient metals (Fig 5.11), that the growth inhibition by 5 μM Co is due to deficiencies of these metals. The results were qualitatively similar in plants grown without Fe with one major exception; the content of Mn, Cu and Zn in leaves was increased by 0.5 μM Co (Fig 5.12). This was particularly striking in the case of Mn. The content of all of these metals based on root DM (an indication of root uptake) was also higher. Thus it appears that low concentrations of Co cause both an increase in root absorption of Mn, Zn and Cu and an increase in their transport to the shoot. The fact that this did not occur to a significant extent in plants supplied with Fe suggests that Fe nullifies this role. It is difficult however to even speculate on the molecular basis for this phenomenon.

5.4.2.2 Macronutrients

As with the micronutrients, it seems unlikely that growth inhibition by Co is due to induced deficiencies or toxicities of macronutrients. Even at the highly toxic concentration of 50 μM Co, the perturbations of nutrient content were generally small (Fig 5.13). The major exception was S whose content in leaves was increased markedly, but toxicity of S seems remote.

5.4.3 The role of phytochelatins

The interaction of Co with S and with S-containing compounds has arisen several times in this and the preceding chapters. The possibility that cysteine-rich transport proteins may mediate Co uptake was raised in chapters 3 and 4, and the strong effect of Co on S transport to leaves was the dominant feature of the changes in nutrient balance. S is a key component of glutathione, which acts as a storage pool or buffer for intracellular S (Schutz et al. 1991).

Glutathione in turn is a precursor of phytochelatins, which are important in the detoxification of high levels of metals (see Rauser 1990 for review). Certain metals induce phytochelatin synthesis but not by others (Tuckendorf and Rauser 1990). It seems reasonable to propose that the increase in leaf S content is due to transport of phytochelatin-bound Co from the roots. The question also arises whether the incorporation of large amounts of S into phytochelatins might lead to a deficit of S for other essential functions in the leaf. The main consequence of S deficiency is inhibition of protein synthesis (Freney et al. 1978) which appears visually as chlorosis of leaves. This needs to be investigated further.

CHAPTER 6 Comparative Effects Of Co And Other Trace Metals On Growth And Nutrient Status Of Mung Beans

6.1 INTRODUCTION

Trace metals (essential or non-essential) exert toxic effects on plants in excess, and the mechanism and extent of the toxicity may vary with the kind of metals and with the kind of plants (Hewitt and Nicholas, 1963, Woolhouse, 1983). The toxicity could be attributed to the inactivation of enzymes by combination of the trace metals with -SH group in the proteins, in which case the affinity of the trace metals to -SH group should govern the relative toxicity of the trace metals at equivalent internal concentrations. This assumption is supported by evidence but not in all the cases (Hewitt and Nicholas, 1963, Madsen, 1963). While it is now the most reasonable explanation for the toxicity of trace metals to plants, more evidence is needed for the involvement of -SH groups.

The toxicity of trace metals is also related to the inhibition of nutrient uptake and/or function, for example Ca (Veltrup, 1981), and Fe (Wallace et al., 1992, Ma and Nomoto, 1993), although it is not clear whether they are the result or the cause of the toxicity of the trace metals. Much attention has been paid to the research on trace metal induced Fe deficiency, since it is related to chlorophyll synthesis and therefore photosynthesis in plants (Hewitt and Nicholas, 1963). While the trace metal induced Fe deficiency and its visual symptoms (leaf chlorosis) are well known, the effect of plant Fe status on the uptake of other nutrients (macro or micro) appears not to have been investigated.

In an attempt to define the nature of the toxicities caused by excess trace metals, the effects of Co on growth and mineral nutrient status were compared with those of a range of other trace metals

6.2 MATERIALS AND METHODS

Mung bean plants were cultured in ¼ modified Hoagland's solution for 1 week after which other trace metals were added. Solutions with 25μM $FeCl_3$ (+Fe) or without $FeCl_3$ (-Fe) were included as controls. Extra Cu was added to the Cu treatment. At the end of the treatment period, 12 seedlings from each treatment were harvested randomly to measure the growth of individual plants, including side root number, FW and/or length of leaves, hypocotyl, epicotyl and roots. The FW and DM of leaves, stems and roots were obtained from the average of three replicates consisting of 12 plants each, and the dried samples were analysed by ICP-AES.

6.3 RESULTS

6.3.1 Physiological effects of trace metals in mung bean

6.3.1.1 Effects of trace metals on the growth of mung bean

Cd, Co, Cu and Hg inhibited the growth of mung bean at 5 μM but Ni had little effect compared to the control. The FW and DM of roots, stem and leaves were all inhibited by the four metals except Ni (data not shown) in a similar pattern to the DM of whole seedling (Fig 6.1). The reduction of DM of whole seedlings by the metals is listed in the following series with the inhibition decreasing from the left to the right:

Cd (-57%) > Cu (-43%) > Hg (-35%) > Co (-22%) >Ni (-1%),

Where the percentage (in brackets) represents the decrease by the metal compared to the control.

The elongation of the main root was inhibited by Co, Ni, Hg, Cu and Cd 11%, 12%, 33%, 36% and 41% respectively compared to the control at 5 μM (Fig 6.2a). The elongation and/or initiation of lateral roots was depressed by Ni, Co, Hg, Cd and Cu 3%, 18%, 34%, 72% and 75% respectively compared to the control (Fig 6.2b). The appearance of roots in the presence of the trace metals is shown in Table 6.1. Except for Ni, the metals induced

