MECHANISMS OF COPPER UPTAKE AND TRANSPORT IN PLANTS

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ABSTRACT

The Cu concentration in plants varies considerably between species. This suggests different abilities to either absorb Cu from soils or translocate Cu from root to shoot. The main objective of the thesis was to provide a fuller understanding of the mechanisms of Cu uptake and transport in plants which may lead to the development the strategies to improve Cu uptake by pasture crops.

Glasshouse experiments with the Cu hyperaccumulator *Haumaniastrum katangense* showed that Cu hyperaccumulation in shoots was not found. It was concluded that *H. katangense* plants tested in these experiments were Cu tolerant rather than having hyperaccumulator status. The mechanism of high tolerance to Cu could be due to the restriction of Cu transport from roots to shoots.

Nutrient solution culture experiments with the Ni hyperaccumulator plants *Alyssum bertolonii* and *Berkheya coddii* showed that co-hyperaccumulation of Cu and Ni did not exist. *Alyssum bertolonii* was not a Cu-tolerant plant, whereas *B. coddii* exhibited a much greater degree of tolerance to this metal, and the tolerance of *B. coddii* to Cu was not at the expense of Ni uptake. It was concluded that *B. coddii* should be considered as a possible plant for phytoremediation of soils contaminated with both Cu and Ni and it is recommended that field trials be carried out to establish this potential.

NFT nutrient solution culture experiments showed that a large proportion of total Cu uptake by chicory and tomato plants was retained by roots except when plants were grown in the basal nutrient solution (0.05 mg Cu L^{-1}). Copper retention by roots, limited Cu translocation to xylem and shoots. Large differences between measured and predicted Cu accumulation by shoots of tomato and chicory suggested that some xylem-transported Cu is recirculated to roots via the phloem.

A Cu speciation study showed that more than 99.7% of total Cu in tomato and chicory xylem sap was in a bound form. Increased Cu concentrations in the rooting media induced selective synthesis of certain amino acids which include NA, His, Asn and Gln, all of which have high stability constants with Cu.

Nicotianamine and His have the highest binding constants for Cu and the concentrations of NA and His in chicory and tomato xylem saps can account for all the bound Cu carried in the sap.

Copper recirculation within plants was demonstrated by an experiment with hydroponically grown tomato plants in a split-root system. Significant amounts of Cu were translocated from roots bathed in a solution of high Cu concentration to another half root system exposed to low Cu. Shoot Cu concentrations were positively correlated to plant water use (mL g^{-1} DM). A Cu recirculation model was suggested.

Efforts have been made to develop the strategies to improve Cu uptake by pastures. The initial uptake of Cu from $CuSO_4$-fertilised soil can be increased by 10-21 % by addition of His and casein. Casein was generally more effective at increasing plant Cu uptake than His and other amino acids.

The $Cu(OH)_2$-based fertiliser was less effective than the $CuSO_4$-based fertilisers in supplying Cu to ryegrass grown in pots of Ashhurst stony silt loam and Wairoa pumice soil. In general, among the three $CuSO_4$ fertilisers, Ca-caseinate-$CuSO_4$ resulted in higher Cu uptake by ryegrass grown in both soils.

The factors constraining Cu uptake by ryegrass plants from Cu-fertilised soils were elucidated. Linear relationships between ryegrass Cu uptake and total soil solution Cu concentration were soil type dependent, despite each soil having similar soil solution Cu concentrations. Between 98.5-99.5% of the soil solution Cu was in complexed forms. No relationship between the Cu^{2+}concentration in soil solution (expressed as pCu^{2+}) and Cu concentration in plants was found. Free Cu^{2+} concentrations in soil solution were sensitive to pH change. The extent of the increase in free Cu^{2+} concentration per unit decrease in pH was dependent on soil type. It is suggested that the rate of Cu uptake by plants is likely to be dependent on both the concentration of organically complexed Cu in the soil solution and the stability of this complex to pH change.

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 2

COPPER UPTAKE BY THE HYPERACCUMULATOR PLANT *Haumaniastrum katangense*

CHAPTER 3

COPPER UPTAKE BY THE NICKEL HYPERACCUMULATORS *Berkheya coddii* AND *Alyssum bertolonii*

CHAPTER 4

COPPER UPTAKE AND TRANSLOCATION IN CHICORY (*Cichorium intybus* L. cv Grasslands Puna) AND TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS GROWN IN NFT SYSTEM. I. COPPER UPTAKE AND DISTRIBUTION IN PLANTS

CHAPTER 5

COPPER UPTAKE AND TRANSLOCATION IN CHICORY (*Cichorium intybus* L. cv Grasslands Puna) AND TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS GROWN IN NFT SYSTEM. II. THE ROLE OF NICOTIANAMINE AND HISTIDINE IN XYLEM SAP COPPER TRANSPORT

CHAPTER 6

AN INVESTIGATION OF COPPER UPTAKE AND REDISTRIBUTION WITHIN TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS

CHAPTER 7

EFFECTS OF AMINO ACIDS AND CASEIN ON COPPER UPTAKE FROM SOIL BY CHICORY (*Cichorium intybus* L. cv. Grasslands Puna)

CHAPTER 8

AGRONOMIC EFFECTIVENESS OF SELECTED FERTILISERS IN SUPPLYING COPPER TO PERENNIAL RYEGRASS

(*Lolium perenne* cv. Nui)

CHAPTER 9
LINKING PLANT COPPER UPTAKE AND SOIL SOLUTION COPPER POOLS IN COPPER-FERTILISED SOILS

CHAPTER 10
SUMMARY

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 9

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 9

LIST OF ABBREVIATIONS

3 Mh	3-methylhistidine
Aaba	α-aminobutyric acid
AAD	α-aminoadipic acid
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
β-Aib	β-aminoisobutyric acid
Carn	carnosine
Cyst	cystathionine
DM	dry matter
EDTA	ethylene-diaminetetraacetic acid
FAAS	flame atomic absorption spectrometry
Gaba	γ-aminobutyric acid
GFAAS	graphite furnace atomic absorption spectrometry
Gln	glutamine
Glu	glutamic acid
His	histidine
Hyp	hydroxyproline
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
NA	nicotianamine
NFT	nutrient film technique system
Orn	ornithine
Phe	phenylalanine
PITC	phenylisothiocyanate
Pro	proline

RP-HPLC	reversed phase high performance liquid chromatography
Ser	serine
Thr	threonine
Tyr	tyrosine
Val	valine
WUE	plant water use efficiency (g DM L^{-1} H_2O)

CHAPTER 1

GENERAL INTRODUCTION

1.1 COPPER IN SOIL-PLANT-ANIMAL SYSTEMS

Following identification of copper (Cu) as an essential element for higher plants and animals in 1931 (Sommer, 1931), numerous studies both by pure and applied biologists have greatly increased our knowledge of this metal in nature.

Copper is one of the eight essential micronutrients for all higher plants, being a component of the protein structure of a range of enzymes involved in electron transport and redox reactions in mitochondria, chloroplasts, cell walls and the cytoplasm of plant cells (Marschner, 1986; Welch, 1995). A common characteristic of heavy metals in general, regardless of whether they are biologically essential or not, is that they may already exert toxic effects in low concentrations compared with macro-nutrients (Verkleij and Schat, 1989). In the case of Cu, it is toxic for most plant species, at concentrations higher than 20-50 mg kg^{-1} dry matter (DM) (Asher, 1991; Gartrell, 1981; Robson and Reuter, 1981; Welch, 1995). Timperley et al. (1970) concluded that the Cu concentrations of most plants tend to be internally rather than externally regulated, so that Cu concentrations in plants tend to be relatively constant, generally between 5-20 mg kg^{-1} DM, irrespective of the available Cu concentration in the rooting media. The concentrations of Cu in soil solutions, however, are much lower (5-200 ppb). These concentrations would not test internal plant regulation of Cu (Jarvis and Whitehead, 1981, 1983; Welch, 1995; Whitehead, 1987).

The basic growth responses of plants to increasing metal concentration in rooting media result in characteristic uptake response curves, the phases of which correspond to ranges of deficiency (in the case of essential elements), tolerance and toxicity (Fig. 1.1). Such generalisations apply equally to metal-tolerant and non-metal-tolerant plants (Baker and Walker, 1989). Baker (1981) reviewed the biogeochemical data for different metals and plant species, and suggested that different patterns of metal uptake could be related to different mechanisms of metal tolerance at the whole plant level. *Excluder*, *indicator* and *hyperaccumulator* were defined as the three basic strategies of metal uptake by plants in relation to substrate concentrations (Fig. 1.2). Most plants adopt the "exclusion" strategy to avoid heavy-metal toxicity, by both excluding the metals from entering the roots and by restricting transport from roots to shoots (Baker and Walker, 1989; Robinson, 1997).

Copper deficiency in grazing animals is a global issue, whether caused by low Cu in forage (primary deficiency) or by dietary interaction with other elements, eg. Sulphur (S), molybdenum (Mo) and/or iron (Fe) (secondary deficiency) (Cunningham, 1946, 1950; Cunningham et al., 1959; Cunningham and Perrin, 1946; Grace, 1983; Smart et al., 1992). In the past 20 years, over 3,000 research reports on both primary and secondary Cu deficiency in cattle have been published. The first indication that Cu deficiency was a problem in grazing animals was noted in the U.S.A. in the early 1930's, but it was not until the 1940's that the Cu-responsive disorders such as enzootic ataxia or swayback in lambs (Fig. 1.3) and peat scours in cattle (Fig. 1.4) were reported in New Zealand (Grace, 1983; Grace et al., 1998; Korte et al., 1996). Deficiency can be prevented by suitable Cu supplementation strategies such as treatment with long acting CuO needles and Cu injections (Grace, 1994), which cost the farming industry several million dollars in animal remedies each year.

In New Zealand primary Cu deficiency in ruminants can occur as a result of ingesting pasture containing less than 3 to 4 mg Cu kg^{-1} DM (Grace, 1983). Overcoming the problem with copper fertilisers has met with mixed success, with generally a low recovery of Cu in pasture plants. Low uptake of Cu into shoots of plants is partly due to Cu being strongly bound to soil organic matter and partly because Cu, absorbed by the roots in high amounts, is only poorly translocated to the shoot (Jarvis and Whitehead, 1981; Whitehead, 1987). Improved translocation of Cu from root to shoot may enhance Cu uptake in pasture grasses and legumes.

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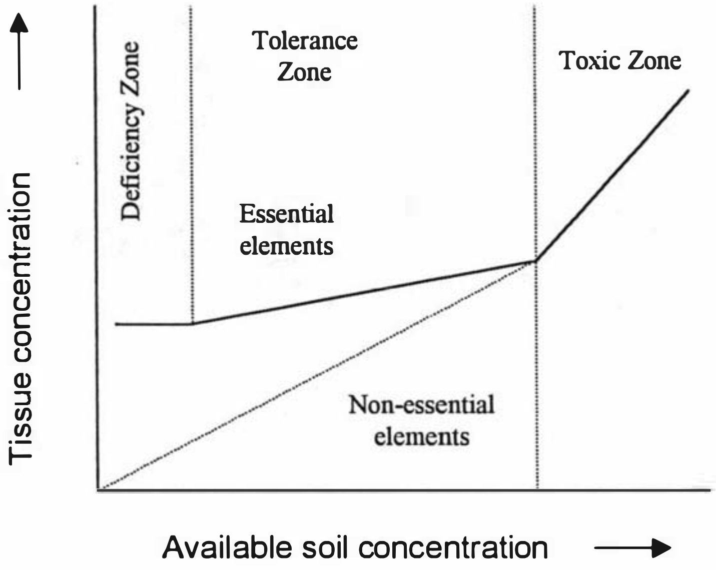

Fig. 1.1 Generalised uptake curves for essential and non-essential elements over the full range of soil concentrations (from Berry, 1986).

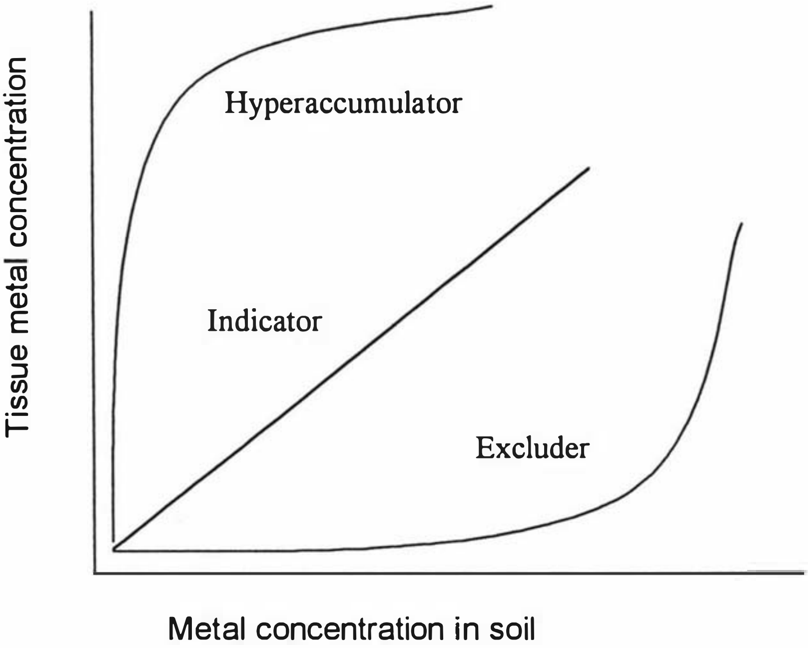

Fig. 1.2 The possible uptake response of plants to heavy metals in soils (from Robinson, 1997).

Fig. 1.3 Copper deficiency in lambs. Note the characteristic position of a lamb with enzootic ataxia or swayback (from Grace, 1983).

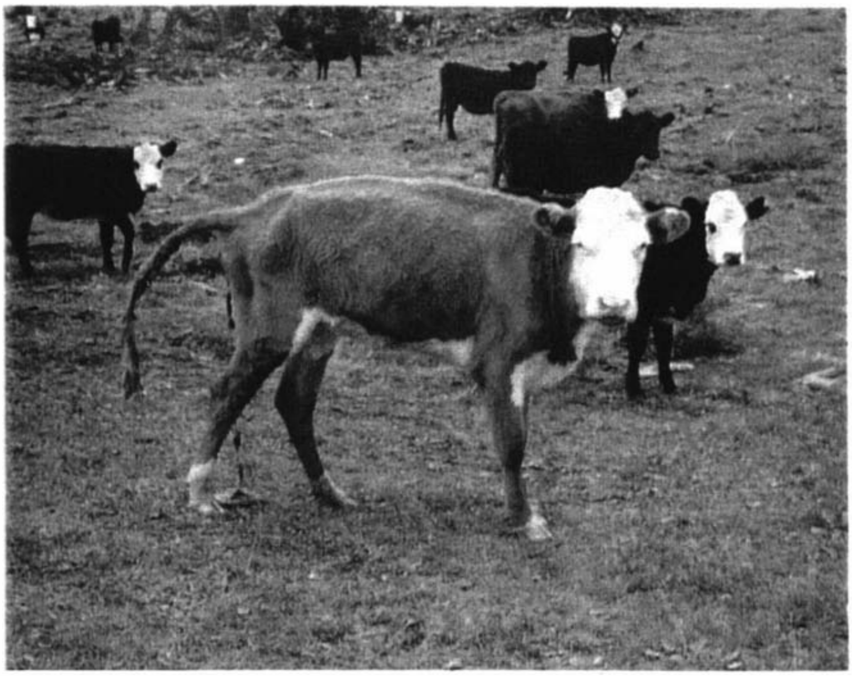

Fig. 1.4 Copper deficiency in cattle. Note the pale coat colour, poor body condition and diarrhoea (from Grace, 1983).

Examples of efficient translocation of Cu from root to shoot may be found with hyperaccumulator plants. *Haumaniastrum katangense, Haumaniastrum robertii* and *Vigna dolomitica* are capable of accumulating more than 1,000 mg Cu kg^{-1} in their shoots, and presumably have efficient mechanisms of Cu translocation. They are known as Cu hyperaccumulators (Brooks, 1977; Brooks et al., 1980, 1987). The mechanisms whereby these plants, evolved to grow in deposits of Cu ore, have efficient translocation processes from root to shoot are only partly understood. A common factor characteristic of all species of hyperaccumulator so far examined, is that metal concentrations in shoots are significantly greater than in roots (Baker et al., 1994). This contrasts markedly with metal partitioning in non-hyperaccumulator plants in which a higher concentration of metal is generally found in the roots, especially under high rooting media Cu concentrations. Moreover this suggests that a critical mechanism for hyperaccumulation occurs at some stage during the process of root-shoot translocation of metals.

There are many rate-limiting steps along the route of metal translocation to shoots (Kochian, 1991; Salisbury and Ross, 1992; Welch, 1995), such as root to xylem (McGrath, et al., 1999), xylem loading (De Boer and Wegner, 1997), xylem transport (Krämer et al., 1996; Tolra et al., 1996) and shoot sequestration (Lasat et al., 1996; Shen et al., 1997; Vázquez et al., 1994). Any of these steps may have been modified to contribute to the hyperaccumulation phenotype.

Once in the xylem, metal transport to the shoots is facilitated by metals complexing with complexing ligands in the xylem sap. Tolra et al. (1996) hypothesised that the xylem sap of hyperaccumulator plants may carry higher concentrations of organic acids than non-accumulator plants or may contain other novel compounds which have high association constants for metal complex formation. Either of these features could act to enhance the mobility of metals in the xylem and to increase the chemical gradient for metal ions, thereby increasing the strength of the xylem as a sink for metal ions. In fact the amino acid histidine, which has a very high stability constant for nickel (Ni) binding, has been implicated as a dominant agent involved in both Ni tolerance and hyperaccumulation by the species *Alyssum lesbiacum* (Krämer et al., 1996). They found elevated levels of histidine in the xylem sap of *A. lesbiacum* following exposure to Ni and determined that a Ni-histidine complex formed the majority of the complexed Ni species in the xylem sap. Moreover, they were able to significantly enhance Ni uptake and tolerance by the non-accumulator *A. montanum* through foliar and root applications of histidine.

1.2 HYPOTHESIS

No information on xylem and/or phloem ligands involved in Cu transport in hyperaccumulator plants is available so far. However it is not unreasonable to assume that the xylem sap of Cu hyperaccumulator plants may carry higher concentrations of organic acids than non-accumulator plants, or may contain other novel compounds which have high association constants for metal complex formation. Identification of these Cu complexing ligands may allow knowledge to be applied to non-accumulating plants (such as pastures) to enhance Cu uptake.

1.3 AIMS OF THE STUDY

The broad aim of this present study was to investigate the mechanisms of Cu uptake and transport in plants, which could lead to a fuller understanding of the strategies for enhancement of plant Cu uptake.

1.4 STRUCTURE OF THIS THESIS

Chapter 1

Chapter 1 serves as an over view of the study. The literature was briefly reviewed (more detailed reviews are discussed in the relevant individual Chapters), the hypothesis and the aim of the study was described, and the directions of the study (Fig. 1.5) are presented.

Chapters 2 and 3

Several experiments were conducted to investigate Cu uptake and translocation in a Cu hyperaccumulator plant *H. katangense* (Chapter 2). Unfortunately, under glasshouse conditions, Cu hyperaccumulation in plants was not found. It has been reported that some metal hyperaccumulating plants were capable of hyperaccumulating more than one metal, i.e. co-hyperaccumulation (Fielder, 1985; Hajar, 1987; Homer et al., 1991; Reeves and Baker, 1984). Copper uptake by two Ni hyperaccumulators was studied, but no co-hyperaccumulation of Cu was found (Chapter 3).

Chapters 4 - 6

Copper uptake and transport in tomato plants has been well documented (Mazhoudi et al., 1997; Ouariti et al., 1997; Pich and Scholz, 1996). Chicory, a pasture plant, is recognised as having higher Cu concentration in shoots than does ryegrass or clover (Thomas et al., 1952; Thomas and Thompson, 1948). In Chapters 4, 5 & 6, studies focus on the mechanisms of Cu uptake and transport in tomato and chicory plants.

Hydroponic plant culture in nutrient film technique (NFT) and static culture systems experiments were conducted to study Cu uptake and distribution in chicory and tomato plants (Chapter 4), the chemical speciation of Cu in xylem saps (Chapter 5), and Cu recirculation within tomato plants (Chapter 6).

Chapters 7-9

A preliminary experiment was conducted to test the hypothesis that Cu uptake in soil grown plants can be increased by addition of amino acids and casein (Chapter 7). A further experiment was conducted to compare the agronomic effectiveness of the selected Cu fertilisers (Chapter 8). A preliminary hypothesis describing the mechanisms of plant uptake of Cu from soils is advanced in Chapter 9.

Chapter 10

The results from this thesis are summarised in Chapter 10, and future studies are suggested.

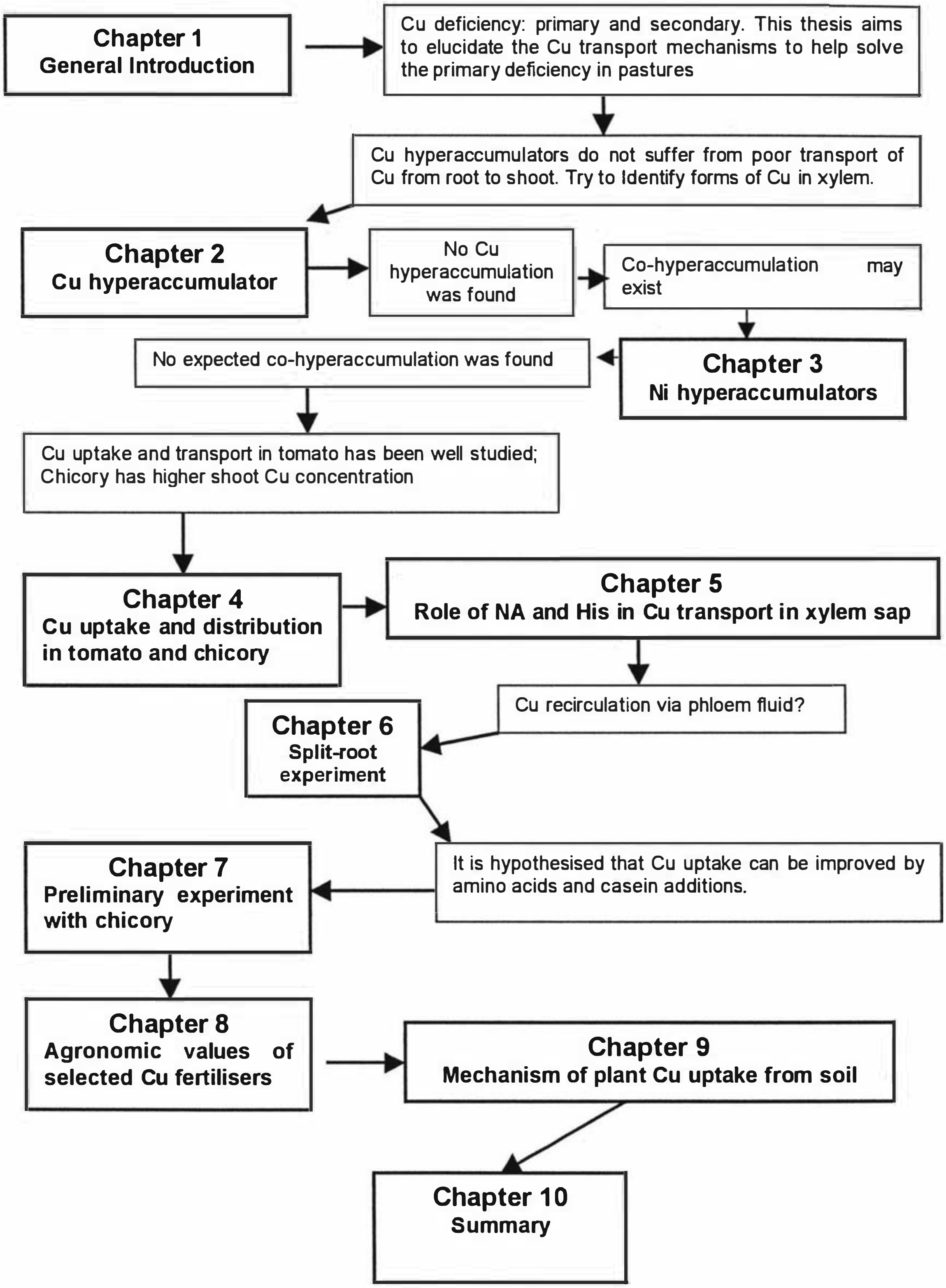

Fig. 1.5 Diagram of the structure of the thesis.

CHAPTER 2

COPPER UPTAKE BY THE HYPERACCUMULATOR PLANT *Haumaniastrum katangense*

2.1 INTRODUCTION

The phenomenon of plants accumulating inordinate concentrations of heavy metals was termed *hyperaccumulation* by Brooks et al. (1977). In this benchmark paper, the threshold for a nickel (Ni) hyperaccumulator plant was set at 1,000 mg Ni kg^{-1} DM (0.1%). In the case of Zinc (Zn), the threshold was later set at 10,000 mg Zn kg^{-1} DM (1%) (Reeves and Brooks, 1983). These values were used because they represent a concentration about ten times greater than the highest levels found in 'ordinary' non-hyperaccumulator plants (Brooks et al., 1977). Reeves et al. (1995) considered that the minimum level for cadmium (Cd) hyperaccumulator should be lowered to 100 mg kg^{-1} DM and that the limit for manganese be raised to 10,000 mg kg^{-1} DM to be consistent (Table 2.1).

Since the discovery of the world's first 'nickel plant' in the late 1940s, the number of Ni hyperaccumulators identified has now exceeded 400 (Baker and Brooks, 1989; Brooks, 1987; Reeves et al., 1995, 1996). There are also hyperaccumulators of Cd, Co, Cu, Mn, Se and Zn (Baker and Brooks, 1989; Brooks et al., 1980; Brooks and Robinson 1998a, b; McGrath, 1998; Reeves et al., 1995).

The first workers to present data indicating hyperaccumulation of Cu were Duvigneaud and Denaeyer-De Smet (1963) who reported values of 1,200, 1,660, and 1,960 mg Cu kg^{-1} DM for *Ascolepis metallorum, Silene cobalticola, and Haumaniastrum robertii*, respectively. To date, at least 25 species of Cu and cobalt (Co) hyperaccumulators have been recorded in families as diverse as the Cyperaceae, Lamiaceae, Poaceae and Scrophulariaceae (Baker and Brooks, 1989; Brooks et al., 1980; Brooks and Robinson 1998a. b; Malaisse et al., 1994; Nor and Cheng, 1986; Tang et al., 1999). Most of these were found in the south central part of Africa in Zaïre and Zambia on Cu- and Co-rich soils. Of these

Aeollanthus biformifolius and *Haumaniastrum katangense* appear to accumulate most Cu.

Table 2.1 Normal elemental concentrations in plants and lower limit for hyperaccumulator plants (from Reeves et al., 1995).