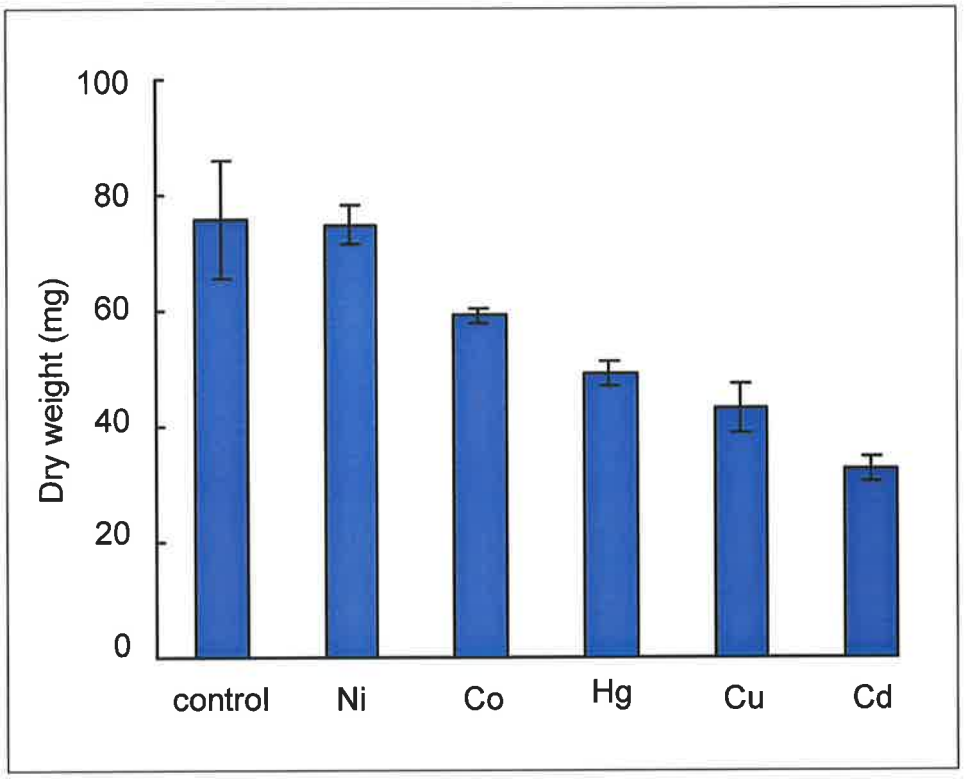


Figure 6.1 Effect of trace metals (5 μM) on the growth of mung bean

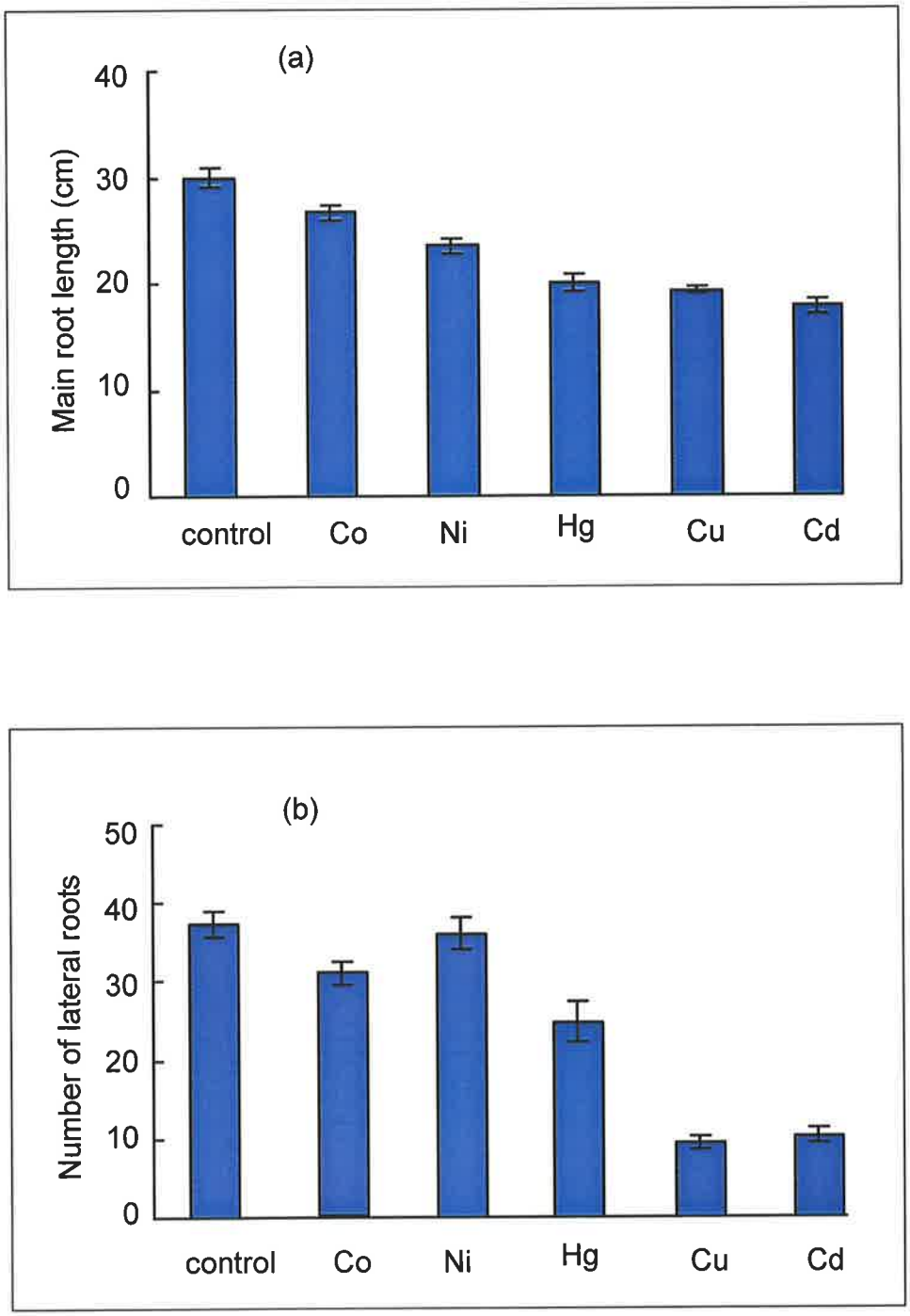


Figure 6.2 Effects of trace metals on (a) the main root length and (b) the number of side roots .

brownness in the roots at 5 μM, similar to Fe-deficiency. Cd induced brownness even at 0.5 μM.

6.3.1.2 Effects of the trace metals on the appearance of seedlings

The appearance of seedlings in the presence of trace metals is recorded in Table 6.1.

Table 6.1 Effects of trace metals on the appearance of seedlings of mung bean

	primary leaves		trifoliate leaves		roots	
treatment	colour	size	colour	size	colour	size
Fe-deficient	slightly yellow	bigger	yellow/dry	small/absent	slightly brown	short 1st and 2nd side roots with tapered ends
control	green	normal	green	normal	white	2nd side roots appeared
Co 0.5 μM 5 μM	green slightly yellow	normal bigger	green yellow/dry	normal smaller/absent	white slightly brown	normal short 1st and 2nd side roots with tapered ends
Ni 0.5μM 5 μM	green green	normal normal	green yellow	normal normal	normal white	normal normal
Hg 0.5μM 5μM	green green	normal smaller	green green	normal smaller	white brown	normal short main and 1st side roots, no 2nd side roots
Cu 0.5μM 5 μM	green green	normal smaller	green yellow	normal smaller or absent	white brown	normal fewer side roots
Cd 0.5μM 5 μM	green green, dehydrated	smaller smaller	yellow absent	smaller or absent	brown brown, soft	short 1st side roots, 2nd side roots absent 2nd side roots absent

At 0.5 μM, Co, Cu and Ni had little effect on the appearance of seedlings. At 5 μM, Co induced chlorosis not only in primary leaves, but also in trifoliate leaves, similar to Fe deficiency. In contrast, Cu and Ni induced chlorosis at 5 μM only in trifoliate leaves. From this point of view, it could be argued that Co is the most powerful metal of those tested in inducing Fe-deficiency symptoms, although it may not be as toxic as Hg, Cu and Cd to growth. Hg did not induce chlorosis at either 0.5 or 5 μM in primary or trifoliate leaves. Cd induced chlorosis in trifoliate leaves at 0.5 μM but not in primary leaves.

6.3.2 Effect of trace metals on the uptake of micronutrients

6.3.2.1 Micronutrients

Fe: Ni, Co, Cu and Hg had little effect or only slightly depressed the content of Fe in the roots at 0.5 μM. At 5 μM, all the trace metals increased the content of Fe in the roots, except Ni, which reduced the Fe content by 24% compared to the control. Cd strongly increased the content of Fe even at 0.5 μM (Fig 6.3a). The increase in the roots may be due to the depression of Fe transport into leaves. Figure 6.3b shows that all the trace metals reduced the content of Fe in leaves. At 0.5 μM the effect of trace metals on Fe content in the leaves decreased in the following series from the left to the right:

Cd (-89%) >= Hg (-85%) > Cu (-54%) >= Co (-59%) > Ni (-29%)

where the percentage is the comparison with the control. At 5 μM, all the trace metals reduced the content of Fe in the leaves to a similar extent, -88 to -79% compared to the control, close to Fe-deficiency (-92%). However, the extent of induction of Fe-deficiency symptoms was different, as shown above (6.3.1.2), suggesting that the induction of Fe-deficiency not only involves the inhibition of Fe transport but also involves a more specific inhibition of Fe function. This agrees with the findings for the inhibition by Co of the synthesis of chlorophyll (Chapter 5).

Mn: Ni increased the Mn content of roots by 24 and 56% at 0.5 and 5 μM respectively compared to the control while Hg increased the content 34% at 0.5 μM but decreased by

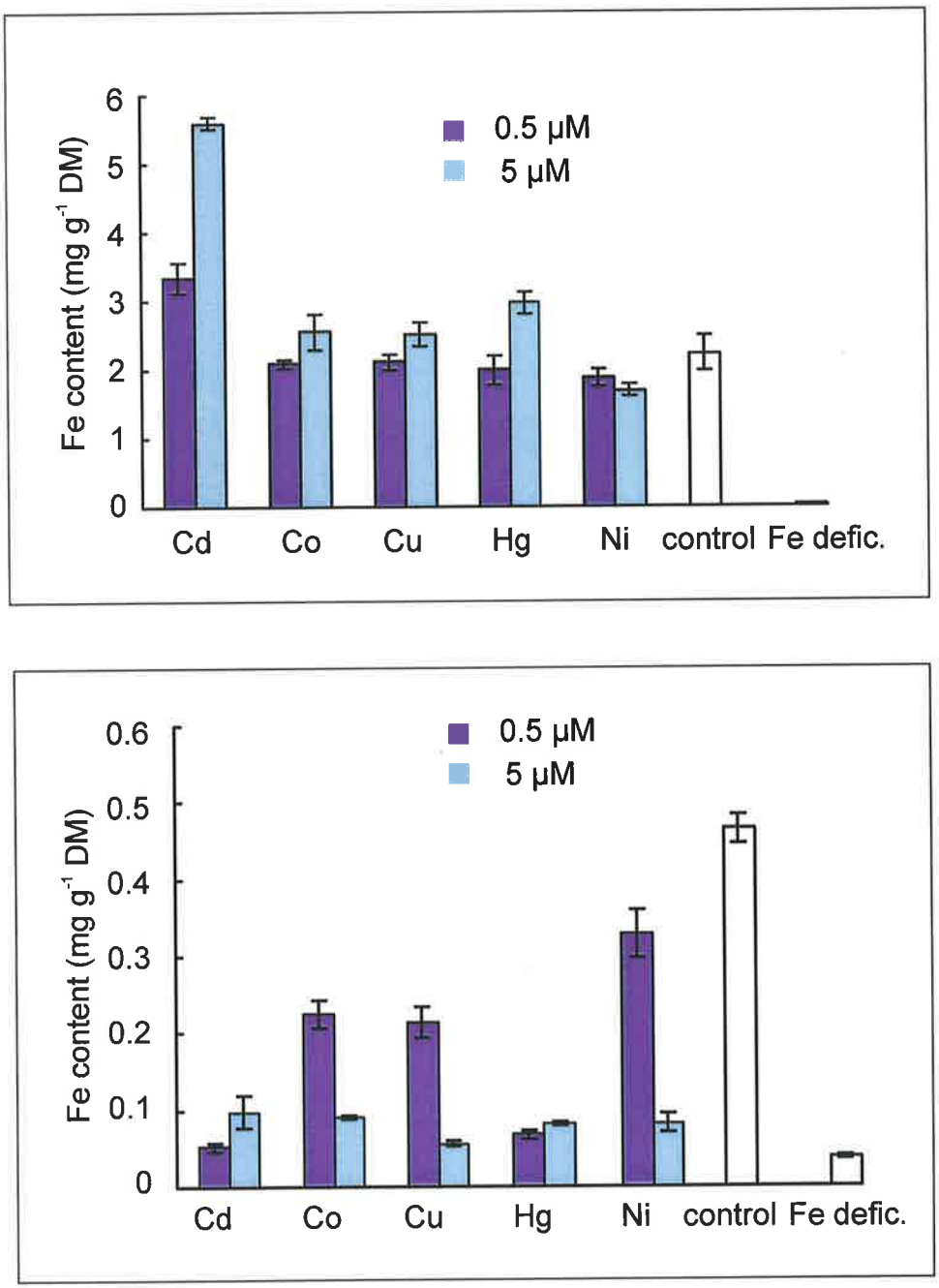


Figure 6.3 Effects of trace metals on Fe content of (a) roots and (b) leaves of mung beans..