Element	Normal Range (mg kg^{-1} DM)	Lower limit for hyperaccumulation (mg kg^{-1} DM)
Cadmium	0.03-20	100
Cobalt	0.05-50	1,000
Copper	1-100	1,000
Manganese	5-2,000	10,000
Nickel	0.2-100	100
Selenium	0.01-10	100
Zinc	5-2,000	10,000

Aeollanthus biformifolius was shown by Malaisse et al. (1978) to contain as much as 13,700 mg Cu kg^{-1} DM (1.37%) in the whole plant. But most of the Cu was in the corms with lesser (though still very high) contents in the aerial parts of the plants. Because the main purpose of this thesis was to study the mechanisms of Cu transport from root to shoot, this plant was of less interest.

Haumaniastrum katangense (S. Moore) P.A. Duvign. & Plancke from Shaba Province, Zaïre, has long been known as a geobotanical indicator of Cu, in which the highest shoot Cu concentration recorded was 9,222 mg kg^{-1} (Brooks et al., 1987; Brooks and Malaisse, 1985; Paton and Brooks, 1996). As a "copper flower", its presence seemed to indicate the existence of a mineralized soil containing anomalous concentrations of Cu and/or Co (Figure 2.1) (Brooks and Malaisse, 1985; Brooks et al., 1992). *H. katangense* has been found to be an accumulator of Cu and/or Co (Brooks, 1977; Brooks et al., 1980, 1987).

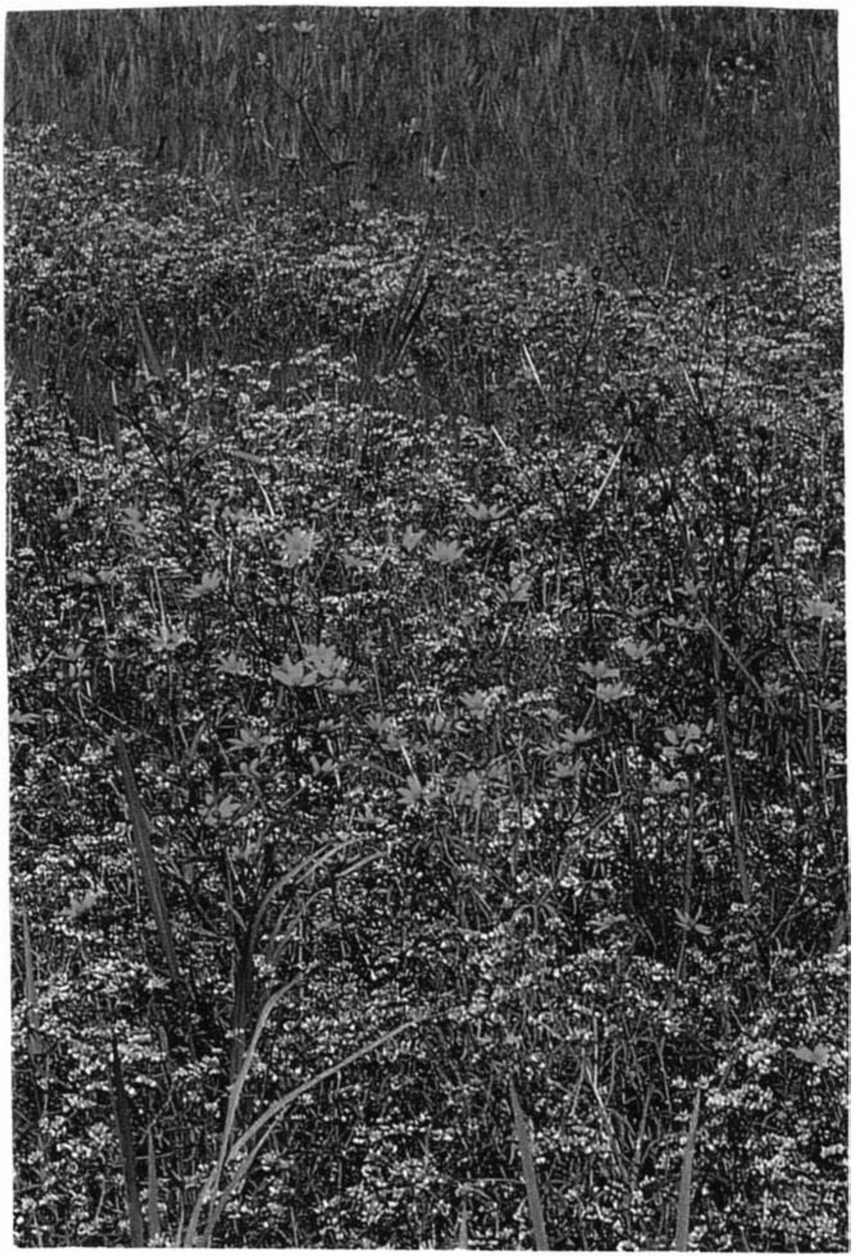

Fig. 2.1 *Haumaniastrum katangense* growing in copper-poisoned soil at Lubumbashi, Zaïre (from Brooks et al., 1992).

There is, however, some doubt about the ability of *H. katangense* to hyperaccumulate copper, as a result of data from greenhouse pot trials. Under greenhouse condition, most reports indicate that *H. katangense* is a Cu tolerant plant rather than a Cu hyperaccumulator plant (Bennett, 1998; Brooks et al., 1980; Malaisse and Brooks, 1982; Morrison et al., 1979). In a recent paper, perhaps the most comprehensive study on *Haumaniastrum* species so far, Paton and Brooks (1996) re-evaluated the ability of two *Haumaniastrum* species, *H. katangense* and *H. robertii,* to accumulate Cu, and their potential as geobotanical indicators of Cu and Co. They analysed a huge number of plant tissue samples collected from Central Africa, and reviewed the results published earlier (Brooks, 1977; Brooks et al., 1987). They concluded that both *Haumaniastrum* species do not always hyperaccumulate Cu and/or Co even in Zaïre, and that *H. katangense* is not an indicator of Cu or Co except perhaps in mineralised of Cu deposits of Zaïre. They suggested that there may be genetic differences between the populations of *H. katangense* that hyperaccumulate Cu and Co and those do not. However, these differences, if they exist, are not apparent from morphology.

The aims of this preliminary study were:

(a) to investigate the ability of *H. katangense*, grown from seeds, to accumulate Cu;

(b) to investigate the Cu complexing ligands in xylem sap of *H. katangense*, (if hyperaccumulation exists) which could lead to fuller understanding of the strategies to increase Cu uptake by pastures.

2.2 EXPERIMENT ONE

2.2.1 Materials and Methods

2.2.1.1 Plant culture and harvest

Seeds of *H. katangense* were germinated in 1:1 (v/v) bark and pumice medium with added "Osmocote" slow-release fertiliser. After four weeks, even-sized seedlings were carefully washed with deionised water and planted into the pots filled with the bark/pumice mixture (control, in 1:1 (v/v) bark and pumice), and the bark/pumice mixture with 500 mg kg^{-1} Cu addition (supplied as $CuSO_4$ powder). There was 500 g of potting mixture per pot. One plant was planted in each pot. There were 10 replicates. All pots were randomised, placed on a bench, and watered using saucers to keep pot capacity with distilled water. During the experimental period, the temperature in the glasshouse was partly controlled (heated at 13 °C, and ventilated at 25 °C). Supplementary lighting was supplied with sodium-vapour lamps to give a 16-h photoperiod. Plants were harvested 10 weeks after transplanting.

At harvest, root and shoot samples were collected. Shoots were washed in deionised water. Roots were washed sequentially in deionised water, a solution of 0.1 *mM* $Ca(NO_3)_2$ and NaEDTA for 30 minutes and finally deionised water for 30 min in each case to remove the Cu attached on root surface. Plant samples were oven-dried at 60°C for 48 hours and dry weights recorded.

2.2.1.2 Plant analysis

Samples of the dried material were digested in concentrated HNO_3 using a block digester with programmed temperature control. The digests were made up to standard volume and analysed for Cu by Flame Atomic Absorption Spectrometry (FAAS) at wavelength 324.7 nm and using an air/acetylene flame (Jarvis, 1980).

2.2.1.3 Extraction of potting mixture solution and analysis

At final harvest, the potting mixtures treated with 500 mg kg^{-1} Cu, as described above, were moistened to pot capacity with distilled water, and then the solutions from these pots were extracted by centrifugation at 10,000 rpm (12,000 RCF) in a refrigerated centrifuge at 5°C (Elkhatib et al. 1987). The solutions were filtered through Millipore filter paper (0.25 μm) to obtain clear solutions.

Potting mixture solution pH was measured with a pH meter (PHM 80 Portable pH Meter), and the total dissolved Cu concentrations in the solutions were measured using graphite furnace atomic absorption spectrometry (GFAAS)

(Sanders, 1982). Briefly, the displaced solutions were evaporated to dryness with concentrated nitric acid to destroy organic matter. The residues were taken up in 0.5 *M* nitric acid and Cu was determined using GFAAS. The conditions used were: dry at 100°C for 40 s, ramp at 20°C for 20 s, char at 500°C for 10s, and atomise at 2700°C for 6s.

2.2.1.4 Data analysis

Differences in mean soil solution Cu concentrations, between treatments, were statistically analysed using analysis of variance (ANOVA), performed by using the SAS GLM procedure (SAS Institute, 1990).

2.2.2 Results and Discussion

2.2.2.1 Copper concentrations in shoots and roots

Copper concentrations in shoots of *H. katangense* plants grown in the potting mixture containing 500 mg kg^{-1} Cu were significantly higher than those of control plants (Table 2.2). Root Cu concentrations showed the same trend, but the differences were much larger (Table 2.2).

Table 2.2 The effect of rooting media (bark/pumice) Cu concentration on Cu accumulation in *H. katangense* plants. Data are the means of ten replicates. Significant differences between means are indicated using significant P values.

Treatment	Cu in shoots (mg kg^{-1} DM)	Cu in roots (mg kg^{-1} DM)
Control	10.5	20.5
500 mg Cu kg^{-1}	16.8	270.3
P	0.05	0.001

The lack of Cu hyperaccumulation reported here is in general agreement with results reported by other researchers who conducted glasshouse pot trials to investigate Cu uptake by *H. katangense*. Morrison (1980) found that leaf Cu concentration of *H. katangense,* grown in a 50% peat and 50% perlite potting mixture with additions of 10-100 mg Cu kg^{-1}, kept in a narrow range from 10-15 mg kg^{-1} DM. Recently, Bennett (1998) found that the Cu concentrations in leaves of *H. katangense* grown in artificial Cu substrates (500 mg Cu kg^{-1}) were about 13 mg Cu kg^{-1} DM, which were well within the range for non-accumulating plants. But she found that the leaf Cu concentration could be increased to 200-300 mg kg^{-1} DM, after the addition of EDTA (2.0 g kg^{-1}). These values still only reach about one fifth of the threshold value for hyperaccumulation of Cu (1,000 mg kg^{-1} DM).

2.2.2.2 The pH and total Cu concentration in potting mix solution

The pH of the solutions extracted from the bark/pumice potting mixture ranged from 5.5-5.7 from both control and 500 mg kg^{-1} Cu treatment. The total dissolved Cu concentration in the solution extracted from the basic potting mixture (control) was about 0.05 mg Cu L^{-1}, and 4.7 mg Cu L^{-1} for the mixture that received Cu treatment (Table 2.3). This indicated that most of the Cu was absorbed by the potting mixture, and that only a small amount remained in solution in free or complexed forms.

Table 2.3 Effect of Cu treatment on pH and total dissolved Cu concentrations in solutions extracted from bark/pumice potting mixture. Data are the means of ten replicates. Significantly different means are indicated using significant P values, or NS if not significant.

Treatment	pH	Total soluble Cu in solution (mg Cu L^{-1})
Control	5.6	0.05
500 mg kg^{-1} Cu	5.7	4.75
P	NS	0.001

2.2.3 Summary

Copper hyperaccumulation was not found when *H. katangense* was grown in a 1:1 ground bark and pumice mixture with 500 mg Cu kg^{-1} addition. Copper was strongly bound to potting mixture and resulted in low total soluble Cu concentrations in the rooting media.

Solution culture techniques were proposed to standardise the Cu concentration. The Cu concentrations in the nutrient solutions could be monitored and adjusted as needed.

2.3 EXPERIMENT TWO

2.3.1 Materials and Methods

2.3.1.1 Plant culture

Seeds of *H. katangense* were germinated in a 1:1 bark and pumice medium. After four weeks, seedlings of even-sized were carefully washed with deionised water, planted into pumice media in 5 cm diameter PVC pots and transferred into PVC channels carrying flowing complete nutrient solution (Nutrient Film Technique, NFT) modified from Urrestarazu et al. (1996). After one week, plant roots had grown through the bottom of the pots, and the pots were then transferred to the experimental NFT system arranged in a complete randomised block design (RCBD) with three replicate blocks of NFT solutions with four Cu concentrations. The basal NFT solution was a modified half-strength Hoagland solution (Hoagland and Arnon, 1941) with 0.05 mg L^{-1} background Cu. The composition of the nutrient solution was: 68 mg L^{-1} KH_2PO_4, 253 mg L^{-1} KNO_3, 590 mg L^{-1} $Ca(NO_3)_2.4H_2O$, 123 mg L^{-1} $MgSO_4.7H_2O$, 11 mg L^{-1} FeEDTA, 613 µg L^{-1} H_3BO_3, 1690 µg L^{-1} MnSO4.H2O, 546 µg L^{-1} $ZnSO_4.7H_2O$, 13 µg L^{-1} $NiCl_2$, 123 µg L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$, 195 µg L^{-1} $CuSO_4.5H_2O$, with additional 0, 5, 10, 20 mg Cu L^{-1} supplied as $CuSO_4.5H_2O$. The pH of the culture solutions was adjusted to 5.5 ± 0.2. During the experimental period, the temperature in the glasshouse was partly controlled (heated at 13 °C, and ventilated at 25 °C). Supplementary lighting was provided by sodium-vapour lamps to give a 14-h photoperiod.