27% at 5 μM. Cd decreased the content at 0.5 and 5 μM by 77 and 82% respectively. Co and Cu had little effect at 0.5 μM but decreased Mn content at 5 μM by 57 and 74% respectively (Fig 6.4a). The effect of the trace metals (5 μM) on Mn content of roots can be expressed in the following series with the inhibition decreasing from the left to the right:

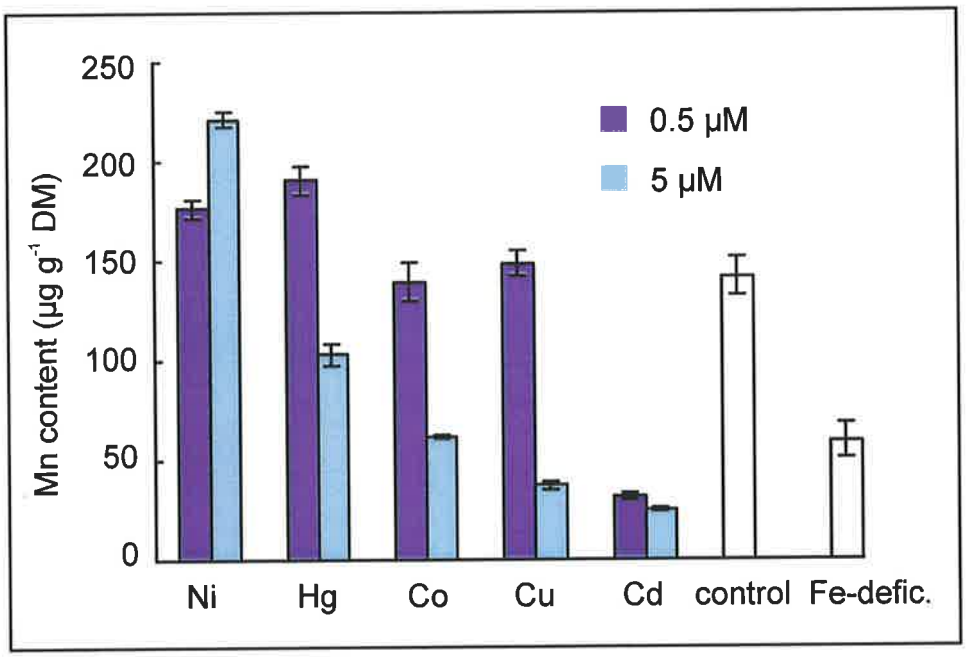
Cd > Cu > Fe-defi. >=Co > Hg > control > Ni

The effect on Mn content in leaves can be expressed as:

Cu > Cd > Ni > control > Co > Hg > Fe-deficiency.

Cd and Cu inhibited Mn accumulation in the roots and transport to the leaves. The latter may simply be a consequence of the lower root content due to the inhibition of Mn uptake into the roots. It is interesting that Ni induced the accumulation of Mn in the roots but inhibited Mn transport to the leaves. In contrast, Co, Hg and Fe-deficiency inhibited the accumulation of Mn in the roots but increased the translocation to the leaves. There appeared to be an inverse relationship between effects of trace metals on root content and leaf content of Mn; lower root content was reflected in a higher leaf content, most obvious in the case of Fe-deficient plants where the leaf Mn content was greatly increased. It may be significant that the primary roles for Mn and Fe in leaves are in photosynthesis and the elevation of Mn may therefore be an attempt to compensate for the lack of Fe.

Mo: Compared to the effects on Mn and Fe content, the effects of the trace metals on Mo content were relatively small. Ni and Co at 5 μM, and Cd and Hg at 0.5 μM increased the Mo content of roots 35, 18, 19 and 38% respectively, but 5 μM Cu decreased the content of Mo 31% compared to the control (Fig 6.5a). The translocation of Mo into the leaves was inhibited by Cd, Cu and Hg both at 0.5 μM and at 5 μM. Ni increased the Mo content 17% at 0.5 μM and decreased 12% at 5 μM. Co decreased the content 19% at 0.5 μM but had no effect at 5 μM. Fe-deficiency had little effect on Mo accumulation in the roots, but decreased Mo translocation to the leaves by 41% (Fig 6.5b).



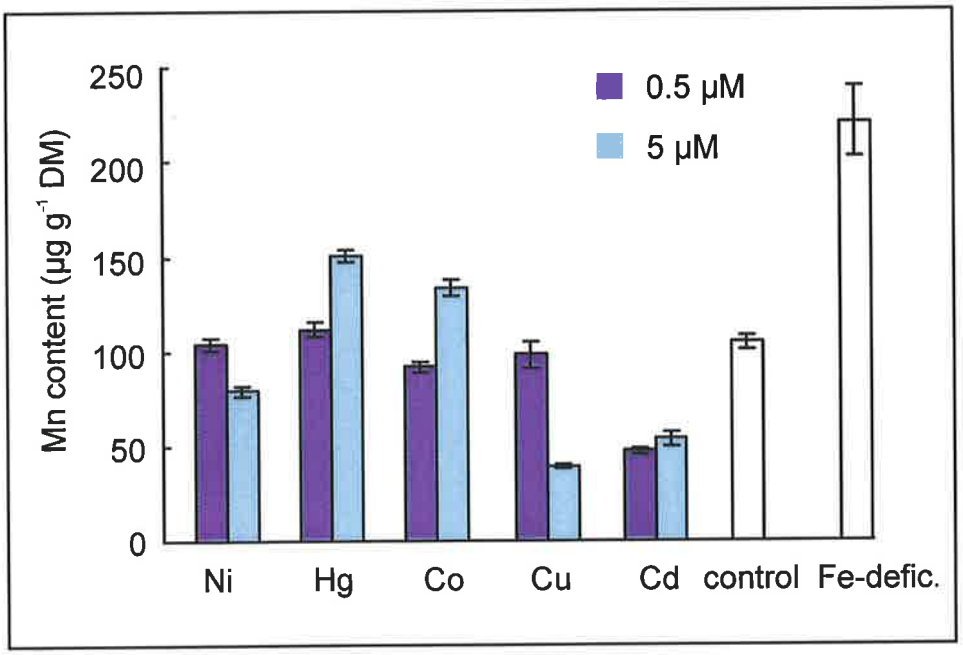


Figure 6.4 Effects of trace metals on the Mn content of (a) roots and (b) leaves of mung beans.

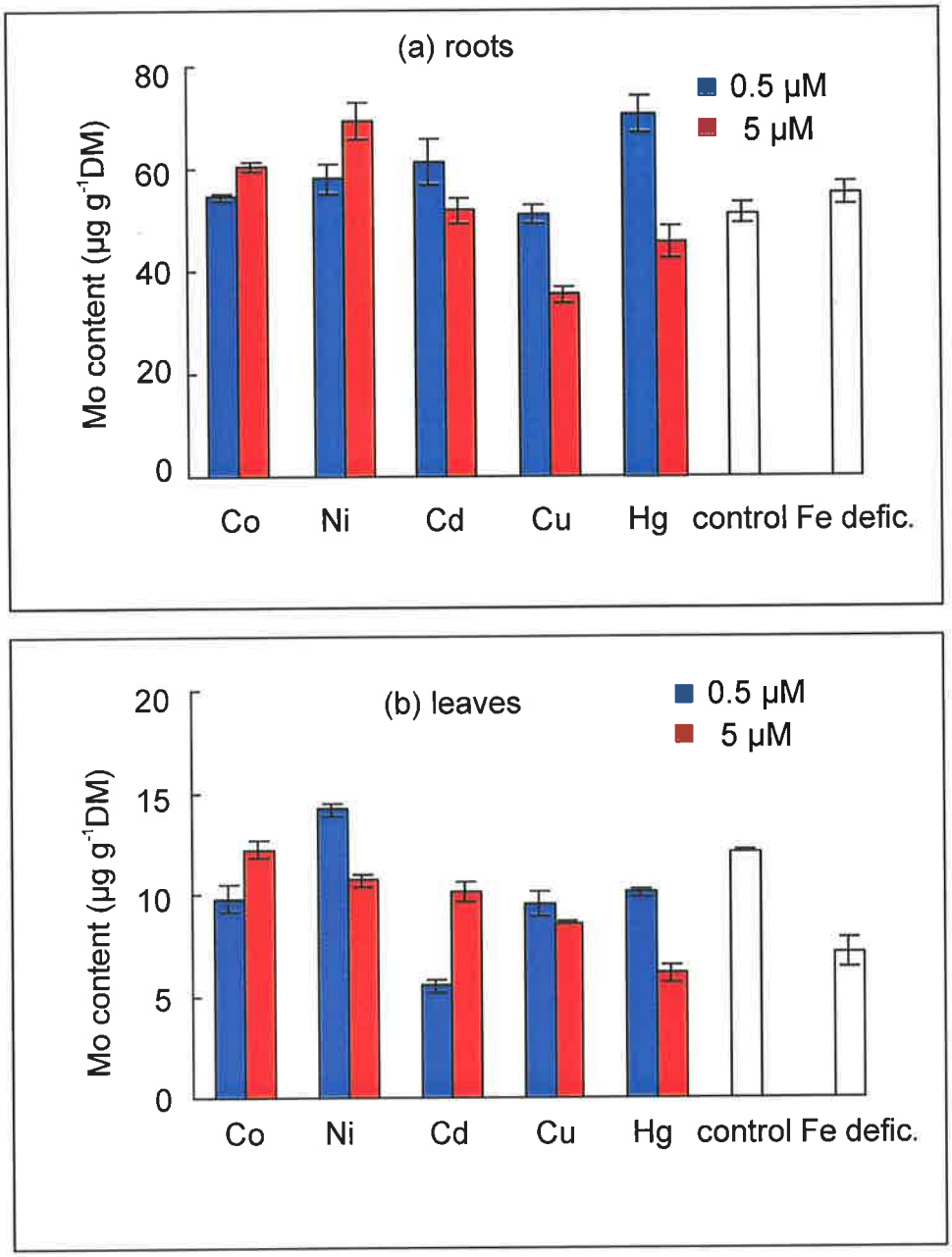


Figure 6.5. Effects of trace metals and Fe deficiency on the Mo content of (a) roots and (b) leaves of mung beans.