The pH and Cu concentration of the culture solutions were monitored and adjusted every 2 days. The NFT solutions were renewed weekly to prevent significant depletion of the nutrients and changes in pH. Plants were grown for 8 weeks before harvest.

Plant samples were collected as two parts: roots and shoots. Shoots were washed in deionised water. Roots were washed as described in Section 2.2.1.1. Plant samples were oven-dried at 60 °C for 48 h. Dry weights were recorded.

2.3.1.2 Analysis

2.3.1.2.1 Plants

Copper concentrations in nitric acid digests of shoots and roots were measured using FAAS (see Section 2.2.1.2 for details).

2.3.1.2.2 Nutrient solution

Briefly, the nutrient solutions were evaporated to dryness with concentrated nitric acid to destroy organic matter. The residues were taken up in 0.5 M nitric acid and Cu was determined using FAAS.

2.3.1.3 Data analysis

Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using the SAS GLM procedure (SAS Institute, 1990).

2.3.2 Results and Discussion

2.3.2.1 Plant growth

The results showed that Cu had no significant effect on plant dry weight of *H. katangense* when Cu concentration in the culture solution increased from 0.05 to 20 mg Cu L^{-1} (Table 2.5). This suggested that *H. katangense* show high tolerance to high Cu concentrations in rooting media. Growth inhibition and reduction of biomass production are general responses of higher plants to heavy metal toxicity, and the extent of inhibition and reduction are different between plants with different tolerance to heavy metals (Baker and Walker 1989, 1995). Though no experiments on Cu uptake by *H. katangense* using solution culture technique have been reported, Morrison (1980) found that the tolerance level of *H. katangense* for Cu was 1,380 mg Cu kg^{-1} in potting mixture. It was found that *H. katangense* shows an optimum growth on soils containing 1,000 mg Cu kg^{-1} (Malaisse and Brooks, 1982).

2.3.2.2 Copper uptake

Both root and shoot Cu concentrations significantly increased with the increases of Cu concentration in nutrient solutions (Table 2.5). But no hyperaccumulation of Cu in *H. katangense* plants was found. These results are consistent with those of the first experiment (section 2.2, this Chapter) and generally in agreement with Morrison (1980) and Bennett (1998). Root Cu concentrations were much higher than those of shoots (Table 2.5), and can well exceed the threshold set for Cu hyperaccumulator (1,000 mg kg^{-1} DM). From this point of view, the mechanism of Cu tolerance in *H. katangense* is mainly achieved by restricting Cu transport from root to shoot; or by very high efficiency of Cu recirculated via phloem. To my knowledge, this is the only study so far that has been conducted by growing *H. katangense* in solution culture system, and examining shoot and root Cu concentrations.

Table 2.4 Effect of Cu concentration in NFT solution on *H. katangense* plant growth. Dry biomass data are the means of three replicates. Relative growth is expressed as the dry weights of shoots or roots of treated plants as a percentage of plants in 0.05 mg Cu L^{-1}.

Cu in culture solution (mg Cu L^{-1})	Dry biomass (g $plant^{-1}$)		Relative growth (% of control)	
	Shoot	Root	Shoot	Root
0.05	2.26	0.98	100	100
5	2.18	0.93	96	95
10	2.09	0.89	92	91
20	1.99	0.81	88	83
P	NS	NS		

NS — no significant difference.

Table 2.5 Effect of Cu concentration in NFT solution on shoot and root Cu concentrations of *H. katangense* plants. Data are the means of three replicates. Significantly different means are labelled with different letters. Relative increase rate of Cu concentration is determined by expressing the concentrations of shoots and roots of treated plants relative to plants in 0.05 mg Cu L^{-1}.

Cu in culture solution (mg Cu L^{-1})	Cu concentration (mg Cu kg^{-1} DW)		Relative increase rate	
	Shoot	Root	Shoot	Root
0.05	11.0 d	25.6 d	1	1
5	35.3 c	333.8 c	3.2	13.0
10	60.1 b	2538.2 b	5.5	99.1
20	75.6 a	2968.3 a	6.9	115.9
P	0.01	0.01		

2.3.3 Summary

Copper hyperaccumulation in shoots of *H. katangense* plants was not found under the nutrient solution culture conditions used. Copper availability in the rooting media is not the only factor limiting plant Cu uptake. Copper hyperaccumulation actually exists in tissue samples collected from the field where there is a high Cu content in soils, though this does not always happen. This may be something particular to the special sites where the hyperaccumulation was observed. Fortunately, Professor R R Brooks obtained some soils from Zaïre in which the hyperaccumulation of Cu by *H. katangense* was found. So a pot trial (section 2.4) was conducted to examine whether our seed material of *H. katangense* possessed the ability to hyperaccumulate Cu in shoots when grown in this soil.

2.4 EXPERIMENT THREE

2.4.1 Materials and Methods

Even-sized *H. katangense* seedlings were transplanted into pots containing 200 g soils from Zaïre. Two plants each pot. Only two replicates were used because of the limited amount of soil available.

Plant growth and harvest were as described in Section 2.2.1.1, The plants were harvested at 8 weeks after transplanting. Cu concentrations in nitric acid digests of shoots and roots were measured using FAAS (see Section 2.2.1.2).

2.4.2 Results and Discussion

Again, no Cu hyperaccumulation in shoots was found. The mean Cu concentration of shoots and roots were 35 mg Cu kg^{-1} DM and 1,200 mg Cu kg^{-1} DM respectively. This is similar to the results from the nutrient solution culture experiment. There would appear to be at least 3 reasons to explain the lack of Cu hyperaccumulation by *H. katangense* in the present studies. Firstly, there may be genetic differences between the populations of *H. katangense* grown on these soils in Zaïre in the field and those used here. Secondly, the soils used in this research were stored which may lack the microrrhizal infection vigour present in the field soils, and this may affect Cu uptake and transport by *H. katangense* plants. Thirdly, differences in micro-climates where water use efficiency (g DM L^{-1}) is extremely low in the native environment compared with the glasshouse conditions used in this experiment.

2.5 CONCLUSIONS

From above three experiments, it is concluded that *H. katangense*, at least as far as our seeds were concerned, is not a Cu hyperaccumulating plant. It is difficult at present for researchers to obtain plant and soil materials, especially from Zaïre, due to civil unrest. Furthermore, it is not easy to grow *H. katangense,* and the plant sizes were uneven. There is also the difficulty of collecting xylem sap from *H. katangense* plants. So no further study was conducted on this species in this thesis. If further studies are under taken with *H. katangense* then the roles of plant genetics, microrrhizal infection and low water use efficiency should all be examined as factors which might contribute towards the observed field hyperaccumulation of Cu. Steps must also be taken to preclude the possibility of soil contamination occurring.

In the next chapter (Chapter 3), Cu tolerance and uptake by two Ni hyperaccumulating plants was studied to examine possible co-hyperaccumulation.

CHAPTER 3

COPPER UPTAKE BY THE NICKEL HYPERACCUMULATORS *Berkheya coddii* AND *Alyssum bertolonii*

3.1 INTRODUCTION

Hyperaccumulation of Cu by *Haumaniastrum katangense* was not observed in experiments summarised in Chapter 2. In this chapter, Copper tolerance and uptake by two Ni hyperaccumulating plants is examined.

The ability of plant species to tolerate a combination of heavy metals can be achieved either by co-tolerance or multiple metal tolerance. Genetically, co-tolerance is achieved by pleiotropy, where the same gene(s) produces tolerance to both metals. Multiple metal tolerance is produced when genes for several tolerances have spread independently through the plant population (Tilstone and Macnair, 1997). It was initially reported that tolerance to one metal does not confer tolerance to others (Gregory and Bradshaw, 1965; Turner, 1969). The tolerance, however, is not necessarily metal specific depending on the mechanism of tolerance. Nickel tolerance can occur in a zinc tolerant population of *A. capillaris*, despite the absence of high soil nickel contamination from the parent environment (Bradshaw et al., 1965). Furthermore, Allen and Sheppard (1971) found that in different individuals of *M. gutatus* from a number of different copper mine sites, tolerance to Zn, Pb and/or Ni also occurred, suggesting co-tolerance between Cu and other metals in *Mimulus*. A low level of tolerance to other heavy metals in Cu tolerant populations of A. capillaris was also observed (Symeonidis et al., 1985). Von Frenckell-Insam and Hutchinson (1993a) suggested that at the population level that Cu-Ni co-tolerance in *D. cespitosia* is due to multiple tolerance, and Ni tolerance can give tolerance to Zn by co-tolerance (Von Frenckell-Insam and Hutchinson, 1993b).

The ability of a plant to take up unusually high levels of more than one metal (co-hyperaccumulation) was observed in *T. goesingense* by Reeves and Baker (1984). They attributed this behaviour to the existence of a non-specific metal detoxification system. From soil culture experiments, Hajar (1987) showed that *Thlaspi alpestre* L., a coloniser of lead mine wastes, was tolerant to Ni, Co, Mn, Cd, Cu, Fe, and Mo. Specimens accumulated metal over the range 1,000-50,000 mg kg^{-1} DM in either roots or shoots or both. Co-accumulation also was found for the Ni hyperaccumulator plant *Alyssum troodii*, which accumulated 2,000 mg Ni kg^{-1} DM and 1,000-8,000 mg Co kg^{-1} DM in shoots (Homer et al., 1991).

B. coddii has been studied extensively because of its potential for phytoremediation and phytomining of Ni (Mesjasz-Przybylowicz, 1998; Robinson et al. 1997b). Morrey et al. (1989) found high tolerance to Ni and Cr in *B. coddii* plants grown in the soils containing elevated concentrations of both elements. Gabbrielli et al. (1991) found that *A. bertolonii* is cotolerant of Co and Zn. But so far no co-accumulation or co-tolerance Cu in these two Ni hyperaccumulator plants was reported. One of the aims of this study was to investigate whether *Alyssum bertolonii* and *Berkheya coddii* can hyperaccumulate Cu.

Another aim of this study was to investigate the phytoremediation potential of *A. bertolonii* and *B. coddii* for soils contaminated with Cu as well as Ni. An initial study determined the tolerance of *Alyssum* and *Berkheya* to increasing Cu concentrations in the rooting media. A second experiment examined the ability of the more Cu-tolerant *Berkheya coddii* to simultaneously take up both Cu and Ni.

3.2 MATERIALS AND METHODS

3.2.1 Experiment one

3.2.1.1 Plant culture

B. coddii and *A. bertolonii* plants were grown in a NFT system as described in Section 2.3.1.1.

3.2.1.2 Plant harvest

Plants were grown for 12 weeks before harvesting and then roots and shoots samples were collected. Shoots and roots samples were washed as described in Section 2.2.1.1. Plant samples were oven-dried at 60°C for 48 hours and dry weights recorded.

3.2.2 Experiment two

Experimental details were as described in Experiment one (see Section 2.2.1.1 for details) A modified half strength Hoagland solution was used as the base (control) solution (See Section 2.3.1.1 for details). Only *B. coddii* was used as the test plant. There were four treatments:

T_1 — base solution;
T_2 — base solution + 10 mg Cu L^{-1};
T_3 — base solution + 10 mg Ni L^{-1};
T_4 — base solution + 10 mg Cu L^{-1} and 10 mg Ni L^{-1}.

3.2.3 Analysis

3.2.3.1 Nutrient solution

Copper and Ni concentrations in nutrient culture solutions were monitored (see Section 2.3.1.2.2 for method) and adjusted back to initial concentration by addition of $CuSO_4$ and $NiSO_4$ solutions.

3.2.3.2 Plants

Copper and Ni concentrations in nitric acid digests of shoots and roots were measured using FAAS (see Section 2.2.1.2 for details).

3.2.4 Data analysis

Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using the SAS GLM procedure (SAS Institute, 1990).

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of Cu on plant growth and biomass production

Biomass production of *A. bertolonii* was significantly reduced with increasing concentrations of Cu in the nutrient solution (Table 3.1). Obvious Cu toxicity symptoms were observed in plants grown in nutrient solutions containing 5 and 10 mg L^{-1} Cu. No plant survived when the Cu concentration in the nutrient solution reached 20 mg Cu L^{-1}.

Copper toxicity symptoms (wilting and chlorosis) were observed in *B. coddii* plants grown in nutrient solutions containing 20 mg L^{-1} of this element. At rates of 5, 10 and 20 mg L^{-1}, the plant growth rates were 96.4%, 92.2% and 17.9% respectively relative to the control (Table 3.1). Copper had no significant effect on biomass production of *B. coddii* over the range of 0.05 to 10 mg L^{-1}.

Growth inhibition and reduction of biomass production are general responses of higher plants to heavy metal toxicity, and the extents of inhibition and reduction are different between plants with different tolerance to heavy metals (Baker and Walker, 1989). In the present study, significant depression of both shoot and root dry biomass production in Cu-treated *A. bertolonii* plants was observed (Table 3.1). This effect varied as a function of the Cu concentration in the culture solution. Punz and Sieghardt (1993) demonstrated that, in higher plants, root growth is particularly sensitive to the presence of metal toxins. Root biomass, length, numbers and rates of root elongation have all been employed to measure plant tolerance to heavy metals (Baker and Walker, 1989). In our study, the data (Table 3.1) show that the growth of the roots and shoots of *A. bertolonii* are equally sensitive to external Cu concentrations. Plant growth (shoot + root) performance, expressed as growth rate relative to background (0.05 mg Cu L^{-1}, Table 3.1), was substantially reduced when the Cu concentration in the nutrient solution reached 5 mg L^{-1}. This suggests that *A. bertolonii* does not have a high tolerance to Cu. It has been reported elsewhere (Gregory and Bradshaw, 1965; Turner, 1969) that tolerance to one metal does not necessarily confer tolerance to others. However, Homer et al. (1991) found that the Ni hyperaccumulator *A. troodii* was tolerant of both Cu and Co.