Cu: Cu accumulation in the roots was increased by Cd and by Hg at the higher concentration but the effects of the other treatments on root Co content were relatively minor (Fig 6.6a).

None of the metals had large effects on Cu translocation to leaves, except that Fe deficiency resulted in a marked increase in leaf Cu (Fig. 6b).

Zn: Hg, Ni, Cd, Co and Fe-deficiency tended to increase the root Zn content, while Cu reduced the content (Fig. 6.7a). Leaf Zn content was also increased by Fe deficiency but was reduced by Cd, Cu and Ni at certain concentrations (Fig 6.7b).

6.3.2.2 Macronutrients

Ca: Co and Ni had little effect on the Ca content of either roots or leaves (Fig 6.8). Cd caused an increase in root Ca and a decrease in Ca in the leaves, which is consistent with inhibition of transport to the leaves rather than any effect on Ca uptake into the roots. A similar but less obvious pattern was seen with Hg and Cu (Fig 6.8).

Mg: Mg accumulation in the roots and transport into the leaves were less affected by the trace metals than those of Ca, and only the results for the trace metals at 5 μM are presented in Fig 6.9. All the trace metals except Ni, depressed Mg accumulation in the roots (Fig 6.9a) while the leaf content was broadly similar for all treatments except Cu, which reduced the Mg content by 35%.

K: K accumulation in the roots was reduced by approximately 50% by 5 μM Cu and Cd, slightly reduced by Hg, Co and Fe deficiency and unaffected by Ni (Fig. 6.10a). Leaf K content was not affected by any of the metal treatments except Cu (lower) and by Fe deficiency (higher) (Fig. 6.10b).

P: Cd and Ni did not significantly affect by Fe deficiency or The P content of roots. Co, Hg and Cu reduced the P content of roots (Fig. 6.11a). None of the metal treatments had a significant effect on leaf P content (Fig. 11b).

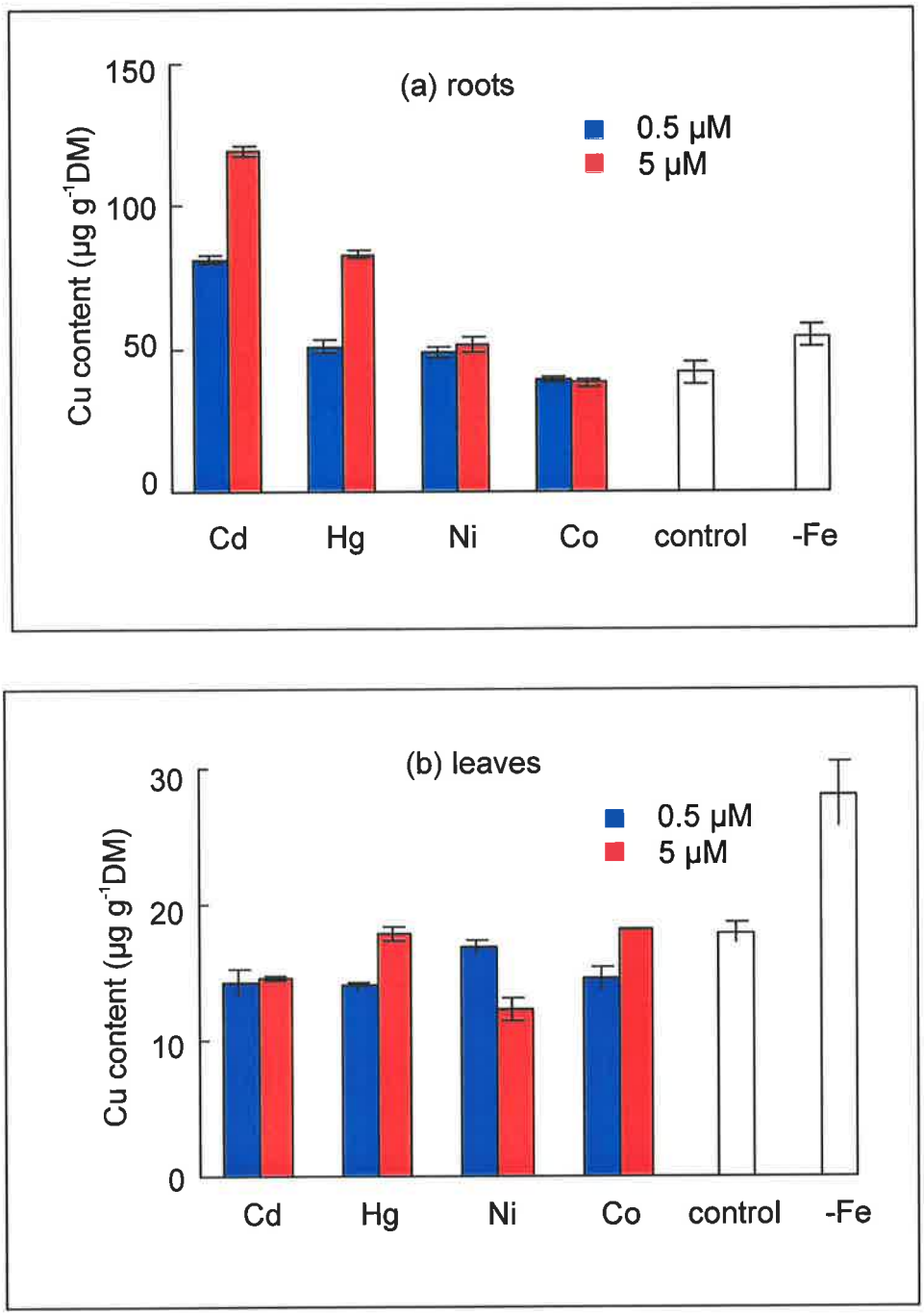
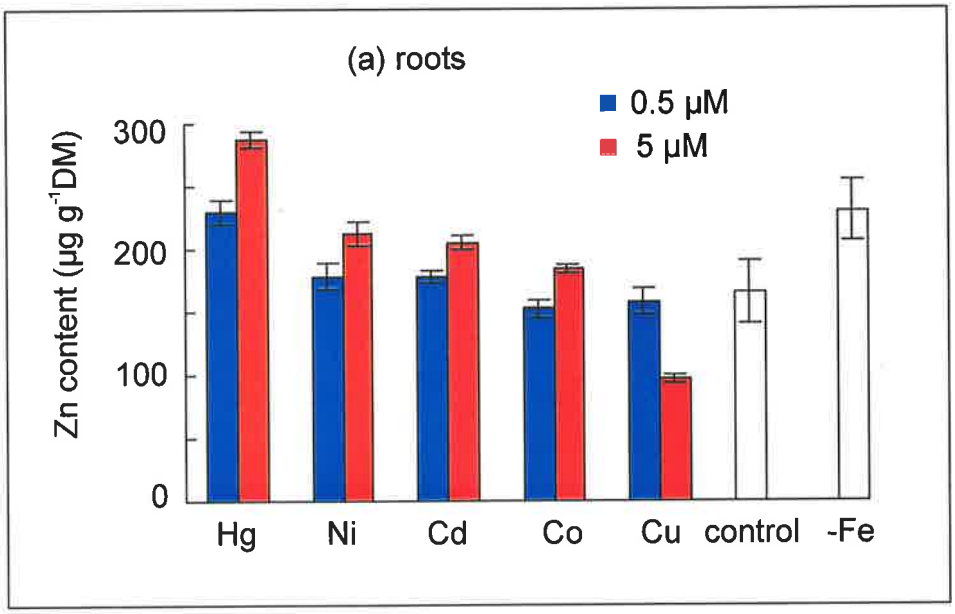


Figure 6.6. Effects of trace metals on Cu content of (a) roots and (b) leaves of mung beans.



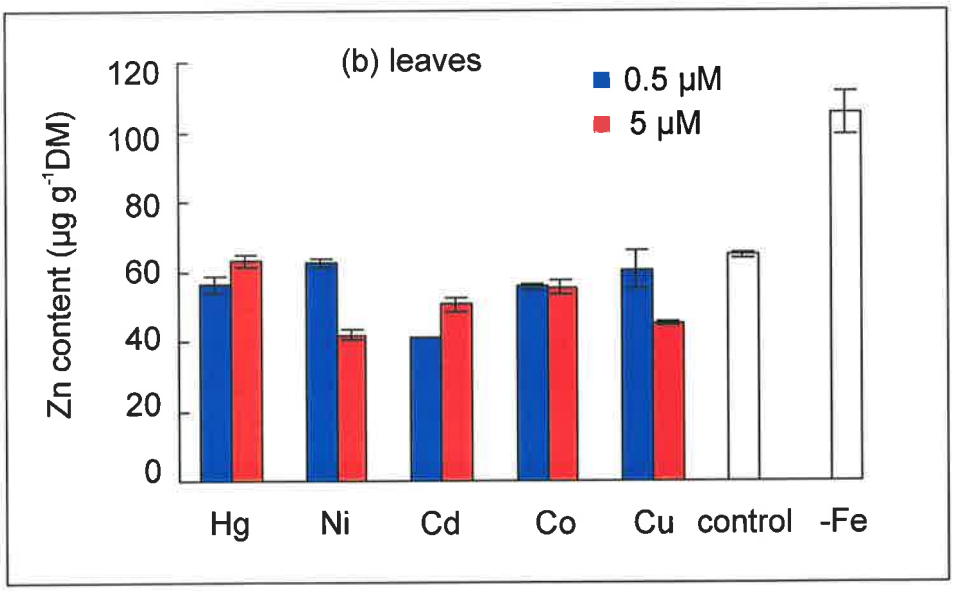


Figure 6.7 Effect of trace metals and Fe deficiency on the Zn content of (a) roots and (b) leaves of mung beans.

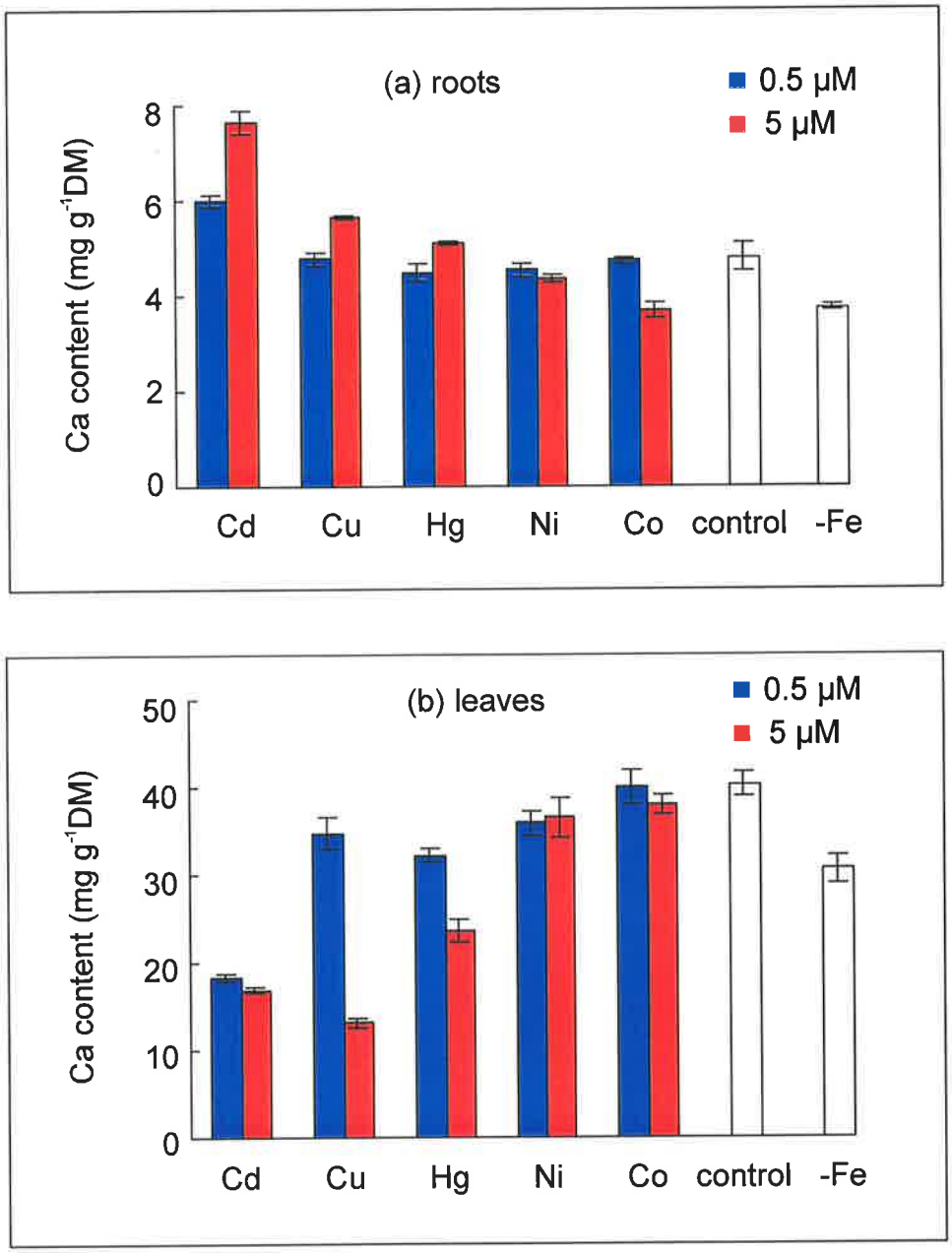


Figure 6.8 Effects of trace metals and Fe deficiency on Ca content of (a) roots and (b) leaves of mung beans.

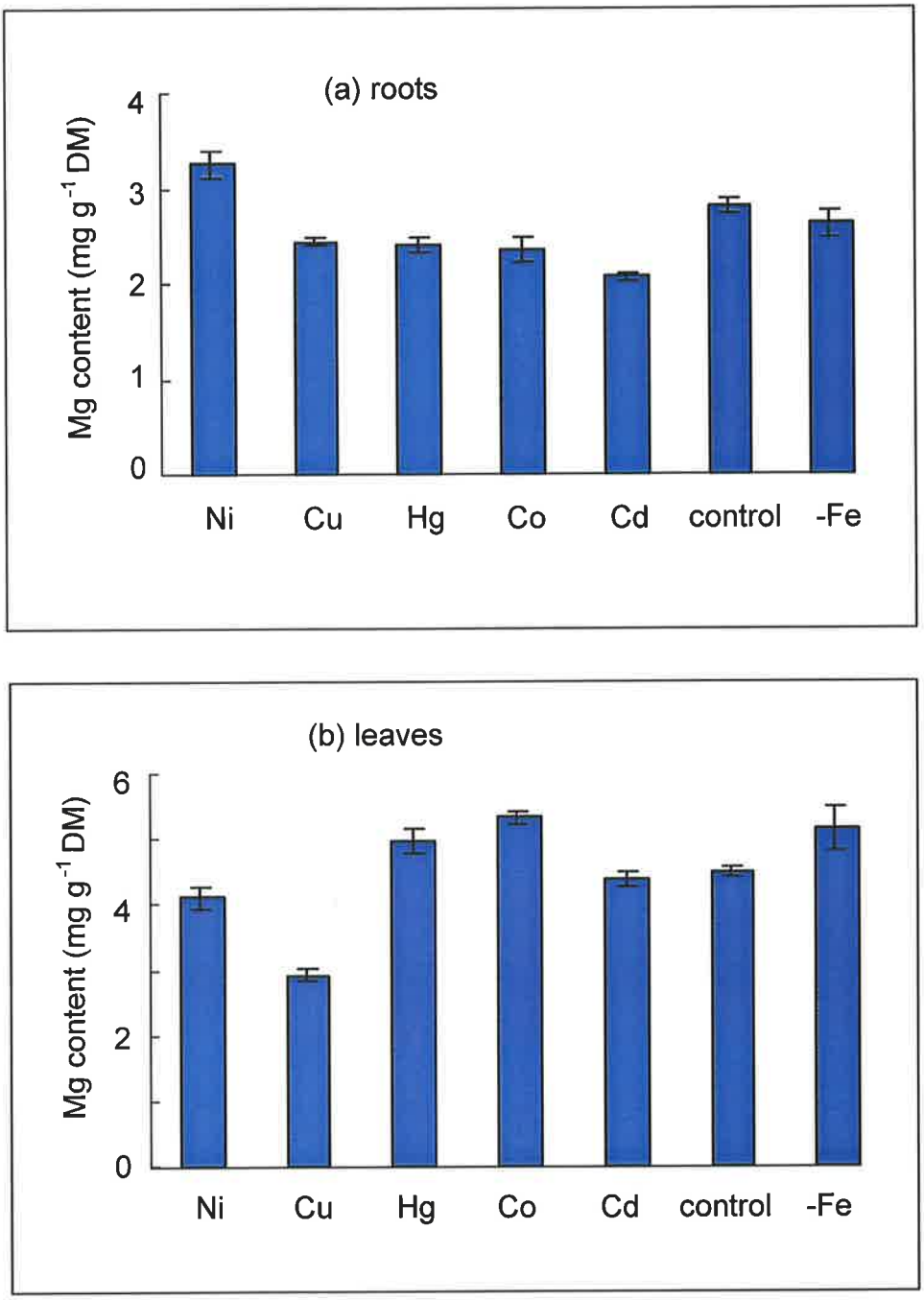
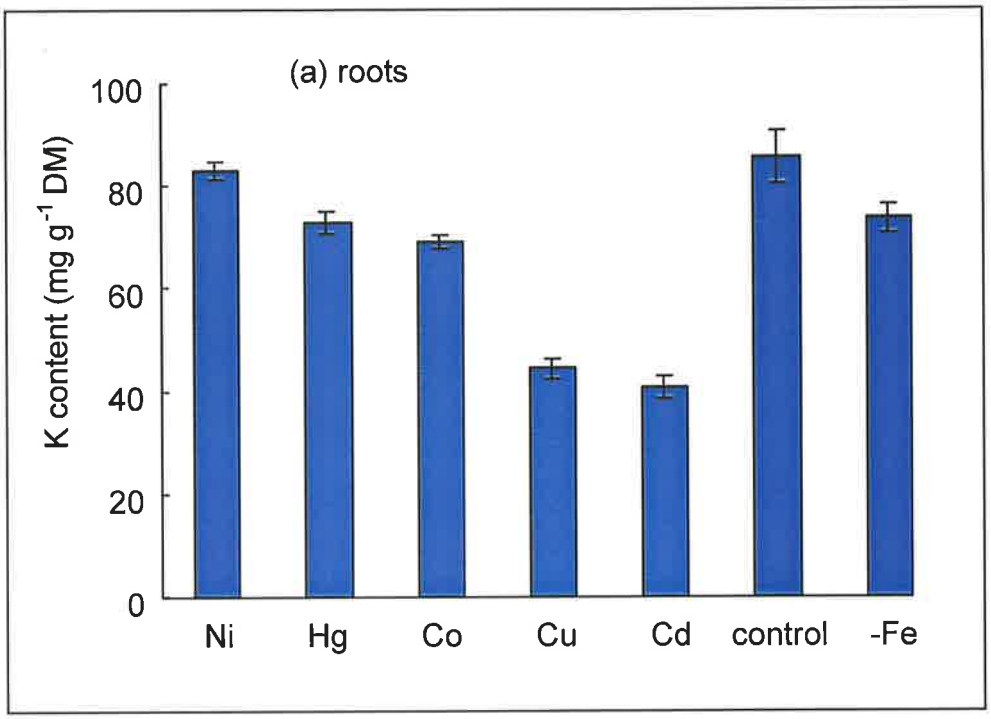