In the case of *B. coddii*, we found no significant decrease in shoot and root biomass production in plants growing in nutrients containing up to 10 mg Cu L^{-1}. This suggests that *B. coddii* displays high tolerance to Cu.

Table 3.1 The effects of Cu on biomass production of *B. coddii* and *A. bertolonii*. Data are the means of three replicates. Significantly different means are represented by labels a, b and c as determined by ANOVA.

Solution Cu (mg Cu L^{-1})	Shoot dry weight (g)	Root dry weight (g)	Relative % growth*
B. coddii			
0.05	33.86a	8.56a	-
5	32.69a	8.20a	96.39
10	31.11a	7.99a	92.20
20	5.33b	2.25b	17.87
P	0.01	0.01	
A. bertolonii			
0.05	17.11a	3.57a	-
5	6.97b	1.13b	39.19
10	1.69c	0.57c	10.89
20	-	-	-
P	0.01	0.01	

*Relative to whole plant (shoot + root) growth in 0.05 mg Cu L^{-1} (background).

3.3.2 Copper uptake by plants

Copper concentrations in shoots and roots of both species significantly increased with increasing Cu concentrations in the nutrient solutions. For both species, Cu concentrations in roots were much higher than in shoots (Table 3.2). When Cu in the NFT solutions increased from 0.05 to 20 mg L^{-1}, the Cu concentrations in *B. coddii* shoots and roots increased by a factor of over 10-fold (14 to 150 mg kg^{-1} DM and 50-fold (20 to 1000 mg kg^{-1} DM) (Table 3.2). The corresponding values for *A. bertolonii* were 5.7-fold (15 to 85 mg kg^{-1} DM) and 28.3-fold (from 30 to 850 mg kg^{-1} DM), respectively. The Cu concentrations in shoots of both species were in the range of 14-150 mg kg^{-1} DM.

Table 3.2 Copper concentrations in shoots and roots of *B. coddii and A. bertolonii* plants grown in NFT solutions containing Cu. Data are the means of three replicates. Significantly different means within columns are represented by labels a, b, c and d as determined by ANOVA.

Cu in NFT solution (mg Cu L^{-1})	Cu concentration (mg kg^{-1} DW) Shoot	Root	Relative increase (%)* Shoot	Root
B. coddii				
0.05	14.1 d	20.3 d	0	0
5	31.2 c	224.1 c	2.21	11.04
10	45.5 b	588.9 b	3.23	29.01
20	150.6 a	1000.3 a	10.68	49.28
P	0.01	0.01		
A. bertolonii				
0.05	15.5 c	30.6 c	1	1
5	38.9 b	500.8 b	2.51	16.37
10	85.8 a	850.1 a	5.54	27.78
20	-	-	-	-
P	0.01	0.01		

*Increase relative to 0.05 mg L^{-1} treatment (background Cu).

Neither plant was capable of taking up Cu to levels comparable to those of Ni (See section 3.3.5 and Fig. 3.1). A similar result has been found for the Ni hyperaccumulator *A. troodii* (Homer et al., 1991) and for *A. pintodasilvae* (Varennes et al., 1996). However, Kumar et al. (1995) found that some cultivars *of Brassica juncea* did accumulate Cr (VI), Cd, Ni, Zn and Cu to similar concentrations in shoots.

3.3.3 Total Cu accumulation and distribution in plant organs

In *B. coddii* plants, the total content of shoot-harvestable Cu increased up to solution Cu concentrations of 10 mg L^{-1}, and showed a decrease at 20 mg L^{-1} because biomass yields are reduced (Table 3.3).

In the case of *A. bertolonii,* the amount of shoot-harvestable Cu increased up to a solution Cu concentration of 5 Cu mg L^{-1}, and then decreased at 10 Cu mg L^{-1} because of reduced biomass yields (Table 3.3). In both species, roots and whole-plant Cu accumulation showed the same trend as that of shoots. The proportions of total plant uptake that were retained by *A. bertolonii* roots ranged from 30.3% in plants grown in the background solution (0.05 mg Cu L^{-1}) to 76.7% in plants grown with 10 mg Cu L^{-1}. The corresponding values for *B. coddii* ranged from 26.7% to 73.7%. When *B. coddii* plants were grown with 20 mg Cu L^{-1}, 74.3% of the total Cu uptake was retained in roots (Table 3.3). The ability of roots to retain Cu against transport to shoots has also been demonstrated in many species, for example, Mediterranean trees (Arduini et al., 1996), orange (Brams and Fiskel, 1971), tobacco (Struckmeyer et al., 1969), red clover (Hill, 1973) and perennial ryegrass (Jarvis 1978, 1980).

Table 3.3 Total Cu accumulation and Cu distribution in *A. bertolonii* and *B. coddii* grown in an NFT system. Data are the means of three replicates. Significantly different means within columns are represented by labels a, b, c and d as determined by ANOVA.

Solution Cu (mg Cu L^{-1})	Shoot Cu (mg $plant^{-1}$)	Root Cu (mg $plant^{-1}$)	Total Cu (mg $plant^{-1}$)	Cu retained in roots (%)
B. coddii				
0.05	0.49 d	0.18 d	0.67 d	26.7
5	1.02 b	1.81 c	2.83 c	64.0
10	1.42 a	4.00 a	5.42 a	73.7
20	0.81 c	2.34 b	3.15 b	74.3
P	0.01	0.01	0.01	
A. bertolonii				
0.05	0.26 a	0.12 c	0.38 c	30.6
5	0.27 a	0.57 a	0.84 a	67.9
10	0.14 b	0.47 b	0.61 b	76.7
20	-	-	-	-
P	0.01	0.01	0.01	

3.3.4 Co-tolerance of *B. coddii* to Cu and Ni

The shoot dry weights of *B. coddii* were not significantly affected by Cu and Ni concentrations of up to 10 mg L^{-1} either individually or in combination (Fig. 3.1A), and Cu did not significantly affect Ni uptake (Fig. 3.1B). This suggests that *B. coddii* is tolerant to the combination of both Ni and Cu, and that the tolerance to Cu is not at the expense of Ni uptake. The presence of high concentrations of Ni had, moreover, no significant effect on Cu uptake (Fig. 3.1C). Tilstone and Macnair (1997) proposed that the ability of a plant species to tolerate a combination of heavy metals could be achieved either by co-tolerance or by multiple-metal tolerance. A multiple-metal tolerance mechanism was found for *Mimulus guttatus* (Tilstone and Macnair, 1997). The ability of a plant to take up unusually high levels of more than one metal (co-accumulation) was also observed in *Thlaspi goesingense* by Reeves and Baker (1984) and some cultivars of *Brassica juncea* (Kumar et al., 1995). The results of the present study show that the ability of *B. coddii* to tolerate a combination of both Ni and Cu is more likely to be attributed to multiple-metal tolerance. It is noteworthy that *B. coddii* not only has the ability to tolerate a combination of both Cu and Ni, but also can accumulate Cu to a significant degree in its shoots because of a high biomass coupled with a moderate Cu concentration in its shoots. Cu bound to soil organic matter (SOM) ceases to be phytotoxic (Pare et al. 1999) because of the very high stability constant of the organometallic complex so formed. It may be significant that the more Cu-tolerant *B. coddii* accumulated this metal in its root system. If this happens when *B. coddii* grows in soils, as roots decompose, root-bound Cu will enter the SOM-bound fraction, and may provide a mechanism for decreasing the amount of phytotoxic Cu.

3.3.5 The potential role of *B. coddii* in phytoremediation of soils contaminated with both Cu and Ni

One of the most important consideration in phytoremediation of contaminated soils is that the plant should translocate metals from the soil to the aerial parts allowing a significant quantity of metal to be removed from the soil with each crop (Robinson et al., 1998). According to Robinson *et al.* (1997a), two crops of *B. coddii* would be sufficient to reduce the Ni content of moderately contaminated soils (100 mg kg^{-1}) to below the EU guideline of 75 mg kg^{-1} and only 4 crops would be required to remediate a 250 mg kg^{-1} Ni-polluted soil. In many cases, however, metal pollution of soils is a multi-element problem (Luo and Rimmer, 1995): the most common cause being sewage sludge disposal to land (Alloway and Jackson, 1991). For example in south China, Ni and Cu concentrations in vegetable plots using sewage sludge as fertiliser were found to be >400 mg kg^{-1} and >200 mg kg^{-1}, respectively. Other sources, such as recycling of scrap plastics (Chui et al., 1988) and metal mining wastes (Pierzynski and Schwab, 1993), lead to similar multi-element contamination. Offshore disposal of metal-rich sewage sludge has become illegal in Europe and increased usage on land for fertiliser will continue to increase levels of metals in soils (Punshon and Dickinson, 1997). Co-tolerance of *B. coddii* for Ni accumulation in the presence of high Cu was clearly demonstrated in the nutrient solution culture experiments discussed in the previous sections (Fig. 3.1). The potential of *B. coddii* to be used to phytoextract Ni from polluted soils and whilst simultaneously modifying Cu phytoxicity in the root zone is worthy of further study.

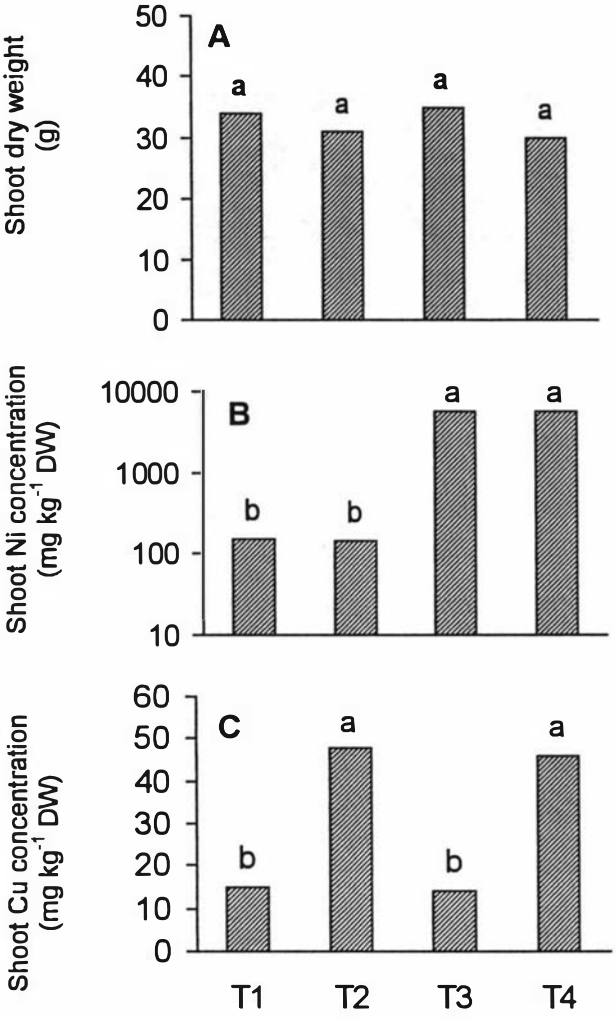

Fig. 3.1 The effect on shoots of *B. coddii* of various Ni and Cu concentrations in the rooting media nutrient solution. (A) The effect of Cu and Ni on shoot dry weight; (B) Ni concentrations in dry shoots as a function of metals in the growth solution; (C) Cu concentrations in dry shoots as in (B). T1 (BS - base [control] solution), T2 (BS + 10 mg Cu L^{-1}), T3 (BS + 10 mg Ni L^{-1}), T4 (BS + 10 mg L^{-1} each of Cu and Ni). Labels a and b indicate significant differences ($P<0.01$) as determined by ANOVA.

3.4 CONCLUSIONS

Neither *A. bertolonii* nor *B. coddii* was capable of co-hyperaccumulating Cu and Ni.

Alyssum bertolonii is not a suitable plant for phytoremediation of both Ni and Cu from contaminated soils because of its low tolerance to Cu. The combination of the high biomass and high Ni content of *B. coddii*, together with its tolerance of high Cu concentrations in the rooting media and moderate accumulation of Cu in shoots, should render this species a potential means for phytoremediation of soils contaminated with both Ni and Cu. It is recommended that further field trials be carried out to determine the true potential of this species for phytoremediation.

CHAPTER 4

COPPER UPTAKE AND TRANSLOCATION IN CHICORY (*Cichorium intybus* L. cv Grasslands Puna) AND TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS GROWN IN NFT SYSTEM. I. COPPER UPTAKE AND DISTRIBUTION IN PLANTS

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4.1 INTRODUCTION

The Cu concentration in shoots of pasture plants varies considerably between species (Beeson et al., 1947; Thomas et al., 1952). This suggests different abilities to either absorb Cu from soil, or translocate Cu from root to shoot. The factors that allow some plants to take up more Cu than others are unclear. Chicory, a forage plant, is recognised as having a high Cu concentration in shoots, and is a valuable forage source of Cu (Thomas, et al. 1952) with Cu concentrations (average 12.5 mg Cu kg^{-1} DM) above those of pasture grasses (average 8.2 mg Cu kg^{-1} DM) and legumes (average 8.7 mg Cu kg^{-1} DM). Higher shoot tissue Cu concentrations must result from greater net translocation of Cu from root to shoot. The effects of Cu concentration in rooting media on growth, Cu uptake and accumulation and distribution in some pasture crops has been comprehensively studied, for example in ryegrass (Jarvis 1978, 1980; Jarvis and Whitehead, 1981; Thornton, 1991; Whitehead, 1987). However, little or nothing has been published about the effects of Cu concentration in the rooting media on plant growth, Cu uptake and distribution in chicory plants.