Figure 6.9 Effect of 5 μM trace metals and Fe deficiency on the Mg content of (a) roots and (b) leaves of mung beans.



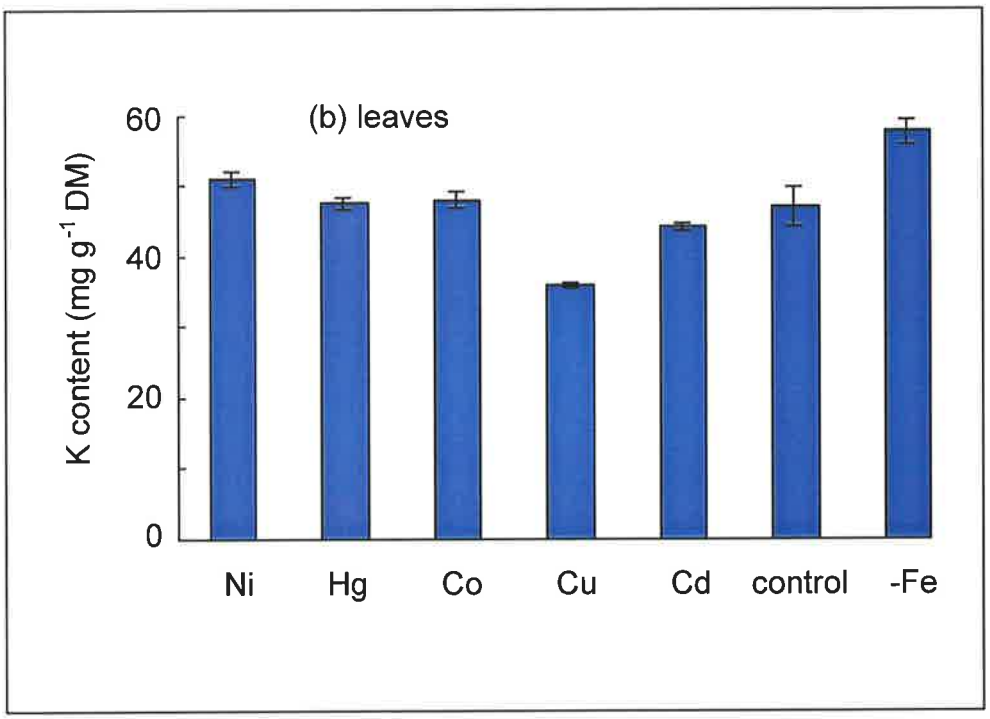


Figure 6.10 Effects of 5 μM trace metals on K content of (a) roots and (b) leaves of mung beans.

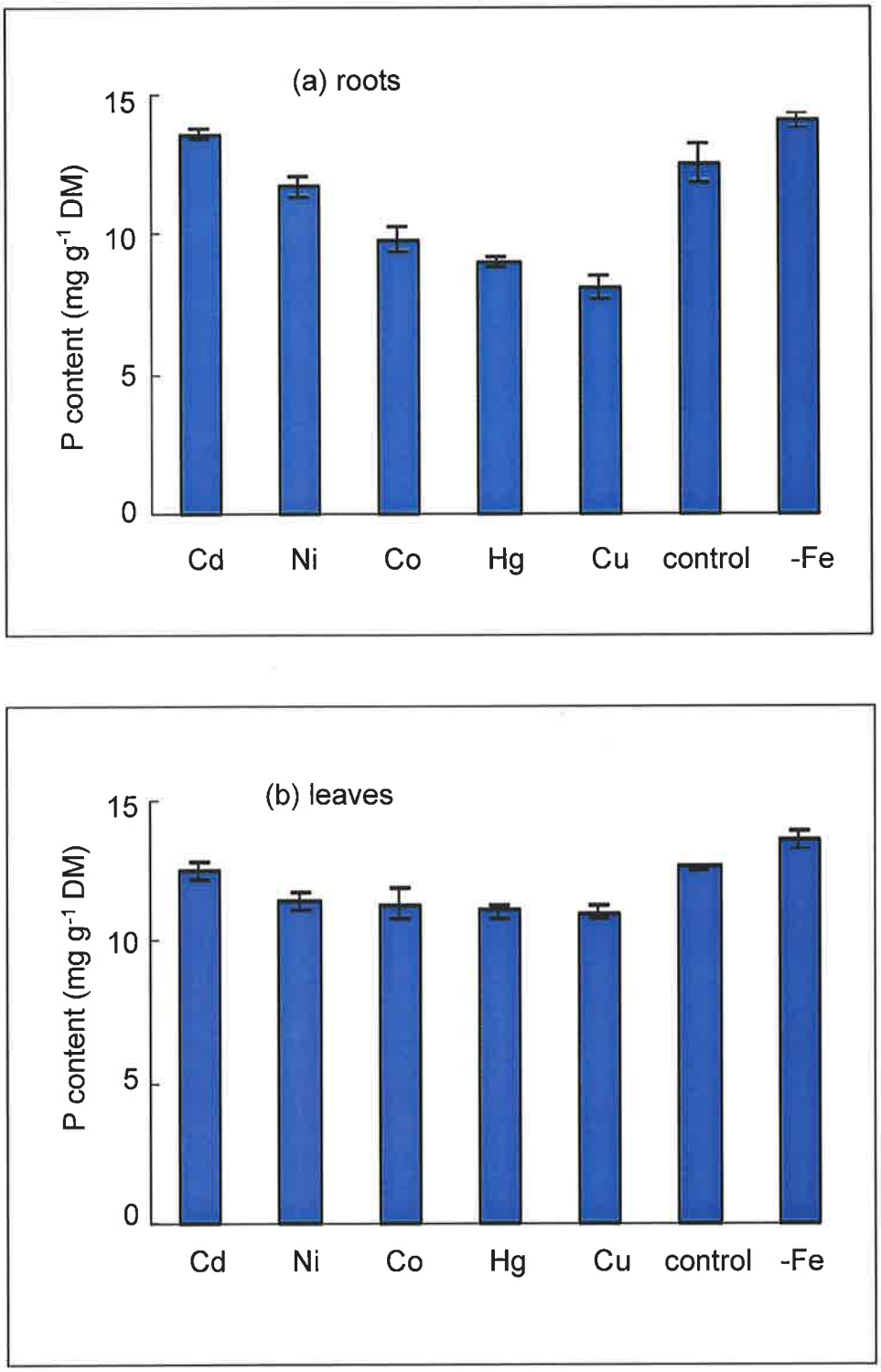


Figure 6.11 Effects of 5 μM trace metals on the P content of (a) roots and (b) leaves of mung beans.