The present study was carried out to test the hypothesis that the raised herbage copper concentrations in chicory are the result of greater xylem sap Cu concentrations. Copper uptake and Cu distribution in tomato and chicory was studied by growing plants in a nutrient film technique system (NFT) with a wide range of Cu concentrations. Tomato was chosen as a comparative species because Cu nutrition in tomato has been well studied, and there is good evidence that Cu translocation in tomato involves complexing with amino acids in the xylem sap (Pich and Scholz, 1996).

4.2 MATERIALS AND METHODS

4.2.1 Plant culture

Chicory (*Cichorium intybus* L. cv. Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv. Rondy) seeds were germinated in 1:1 bark and pumice media. After two weeks, seedlings of even size were carefully washed with deionised water, planted into NFT system as described in Section 2.3.1.1. The NFT solutions were renewed weekly as well as half an hour before plant excision to prevent significant depletion of the nutrients and changes in pH.

4.2.2 Harvest

Plants were grown for 6 weeks before harvest. At harvest, shoots were removed and xylem sap was collected as described below.

4.2.2.1 Plant harvest

Roots and shoots samples were collected, washed and dried as described in Section 2.2.1.1. Plant dry weights were recorded.

4.2.2.2 Xylem sap collection

The chicory stems were severed using a stainless-steel razor blade at about 1 cm above media surface perpendicular to the stem axis. The pots were retained in the flowing NFT solution. To avoid contamination of the xylem exudate with cell sap, the first drop of exudate was rejected. Xylem sap was collected with a micropipette. Tomato xylem sap was collected as described by White et al. (1981b). Plant stems were severed perpendicular to the stem axis with stainless-steel razor blades at about 3 cm above the roots. The decapitated stumps were wiped gently and fitted with Tygon tubing. The stumps were allowed to bleed into glass scintillation vials. Time of day can cause variations in xylem sap Cu concentrations (Hocking et al., 1978), therefore xylem saps were collected between 8 am - 9:30 am. The saps were filtered immediately (Nylon, 0.45 μm) and frozen at - 70 °C until needed.

4.2.3 Analysis

4.2.3.1 Plants

Cu concentrations in nitric acid digests of shoots and roots were measured using FAAS (see Section 2.2.1.2 for details).

4.2.3.2 Cu in xylem sap

Total Cu concentrations in xylem saps were measured using GFAAS (see Section 2.2.1.3 for details).

Free Cu^{2+} concentrations were measured directly with a cupric electrode (Orion 9629BN Ionplus™ Cupric Electrode) which only responds to Cu^{2+} ions in solution (Graham, 1979). The free Cu^{2+} concentration in chicory saps was measured with 2-fold diluted xylem sap because of the limited sap volume.

4.2.4 Data analysis

Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using SAS GLM procedure (SAS Institute, 1990).

4.3 RESULTS

4.3.1 Plant growth

Exposure of tomato roots to 5, 10 and 20 mg Cu L^{-1} reduced the dry weight of tomato plants to 86, 58 and 39% for shoots, and 86, 56 and 37% for roots, respectively, as compared to controls (Table 4.1). The corresponding values for chicory plants were 97, 76 and 41% for shoots, and 97, 79 and 45% for roots, respectively (Table 4.1). Exposure of chicory roots to 5 mg Cu L^{-1} did not cause a significant yield reduction.

4.3.2 Plant Cu concentration

As the Cu concentration in the NFT solutions increased from 0.05 to 20 mg L^{-1}, Cu concentrations in chicory shoots and roots increased 4.1 fold (from 16.0 to 65.3 mg Cu kg^{-1} DM) and 19.4 fold (from 24.5 to 476.0 mg Cu kg^{-1} DM) respectively (Table 4.2). Tomato plant Cu concentrations in both shoots and roots increased 4.2 fold (from 13.1 to 55.1 mg Cu kg^{-1} DM) and 41.7 fold (from 16.9 to 707.7 mg Cu kg^{-1} DM) respectively (Table 4.2). Notably, increases in tomato root Cu concentrations were double those of chicory root when the external solution Cu concentration reached 20 mg Cu L^{-1}.

4.3.3 Plant Cu accumulation

In both plant species, the amount of shoot-harvestable Cu increased up to solution Cu concentration of 10 mg Cu L^{-1}, and then decreased at 20 mg Cu L^{-1} (Table 4.3) because plant yields were reduced (Table 4.1). In chicory plants, root and whole plant Cu accumulation showed these same trends of depression in yield and Cu uptake at solution Cu concentration of 20 mg Cu L^{-1} (Table 4.3). In contrast, tomato plants yields were reduced at solution Cu concentrations of 20 mg Cu L^{-1}, but root and whole plant Cu accumulation continued to increase as a consequence of the high root Cu concentrations (Table 4.3).

4.3.4 Copper distribution in plants

The proportions of total plant uptake that were retained by the root ranged from 33.7% in the plants grown with base solution (0.05 mg Cu L^{-1}) to 73% in chicory plants grown with 20 mg Cu L^{-1}. The corresponding values for tomato ranged from 28.9% to 79.2% (Fig. 4.1).

Table 4.1 Effect of Cu concentration in NFT solution on chicory and tomato plant growth. Dry biomass data are the means of three replicates. Significantly different means are labelled with different letters. Growth rate is expressed as the dry weights of shoots or roots of treated plants as a percentage of plants in 0.05 mg Cu L^{-1}.

Cu in culture solution (mg L^{-1})	Dry biomass (g $plant^{-1}$)		Growth rate (% of control)	
	Shoot	Root	Shoot	Root
Chicory				
0.05	5.96 a	1.98 a	100	100
5	5.80 a	1.93 a	97	97
10	4.51 b	1.57 b	76	79
20	2.43 c	0.90 c	41	45
P	0.01	0.01		
Tomato				
0.05	10.31 a	3.23 a	100	100
5	8.89 b	2.74 b	86	86
10	6.04 c	1.81 c	58	56
20	4.03 d	1.21 d	39	37
P	0.01	0.01		

Table 4.2 Effect of Cu concentration in NFT solution on shoot and root Cu concentrations of chicory and tomato plants. Data are the means of three replicates. Significantly different are labelled with different letters. Relative increase rate of Cu concentration is determined by expressing the concentrations of shoots and roots of treated relative to plants in 0.05 mg Cu L^{-1}.

Cu in culture solution (mg L^{-1})	Cu concentration (mg Cu kg^{-1} DM)		Relative increase rate	
	Shoot	Root	Shoot	Root
Chicory				
0.05	16.0 d	24.5 d	1	1
5	31.6 c	122.0 c	2.0	5.0
10	45.2 b	342.9 b	2.8	14.0
20	65.3 a	476.0 a	4.1	19.4
P	0.01	0.01		
Tomato				
0.05	13.1 d	16.9 d	1	1
5	23.9 c	182.3 c	1.8	10.8
10	40.2 b	397.7 b	3.1	23.5
20	55.1 a	707.7 a	4.2	41.7
P	0.01	0.01		

Table 4.3 Effect of Cu concentration in NFT solution on Cu accumulation and distribution in chicory and tomato plants. The data of shoot, root and total Cu accumulation are the means of three replicates. Significantly different means are labelled with different letters. Theoretical Cu accumulation was calculated as: DM (g) ÷ WUE (g DM L^{-1}) × Xylem sap Cu concentration (mg Cu L^{-1}), where WUE was based on typical values in the literature.

Cu in solution (mg L^{-1})	Actual Cu accumulation (mg Cu plant^{-1})			WUE[1] (g DM L^{-1})	Theoretical Cu accumulation (mg Cu shoot^{-1})
	shoot	root	total		
Chicory					
0.05	0.095 c	0.049 d	0.144 d	5~6.7[2]	0.626~0.834
5	0.183 ab	0.236 c	0.419 c		0.827~1.103
10	0.203 a	0.540 a	0.742 a		0.947~1.263
20	0.158 b	0.430 b	0.588 b		1.215~1.620
P	0.01	0.01	0.01		
Tomato					
0.05	0.134 c	0.055 d	0.189 d	2~8[3]	0.660~2.639
5	0.213 b	0.499 c	0.711 c		0.792~3.169
10	0.243 a	0.719 b	0.961 b		0.837~3.346
20	0.222 ab	0.857 a	1.079 a		1.068~4.274
P	0.01	0.01	0.01		

[1] WUE: water use efficiency (g DM L^{-1}).

[2] Santamaria et al. (1998). Data have been transferred from fresh weight (FW) basis to dry weight basis (DM) by assuming that DM = FW × 10%.

[3] Oertli et al. (1990).

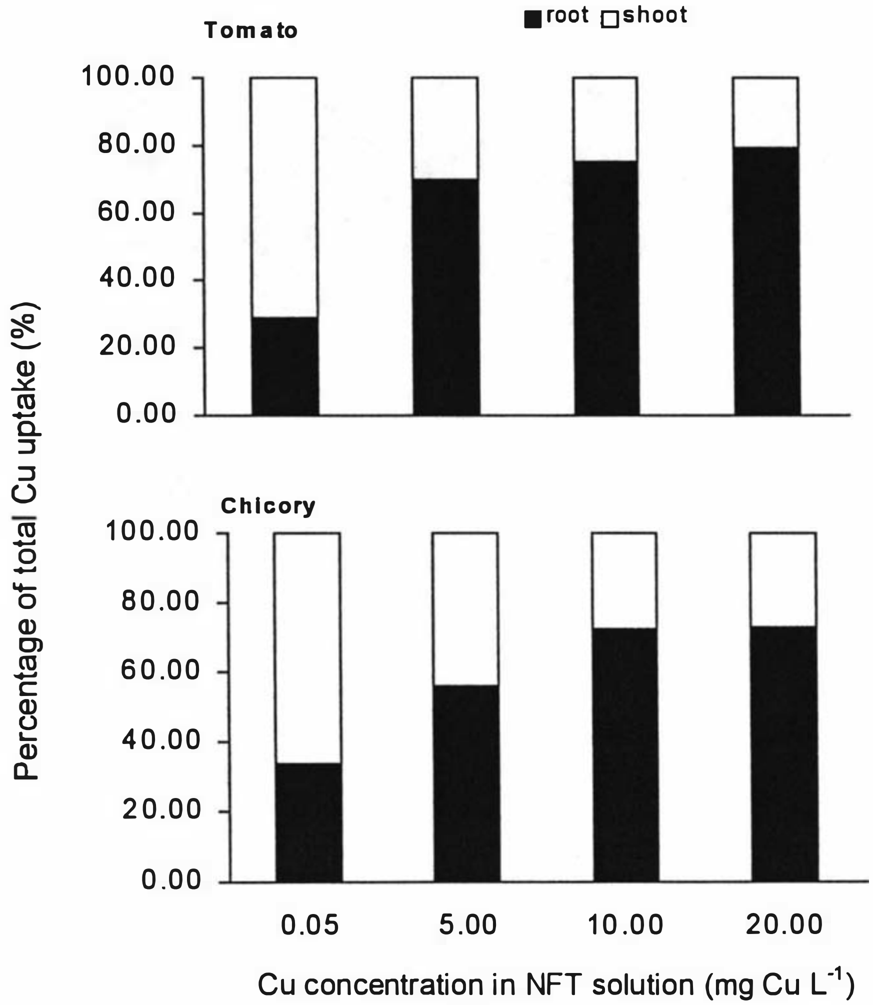

Fig. 4.1 Effect of Cu concentration in NFT solution on Cu distribution in chicory and tomato plants.

4.3.5 Cu in xylem sap

4.3.5.1 Total Cu concentration

Total Cu concentration in the xylem saps of chicory and tomato plants increased with increasing Cu concentration in the nutrient solution (Fig. 4.2). When Cu in the culture solution increased from 0.05 to 20 mg Cu L^{-1}, Cu concentrations in chicory and tomato xylem saps increased 4.8 fold (from 0.700 to 3.333 mg Cu L^{-1}) and 4.1 fold (from 0.512 to 2.121 mg Cu L^{-1}) respectively. At all Cu treatments, total Cu concentrations in chicory xylem sap were higher than in tomato xylem sap.

The rate of increase of root Cu concentrations was higher than that of xylem sap Cu concentrations in both chicory and tomato (Fig. 4.3A). Shoot Cu concentrations were curvilinearly related to xylem Cu concentrations for both chicory ($r^2 = 0.96$, $P < 0.01$) and tomato ($r^2 = 0.99$, $P < 0.001$) (Fig. 4.3 B).

4.3.5.2 Copper forms in xylem sap

More than 99.7% total Cu in the xylem saps of chicory and tomato was in a bound form (Fig. 4.4), based on estimates of free Cu^{2+} ion obtained with the Cu electrode.

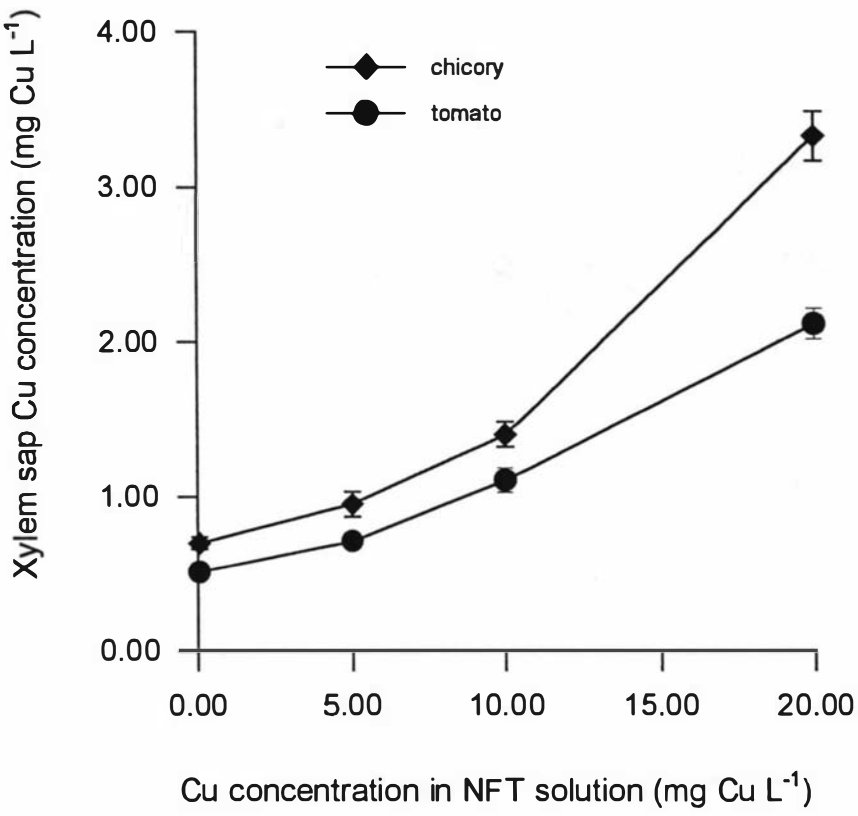

Fig. 4.2 Effect of Cu concentration in NFT solution on Cu concentration in xylem sap of chicory and tomato plants. Data shown are means ± S.E. (n = 3).

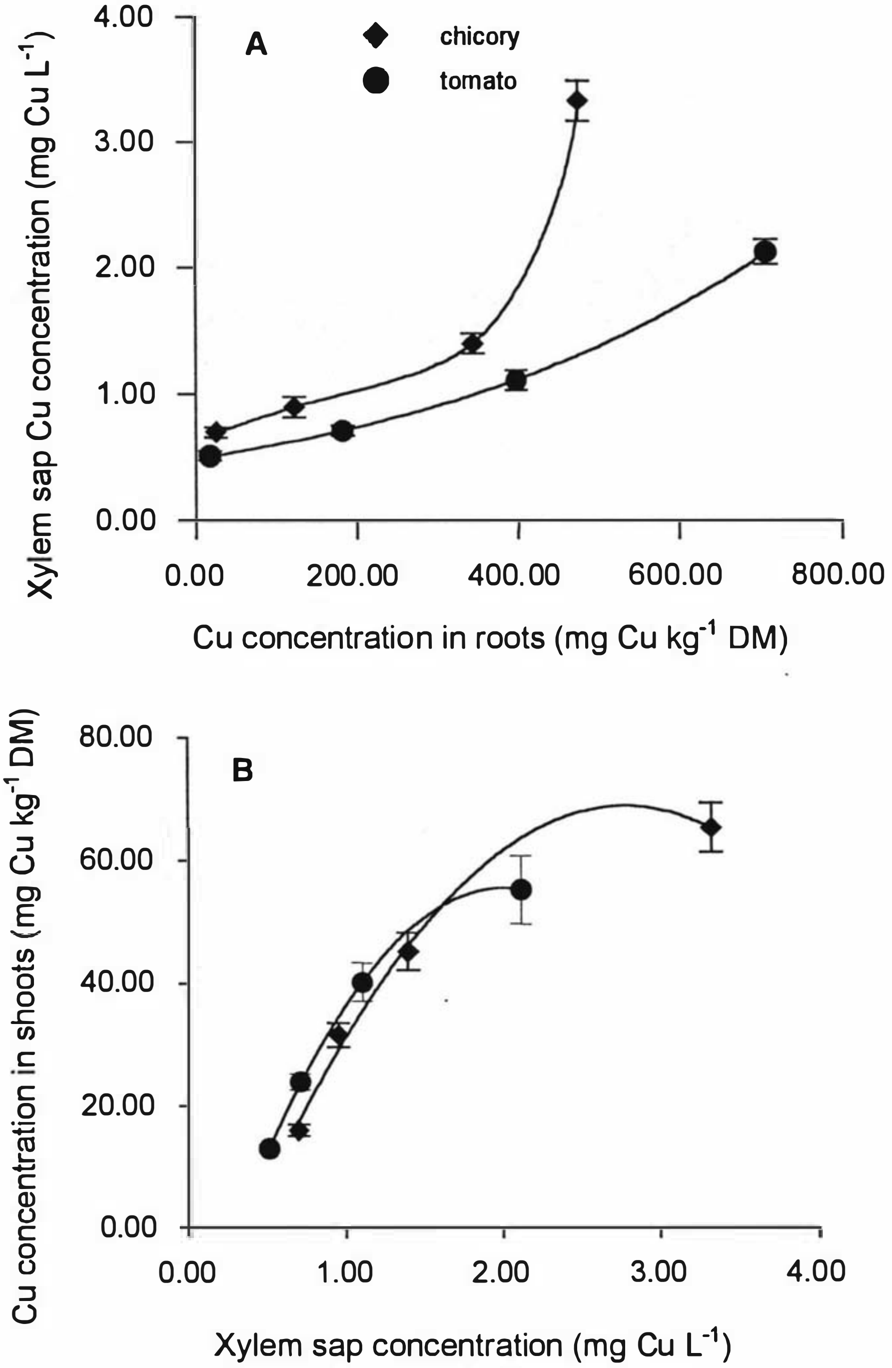

Fig. 4.3 Relationship between Cu concentrations in xylem saps and Cu concentrations in roots (A) and shoots (B) of tomato and chicory plants. Data shown are means ± S.E. (n = 3).

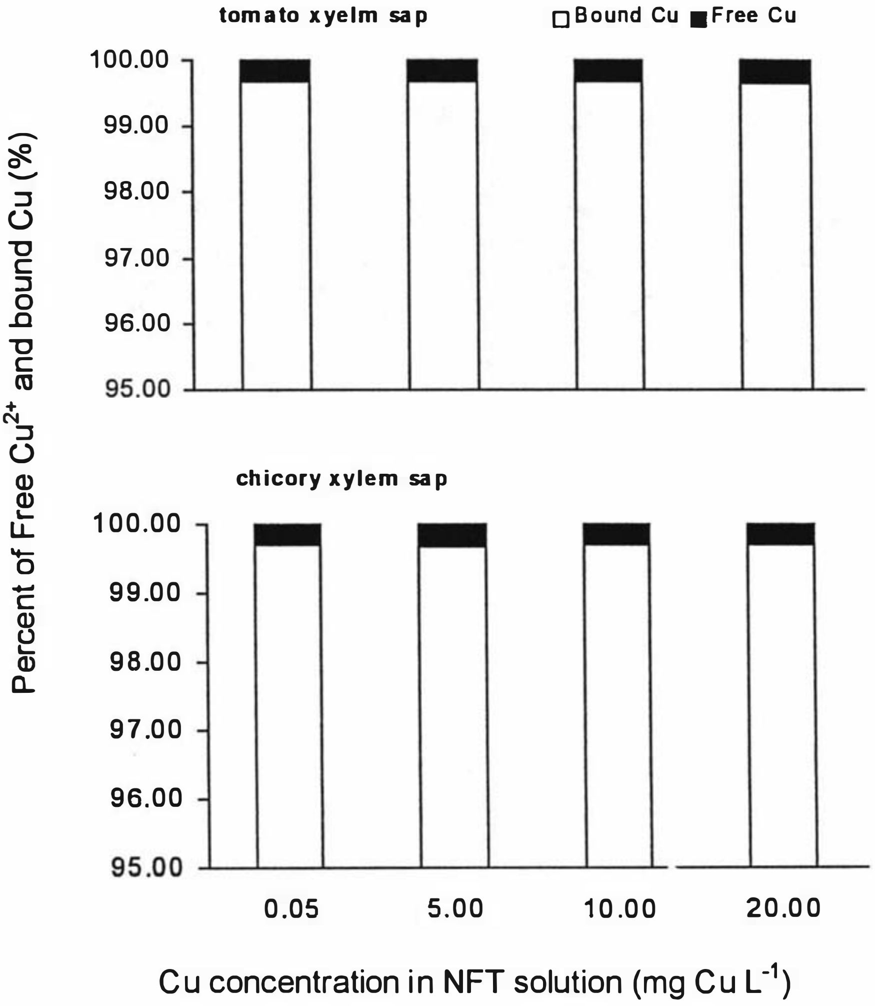

Fig. 4.4 Degree of Cu chelation in chicory and tomato xylem saps.

4.4 DISCUSSION

Growth inhibition and reduction of biomass production are general responses of higher plants to heavy metal toxicity (Baker and Walker, 1989; Woolhouse and Walker, 1981). Similarly, in the present study, significant depression of both shoot and root dry biomass production in Cu treated tomato and chicory plants was observed (Table 4.1), and this effect varied as a function of the Cu concentration in NFT solution. Punz and Sieghardt (1993) demonstrated that, in higher plants, root growth is particularly sensitive to the presence of metal toxins. In our study reported here, the data (Table 4.1) showed that the growth of the roots and shoots of tomato and chicory are equally sensitive to external Cu concentrations.

The concentration of Cu in roots is generally higher than that in shoots but the degree of difference varies markedly with the levels of Cu supply (Jarvis, 1978, 1980). With increasing concentrations of Cu in solution, the concentration of Cu in roots of most species increases much more rapidly than that of tops, for example, tobacco (Struckmeyer et al. 1969), red clover (Hill, 1973), ryegrass (Jarvis 1978, 1980). Our results (Table 4.2) support these previously published results.

Shoot and root Cu accumulation (Table 4.3), the products of dry matter production (Table 4.1) and Cu concentration (Table 4.2), are worth considering separately as shoot Cu represents the amount that can be harvested from chicory and tomato plants, and differential accumulation in roots may indicate the presence of a translocation limiting process. In both plants the amount of shoot-harvestable Cu increased up to solution Cu concentration of 10 mg Cu L^{-1}, and then decreased at 20 mg Cu L^{-1} because plant yields were reduced (Table 4.3). Root Cu accumulation in chicory plants showed the same trend as shoot Cu accumulation. In tomato plants, however, root Cu accumulation continued to increase as a consequence of the high root Cu concentrations (Table 4.3).

An important process limiting the movement of copper, as well as of other metals, to plant shoots, is accumulation in roots. The ability of roots to retain Cu against transport to shoots has also been demonstrated in many species, for example, Mediterranean trees (Arduini et al. 1996), orange (Brams and Fiskel, 1971), tobacco (Struckmeyer et al., 1969), and perennial ryegrass (Jarvis 1978, 1980). This root Cu accumulation was not found in tomato when Cu in rooting media is low (Maier and Cattanni, 1966), but when exposed to excess Cu, high amount of Cu was retained in roots (Mazhoudi et al. 1997; Ouariti et al. 1997). The proportion of the plant's Cu in roots varied greatly with Cu supply. Similar results were found in the present study, when chicory and tomato plants grown in control solution with 0.05 mg Cu L^{-1} (background Cu), only 33.7 and 29.3% were retained in roots by chicory and tomato plants, respectively. However, large proportions of uptake were retained by the roots and reached up to 73% of the total plant copper when the external Cu concentration reached 20 mg L^{-1} (Table 3, Fig. 4.1). In tomato plants, up to 89% of the total Cu uptake were retained by roots when exposed to 20 mg Cu L^{-1} (Fig. 4.1). This indicates that the number of Cu-binding sites on tomato roots must be greater, or Cu binding is stronger, than in chicory roots. The alternative explanation would be that more ligands for Cu capable of not only binding Cu but also facilitating its movement toward the xylem and transport in the xylem sap exist in chicory roots compared to tomato roots (Z. Rengel, person communication).

Because metals move acropetally in the xylem sap, it is reasonable to study chemical species and metal complexing within that system. However there is a lack of information on the complete chemical nature of xylem fluid, as well as possible metal forms at normal and toxic concentrations of heavy metals (White et al. 1981a, b). Most information on xylem solutes is fragmentary. It is very difficult to detect metal species directly *in situ*. Simulation models and theoretical calculations have been the main sources of our knowledge (Senden et al. 1992; Welch 1995). In the present study, the total Cu concentrations in chicory and tomato xylem saps were analysed. Total Cu concentration in xylem sap increased significantly with increasing Cu in the rooting media. However, the root system tended to bind more of the Cu, in effect, buffering the concentration of Cu in the xylem and limiting the supply of Cu to the shoot in both chicory and tomato plants (Table 2, Fig. 4.2). While the Cu supply varied by a factor of 400, the total Cu content of roots varied by a factor of 19.4 for chicory and 88.5 for tomato, but the total Cu concentration in xylem saps varied by only a factor of 4.8 and 4.1 respectively. As root-Cu concentrations increase, xylem sap Cu concentrations increase in an exponential manner (Fig. 4.3A). This exponential trend, particularly in chicory, may indicate weaker binding of Cu by roots as the amount of root bound Cu increases, and/or indicates that as xylem Cu concentrations increase, sites for binding Cu on the surface of xylem vessels become limited. Graham (1979) found similar relationships for sunflower xylem sap.

The total Cu concentration of shoots in tomato and chicory varied by a factor of 4.1 and 4.2 respectively, which was quite close to that of xylem saps. Moreover, a significant diminishing-returns-type relationship existed between xylem sap Cu concentrations and shoot Cu concentrations for both chicory ($r^2 = 0.96$, $P < 0.01$) and tomato ($r^2 = 0.99$, $P < 0.001$) plants (Fig. 4.3B). The greater accumulation of Cu in roots than shoots, and the relative differences in root, shoot and xylem sap Cu concentrations between chicory and tomato plants (shoot Cu: chicory > tomato; root Cu: tomato > chicory) suggest that Cu binding by roots or the inability of xylem to transport Cu are factors influencing Cu transport from roots to shoots (Jarvis, 1980; Jarvis and Whitehead, 1981). However, the diminishing-returns-type relationship between xylem Cu concentration and shoot Cu concentration for both species indicate that some other mechanisms are limiting greater Cu assimilation in shoots as the amount of Cu transported in xylem increased.