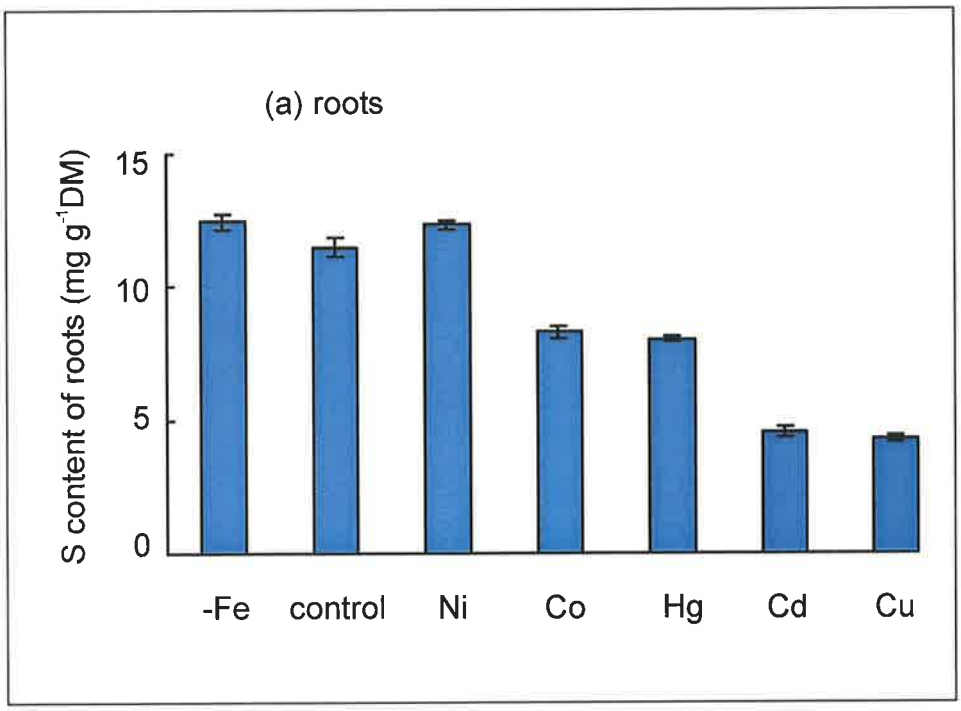
S: S accumulation in the roots was depressed by Co, Hg, Cd and Cu at 5 μM but was not affected by Fe deficiency or by Ni (Fig 6.12a). The effects of the trace metal treatments on leaf S content were small or non-significant except for Co which caused an 86% increase in S in leaves. (Fig 6.12b).

6.4 Discussion

The main purpose of the experiments described in this chapter was to examine the relative effects of other non-essential trace metals at toxic concentrations, on the nutrient balance of mung bean seedlings. The rationale for this is that there may be similarities between these metals that contribute to a common effect on specific nutrient-related processes (e.g. inhibition of Fe transporters). A complicating factor is the different degree of inhibition of growth caused by the different metals and the effects that these may have on nutrient concentrations. Does an increase in nutrient concentration caused by a trace metal treatment arise because uptake of the nutrient was stimulated or because growth was inhibited? It is easier to interpret cases where the nutrient concentration is lower, since this can only occur because of reduced uptake. Equally, caution is needed when interpreting leaf concentrations since these are the end result of a number of processes - uptake, compartmentation within the root, transport into the xylem or phloem and translocation to the leaves.

6.4.1 Relative toxicity of Co.

Mung beans were able to grow relatively well in the presence of 5 μM Co. It is difficult to relate this to soil solution concentrations because of the large differences in availability in different soils. In terms of the effects on growth, Co is less toxic than the other 'heavy metals' Hg, Cu and Cd but more toxic than Ni (Fig. 6.1). The effects of the metals on nutrient balance varied but there were strong similarities with respect to the effects on Fe status of the plants.



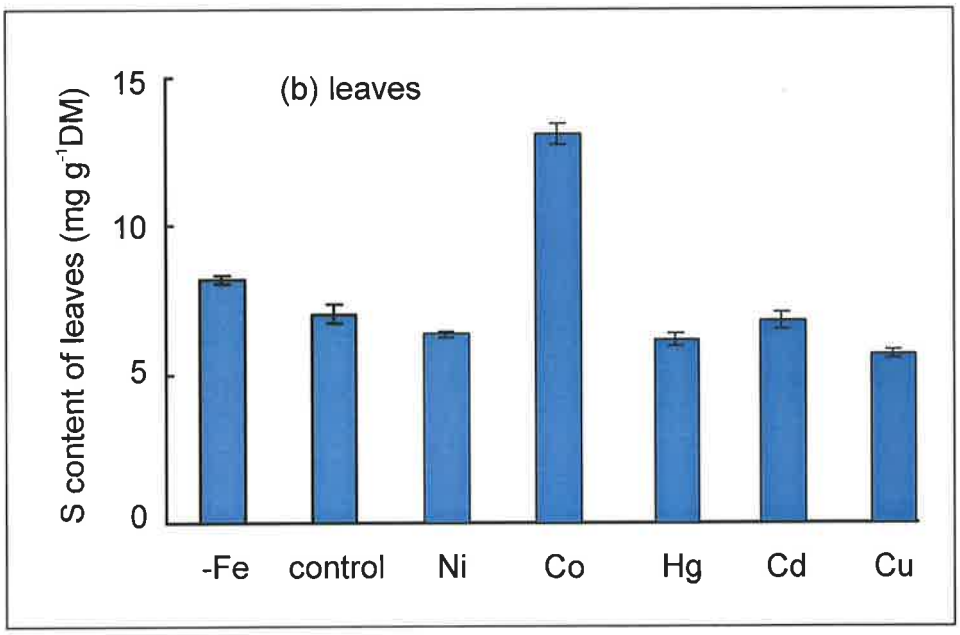


Figure 6.12 Effects of trace metals on S content of (a) roots and (b) leaves of mung beans.

6.4.2. Effect of trace metals on Fe deficiency

It is known that trace metals, Co, Cu, Cd, Cr, Mn, Ni and Zn may induce Fe-deficiency in plants (Hewitt and Nicholas, 1963) although the processes which contribute to the deficiency are not understood. In this study, it was shown that in the presence of Co, Cu, Cd, Ni and Hg, root Fe content remained high and in some cases (e.g. Cd), was higher than the control. This was in contrast to the leaves where Fe content was significantly reduced by all metals, and at 5 μM the content was similar to that in plants which had not been supplied with Fe (Fig. 6.3). These latter plants were deficient in Fe in both roots and leaves (roots 21.6 $\mu g\ g^{-1}$, leaves 37.2 $\mu g\ g^{-1}$), whereas in the metal-treated plants only the leaves were Fe deficient. In the presence of the added metals at 5 μM, the leaf Fe levels were similar to the deficient plants while the root Fe content was similar to or higher than the control, 2,220 $\mu g\ g^{-1}$. Taken together, this argues strongly against the assumption that Fe deficiency is due to inhibition of Fe uptake into the root. The main process that appears to be affected is the translocation of Fe from roots to shoots. Fe is transported from root to shoot in the xylem as a result of transpirational water flow, and has limited phloem mobility (Marschner 1995). Inhibition of transpiration is therefore a possible mechanism for inhibition of Fe transport to the shoots. Although transpiration data were not collected during the growth of the plants, the small effects of the metals on the leaf content of other xylem-mobile elements such as Mn, Cu and Zn (Figs 6.4, 6.6, 6.7) argues against inhibition of transpiration as a major factor in the induction of Fe deficiency in the leaves. There are a number of intermediate steps between uptake of a substance into the root cells and its appearance in the xylem that may be sensitive to inhibition by high concentrations of trace metals. This may involve competition with Fe for efflux from the root cells into the vascular tissue, or disturbance of Fe compartmentation in the root, e.g. by binding to metallothionein-like molecules induced by the toxic metals.

Trace metals are known to inhibit the activity of Fe(III) reductase as reported by Alcantara et al (1994) and this would prevent the reduction of Fe(III) to Fe(II), the form of Fe most

likely involved in membrane transport (Kochian, 1991, Fox et al., 1996), and therefore loading into the xylem. However, in dicots it is likely that most Fe reduction occurs via a membrane reductase which forms part of the uptake complex (Romheld 1987) rather than reduction within the cytoplasm.

The inhibition by Co and other trace metals of chlorophyll synthesis is not well understood. One explanation is that Co, similar to Mn, may be incorporated into protoporphyrin, thus inhibiting the incorporation of Fe (Hewitt and Nicholas, 1963).

6.4.3 Effect of Fe-deficiency on uptake of other nutrients

It is known that Fe-deficiency induces the accumulation of other metals in the upper parts of plants, such as Cu, Mn, Mg, Cd, Zn (Welch et al., 1993, Rodecap et al., 1994, Cohen et al., 1998). This may be due to a greater inhibition of leaf expansion than of uptake and translocation from the root. The micronutrient metals Mn, Cu and Zn showed large increases in leaf content under Fe deficiency whereas the macronutrient metals showed only small increases (Mg, K) or had a lower content (Ca). Anionic nutrients P, S and Mo were largely unaffected by Fe deficiency. There was no real evidence that Fe deficiency inhibited root uptake of these micronutrient metals, just as there was no evidence that high concentrations of trace metals inhibited Fe uptake (Fig 6.3). Thus the major effect of Fe deficiency was to allow a higher accumulation of trace metals in leaves. A simple hypothesis to explain these findings would be that Fe competes with other trace metals for transport from the root cells into the xylem. Under Fe deficiency, loading of other divalent cations would be favoured leading to high concentrations in the leaves. On the other hand, in the presence of adequate Fe but high concentrations of other trace metals, Fe transport to the leaves would be competitively inhibited leading to Fe deficiency in the leaves and normal or slightly elevated Fe concentrations in the roots.

Previous hypotheses to explain increased uptake and translocation of micronutrient metals in Fe deficient conditions have revolved around the induction of higher amounts of Fe(III)

reductase during Fe deficiency (Welch et al., 1993, Romheld and Marschner, 1986, Kochian, 1991). However, recent work by Yi and Guerinot (1996) provided evidence that Fe(III) reductase may not be involved in divalent cation influx. Under Fe-deficiency, a mutant of *Arabidopsis* with impaired Fe(III) reductase accumulated even higher levels of Mn, Cu and Zn than the wild type with normal Fe(III) reductase activity. Another hypothesis is that Fe-deficiency induces the expression of more Fe transporters, which may also facilitate the transport of other divalent cations (Cohen, 1998).

These hypotheses need to be evaluated further but the results from this and Chapters 3 and 5 clearly argue against the specificity of Fe and other trace metal uptake as raised by Cohen et al (1998).

6.4.4 Causes of trace metal toxicities: nutrient imbalance versus direct toxicity

Low foliar Fe was the most obvious disturbance of nutrient balance caused by high concentrations of the trace metals tested, and the visual symptoms in most cases were consistent with Fe deficiency, except for Hg (see Table 6.1). However, it is important not to overemphasise the role of Fe in toxic responses to trace metals. While Fe concentrations were low and comparable to those in plants not supplied with Fe, there was no correlation between Fe content of roots or leaves and the degree of inhibition of growth (compare Figs 6.1, 6.3 and 5.1). There was also no consistent effect of excess trace metals on micronutrient trace metals; the changes in the concentration of most micronutrients would have been insufficient to cause deficiency or toxicity at the levels supplied in the nutrient solution. The same applies to the macronutrients, where the changes in concentration were relatively minor. It seems reasonable to propose therefore, that the toxicities in most cases are due to a combination of Fe deficiency and direct effects of high concentrations of the trace metals. The exact processes which are disrupted by different metals has not been examined here but may involve both direct inhibition of cellular processes (e.g. by non-specific binding to proteins) or by competition for essential binding sites on metalloproteins. The latter process

may explain the high degree of chlorosis in the metal treated plants since other divalent metals may compete with Fe, Mg and Mn for important sites in chloroplastic photosystems.

6.4.5 The specificity of Co on S uptake

It was difficult to isolate specific effects of Co on nutrient content and distribution or on growth responses or toxicity symptoms. In most cases Co was intermediate in causing the various responses. The one exception was in the effect of Co on transport of S into leaves where leaf S content was almost doubled by the addition of 5 μM Co (Fig. 6.12). The reason for this stimulation is unclear since although Co has a high affinity for –S and –SH groups, so do some of the other metals examined such as Hg and Cd (see Fig. 3.13). A Co-specific S-containing complex which either assists in the loading of S into the root xylem or phloem or increases mobility of S is possible but has not been identified.

It may be significant that both root and shoot S content were also enhanced by Fe deficiency (Fig 6.12) in view of the report in yeast that Fe-deficiency resulted in higher levels of reduced sulfhydryl groups at the exofacial plasma membrane surface and higher concentrations of GSH within the cell (Lesuisse and Labbe, 1992). Cohen et al (1998) also noted a 50% increase in reduced sulfhydryls on the root surface under Fe-deficiency compared to that under Fe-sufficiency.

CHAPTER 7 General Discussion

7.1 UPTAKE OF TRACE METALS.

Much mystery and confusion surrounds the precise molecular mechanisms for uptake of trace metals. Speculation about the existence of micronutrient channels has so far not been resolved. Nor has the question of whether there are specific transporters for individual micronutrients. Co should be a good metal with which to begin to examine trace metal uptake because plants do not need Co and its transport should therefore not be dependent on such factors as growth and intracellular demand. The transport experiments in this thesis are relevant here. It was found that there was a high degree of competition for Co uptake that suggests that there exist some fairly non-specific transporters for trace metals. If the inhibition of Co uptake was actually due to competition for uptake sites (as opposed to non-competitive inhibition of the transporter), then there exists a degree of selectivity for different metals. For example Cd was a strong inhibitor of Co uptake while Ca and Mg were poorly competitive. This latter result argues against the leakage of Co, and perhaps other trace metals, through Ca channels. The inhibition of Co uptake by the sulfhydryl reagent NEM suggests that binding to –SH groups in proteins is an important stage in the membrane transport of Co.

Attempts to isolate specific transporters by molecular biological means have not so far been successful in plants. There are however, reports of cloning of metal transporters in yeast. The existence of both specific and non-specific transporters for micronutrient metals is possible; the identification of 3 kinetically independent Co transporters (Chapter 3) is consistent with this. The concentrations of Co used in most transport experiments (0.1 – 1 μM) were designed to be within the range of concentrations which are adequate for other micronutrient metals and would therefore compete with these metals at physiologically relevant concentrations. It would seem more likely that specific transporters would have a high affinity for certain metals and operate to extract selected metals at low concentrations

in order to prevent individual deficiencies. How then would a non-selective uptake system allow control of internal concentrations and maintain nutrient balance? It is known that internal nutrient status has a strong influence on net uptake (Reid 1998). Uptake by a selective transporter could be regulated by controlling influx. This would seemingly not occur for a non-selective transporter since the influx of all metals would also be affected. For non-selective transporters, individual efflux pumps for each trace metal could control net uptake. It was shown that internal Co status only affected Co influx in the high concentration range and this effect may have been indirect, due to the toxicity caused by high uptake. Thus, one reason why some metals are more toxic than others is that there are no efflux pumps which control their internal concentrations.

A model illustrating the possible areas of interaction of Co with the uptake and movement of other trace metals in roots is shown in Fig. 7.1. In the case of Co, much of the disturbance of the internal concentrations of other trace metals could be traced to changes in the transport of these metals from root to shoot. This was particularly obvious in the case of Fe, where uptake to the root was not greatly affected by Co, but leaf concentrations of Fe were low, even at Co concentrations that were not toxic to growth. These results imply that the uptake system for Fe is independent of the uptake system for Co, but they may share a common transport pump for efflux into the xylem.

The characteristics of uptake and of transport from root to shoot were affected differently by other trace metals. In the case of Co, uptake into the root was reduced by most other trace metals but transport to the shoot was possibly only reduced due to the lower concentration in the root. Ni was interesting in that it stimulated Co transport to the shoot.

For some trace metals, Co at low concentrations actually stimulated transport to leaves, particularly in Fe-deficient conditions (Fig. 5.12). This might occur either by displacing these metals from intracellular binding sites, thereby making them more available for loading into the xylem, or by stimulating the synthesis of ligands that aid in their transport as complexes.

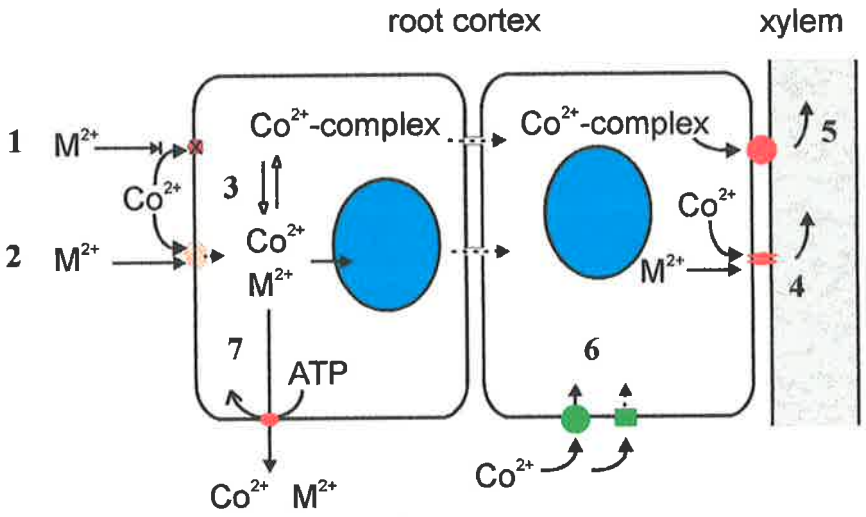


Figure 7.1 Possible pathways for the uptake of Co and for its interaction with other metals (M^{2+}).

1. direct inhibition of transport (non-competitive)
2. competition for uptake of other metals
3. competition for binding to intracellular ligands (e.g. enzyme sites)
4. competition for unloading into the xylem
5. transport of Co as a complex is also possible, especially with ligands containing S or SH.
6. specific active or passive transport systems for Co seem unlikely
7. net uptake may be controlled by regulating efflux rather than controlling influx.

The stimulation of S transport to leaves, and to a lesser extent S uptake into the roots, may indicate that Co moves in the xylem as a complex with S-containing ligands. It was interesting that among the trace metals tested, only Co increased the S content of the leaves.

At the subcellular level, the experiments with *Chara* showed that Co was rapidly transferred to the vacuole and the possibility therefore exists that some of the toxic effects of Co and its disturbance of other trace metal concentrations could be due to Co competing for uptake into the vacuole.

The amelioration of Co toxicity by Ca was suggested to occur via the indirect effects of Ca on membrane surface charge. This explanation was put forward by Kinraide (1994) to account for the effects of a range of treatments on the amelioration of Al toxicity. While the proposal seems theoretically feasible, the whole question of the relevance of surface charge to nutrient uptake needs to be examined in more detail.

The precise mechanism(s) by which Co inhibits growth of mung bean have not been completely established. The most likely cause is through inhibition of Fe transport to the leaves, leading to Fe deficiency in the chloroplast. This is supported by the measurements of leaf Fe concentrations, of Chl a and Chl b content, of photosynthetic efficiency measured by chlorophyll fluorescence, and by the similarity of appearance of leaves of Fe-deficient and Co-treated plants (Chapter 5). Nevertheless, there were morphological differences between Fe-deficient and Co-treated plants that suggested that the two treatments were not synonymous. The possibility that Co inhibited growth be inducing deficiencies of other nutrients appeared to be discounted. Although there were significant changes in the nutrient content, mainly of leaves, it is unlikely that the extent of the changes would have been sufficient to inhibit growth. A further possibility is that Co disrupts metabolism by competing with other trace metals in various intracellular processes. The fact that Co reached high concentrations in tissues, and its known affinity for –SH groups in proteins, is consistent with this proposal.

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