Nutrients move to shoots via xylem flow, so we can estimate the theoretical Cu accumulation by shoots from the product of plant water use efficiency (WUE, kg DM L^{-1}), shoot dry weight (DM, g) and xylem sap Cu concentration (mg Cu L^{-1}). The measured shoot Cu accumulation was much lower than the theoretical Cu accumulation values (Table 3). Possible explanations for the differences between measured and estimated shoot Cu accumulations are that plant water use is abnormally low or that Cu within the shoot can be recirculated to the roots via the phloem. This speculation is not unreasonable, because there is good evidence of Cu and other metal ions are transported in the phloem (Hocking, 1980; Pate 1976, 1980). For example, in tree tobacco plants, total amino acid concentration and Cu concentration in phloem sap are 38.2 and 10.9 times higher than those in xylem sap (Hocking, 1980). Recently, Köckenberger et al. (1997) used non-invasive nuclear magnetic resonance microimaging to demonstrate that water is internally circulated between phloem and xylem in castor bean seedlings. Furthermore, others have shown that nicotianamine (NA), a non-protein amino acid which is ubiquitous in higher plants (Budêšínský et al. 1980), is involved in the intra- and inter-cellular translocation of micronutrient metals after their absorption across the plasma membrane into the cytoplasm (Welch, 1995), and in long-distance translocation of metals in both xylem sap and phloem sap (Pich and Scholz, 1996; Schmidke and Stephan, 1995; Stephan and Scholz, 1993; Welch, 1995). In this study, measurements using a cupric-sensitive electrode showed that in both chicory and tomato xylem sap, more than 99.7% of all xylem sap Cu is in a complexed form. Graham (1979) found a similar result for sunflower xylem sap. Furthermore, Graham (1979) demonstrated that the complexes in xylem sap might be quite strong. However little is known about the chemical species of Cu in xylem sap. This is the subject of Chapter 5.

4.5 CONCLUSIONS

Root binding of Cu, limited Cu transport capacity in xylem, or poor shoot assimilation of xylem transported Cu, slow the increase in Cu translocation to shoots as roots are exposed to increased Cu concentrations. Higher shoot Cu concentrations in chicory compared to tomato were associated with higher xylem Cu concentrations. Unfortunately, as Cu loading in roots increases, shoot dry matter decreases leading to lower values of Cu accumulation in shoots. Theoretical estimations of total Cu uptake by shoots based on plant water use and xylem Cu concentrations are much higher than actual measured Cu accumulation in shoots and this suggests that counter-flow of Cu in phloem to roots may be a significant influence on Cu allocation between shoots and roots. Further investigations of Cu species in the xylem sap are required before research strategies can be designed to improve forage plant's ability to take up Cu.

CHAPTER 5

COPPER UPTAKE AND TRANSLOCATION IN CHICORY (*Cichorium intybus* L. cv Grasslands Puna) AND TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS GROWN IN NFT SYSTEM. II. THE ROLE OF NICOTIANAMINE AND HISTIDINE IN XYLEM SAP COPPER TRANSPORT

Publication arising from chapter:

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5.1 INTRODUCTION

As described in Chapter 4, higher shoot Cu concentrations in chicory compared to tomato were associated with higher xylem Cu concentrations, and almost all Cu in xylem saps was in a bound form. Investigations into the chemical species of Cu in the xylem sap are required before research strategies can be designed to improve the ability of forage plants to take up Cu.

A thorough understanding of Cu uptake and translocation to plant shoots will assist in solving the problem of primary Cu deficiency in grazing ruminants (Liao et al. 1999a, Chapter 7). Mechanisms involved in the translocation of micronutrients from their absorption sites in the roots to different organs of the shoot are only partly understood (Kochian, 1991; Loneragan, 1981; Pich and Scholz, 1996; Salisbury and Ross, 1992; Welch, 1995). In a recent review, McGrath et al. (1999) suggested that there are many rate-limiting steps along the route of metal translocation to shoots.

Firstly, *root to xylem transport*. The majority of elements taken up by plants from soil solution are transported at some point through the cytoplasm of root cells in order to bypass the impermeable casparian strip of the root endodermis. Once in the cytoplasm, metals can be excreted, transported towards the xylem, or shuttled into the vacuole. Significant quantities of metals are stored in the vacuoles of root cells, which effectively immobilises the metals (McGrath et al., 1999).

Secondly, almost nothing is known about the actual mechanisms involved in the *loading or unloading of metals into and out of xylem vessels* within the vascular tissues of higher plants (De Boer and Wegner, 1997; Kochian, 1991; Welch, 1995), in large part due to the practical difficulties involved in isolating xylem parenchyma cells for study (McGrath et al., 1999). These mechanisms are likely to provide an important rate-limiting step in metal transport to the shoots.

Thirdly, little is known about the *soluble forms of micronutrients in xylem sap* (Kochian, 1991; Welch, 1995). Once in the xylem, metal transport to shoots is facilitated by metals complexing with ligands (such as organic acids and amino acids) in the xylem sap, especially for metals such as Cu, as Cu^{2+} is highly toxic to plants. Because metals move acropetally via the xylem fluid, improved understanding of translocation requires study of the chemical speciation and metal complexing within that system. This was the objective of this Chapter.

It was found that, in four dicotyledonous plants, all of the Cu in xylem exudates was present in one or more negatively charged complexes possibly involving amino acids (Tiffin, 1972). Moreover, Graham (1979) showed that more than 99% of total Cu was in a complexed form in sunflower xylem sap. In the first part of this study (Chapter 4), we found similar results for chicory and tomato xylem saps.

The ionic and organic composition of the xylem sap is quite complex, consisting of a wide range of mineral ions (both macronutrients and micronutrients), along with organic acids, amino acids, and other organic ligands that can complex micronutrients and facilitate their movement to shoots (Kochian, 1991). The pH of the xylem sap appears to be regulated in the range of pH 5.2 to 6.0 (Clarkson et al., 1984; Clarkson and Hanson, 1986). The reduction potential of xylem sap is also fairly constant (Marschner, 1986; Morris and Swanson, 1980; Welch, 1986). The chemical-organic environment in the xylem will have a significant influence on metal-complex chemistry as it relates to translocation of micronutrients in the xylem.

Amino acids, which are generally present in higher concentrations than total Cu in xylem sap, can form very strong complexes with Cu (Hocking, 1980; Loneragan, 1981; Walker and Webb, 1981). However, because it is very difficult to detect these complexes directly *in situ*, simulation models and theoretical speciation calculations have been the main sources of our knowledge. White et al. (1981a, b) analysed the chemical composition of tomato and soybean stem xylem exudates and developed a computer model to calculate chemical species of metal in xylem sap. Their findings suggest that Cu is bound to several amino acids, mostly Asn and His in soybean exudate and His, Gln and Asn in tomato exudate.

Beneš et al. (1983) determined the stability constants of nicotianamine (NA) with metal ions, and found that the stability constants are between logK=8.8 (Mn) and logK=18.6 (Cu). Thus, NA could compete with Asn (logK=13.7), Gln (logK=13.4), His (logK=17.5) and other amino acids as a ligand for Cu (May et al. 1977). Complex formation with transition metals is one of the basic functions of NA (Scholz et al. 1988). Working with NA-less mutant and wild type tomatoes, Pich and Scholz (1996) concluded that NA is important in Cu transport in xylem sap of tomato.

The objective of the present study was to investigate the Cu species in xylem sap, in order to understand the mechanisms of copper translocation in chicory and tomato plants. A novel technique using the pH sensitive, Cu-binding signatures of organic ligands was used to distinguish the importance of various amino acids in xylem transport of Cu.

5.2 MATERIALS AND METHODS

5.2.1 Xylem sap collection

Xylem saps used in this study were collected from chicory (*Cichorium intybus* L. cv. Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv. Rondy) plants grown in a nutrient film technique system (NFT), with a range of solution Cu concentrations (0.05-20 mg Cu L^{-1}), as described in Chapter 4. Saps were stored at –70 °C until analysis.

5.2.2 Xylem sap analysis

The xylem sap pH was measured directly with a combination glass microelectrode (White et al. 1981b).

Ionic strength was determined by measuring the electrical conductivity of the xylem sap (Griffin and Jurinak, 1973).

Total Cu and free Cu^{2+} concentrations in xylem saps were measured as described in Chapter 4.

The separation and quantitative estimation of amino acids by high performance liquid chromatography (HPLC) was modified after Fierabracci et al. (1991) and Senden et al. (1992). A Water's HPLC system consisting of two 510 pumps, a 490 programmable UV/Vis detector and an auto-injector were used. A Waters C_{18} Pico-Tag column (15 cm × 3.9 mm I.D.) was used for all separations. The column temperature was controlled at 46 ± 1°C using a column oven. The sample injection volume was 20 μL, and an eluent gradient (Table 5.1) at a flow rate 1 mL min^{-1} was used. The wavelength was set at 254 nm.

Table 5.1 The operating eluent gradient. Eluent A: 0.14 M sodium acetate in water titrated to pH 6.5 ± 0.02 with glacial acetic acid, with the addition of 17.8 mL L^{-1} acetonitrile and 250 μL L^{-1} 10 mM EDTA. Eluent B: 9:8:3 (v/v) of acetonitrile - water - methanol.

Time (min)	Flow rate (mL min^{-1})	A%	B%
initial	1	100	0
6.75	1	97	3
12	1	94	6
15	1	91	9
25	1	66	34
31	1	66	34
34	1	0	100
44	1	0	100
45	1	100	0
50	1	100	0

5.2.3 Free Cu^{2+} concentration/pH titration

The stability of Cu-complexes containing carboxylic acid functional groups is normally sensitive to solution pH. The proportion of total soluble Cu present as Cu^{2+} ions as solution pH varies provides a 'signature' for the Cu-organic complex system. A cupric electrode (Orion 9629BN Ionplus™ Cupric Electrode) was used to measure the free Cu^{2+} concentration in amino acid solutions, plant saps and simulated saps, at varying solution pH values. Xylem sap (5 mL) collected from tomatoes grown in 20 mg Cu L^{-1} NFT treatment (Liao et al. 2000a, Chapter 4) was pipetted into a 10 mL vial. The pH of the solution was increased stepwise from about pH 3 to 6.5 and then decreased through the same pH range, while the solution Cu^{2+} and pH were monitored. Solutions of 0.1 *M* KOH and 0.1 *M* HNO_3 were used to adjust the pH. Since the total volume of these adjustments was less than 0.4% of the total solution volume, no corrections for dilution were made. Because of the small volume of chicory sap collected, 2.5 mL of sap was diluted with deionised water to a final volume of 5 mL and titrated as above.

Solutions of single amino acids and combinations of amino acids at concentrations measured in tomato xylem sap were prepared. $CuSO_4$ was added to give total solution Cu concentrations detected in the plant xylem saps. The ionic strength was controlled at around 60 *mM* (as detected in the xylem saps) using an ionic strength adjuster (5 *M* $NaNO_3$). The simulated sap solutions were titrated as described above. For simulated chicory saps, the free Cu^{2+} concentration vs pH titration experiment was conducted with two-fold diluted simulated chicory saps (amino acids concentrations added were half the concentrations detected in original sap, ionic strength was controlled at 30 *mM*).

To establish the significance the effect of competing cations on Cu complexation in xylem exudates, free Cu^{2+} concentrations, at xylem sap pH, were measured in the following simulated xylem sap solutions to examine possible competition of Zn with Cu for NA and His (Table 5.5). (1) 70 μM NA + 600 μM His; (2) 70 μM NA + 600 μM His + 33 μM Cu; (3) 70 μM NA + 600 μM His + 33 μM Cu + 6.5 μM Zn; (4) 70 μM NA + 600 μM His + 33 μM Cu + 33 μM Zn.

5.2.4 Data analysis

Linear regression and correlation analyses were used to test relations between selected response variables.

5.3 RESULTS

5.3.1 Amino acids in xylem sap

Glutamine (Gln) was the major amino acid present in tomato xylem sap, followed by Asn, His and γ-aminobutyric acid (Gaba) (Fig. 5.1A). The amino acids and amines which showed the greatest relative increase (2.4 to 7 fold) in concentration when exposed to high Cu concentration in the rooting media were: glutamine (Gln), histidine (His), asparagine (Asn), valine (Val), nicotianamine (NA), and proline (Pro) (Table 5.2).

More amino acids were detected in chicory xylem sap than in tomato xylem sap. Gln, again, was the major amino acid present in chicory xylem sap, followed by Asn, Pro, His, Gaba and Glu (Fig. 5.1B). The amino acids and amines which showed the greatest relative increase (4 to 6.4 fold) in concentrations when exposed to high Cu concentrations in the rooting media were: His, ϒ-aminobutyric acid (Gaba), glutamic acid (Glu), leucine (Leu), NA, and phenylalanine (Phe) (Table 5.2).

5.3.2 Free Cu^{2+} concentration/pH titration signatures

5.3.2.1 Tomato xylem sap and simulated saps

The free Cu^{2+} concentration/pH titration curves of tomato xylem sap, and key amino acids detected in xylem sap are presented in Fig. 5.2A. For a single complexing agent, the pattern of titration curve of 70 μM NA with 33 μM Cu was most similar to the curve of tomato xylem sap, especially for pH values below 5, closely followed by 600 μM His,

In order to investigate the relative importance of His and NA on Cu binding in the presence of other amino acids, titration experiments were conducted on the following solutions: **A**: simulated xylem sap (Gln + Asn + His + NA + Pro + Val); **B**: as A without His; **C**: as A without NA; **D**: tomato sap. The pattern of Cu^{2+} release from Cu-complexes of solution A is most similar to solution D (tomato xylem sap), followed by solutions B and C (Fig. 5.2B).

In tomato xylem sap, 460 μM Asn, 600 μM His, 70 μM NA, 718 μM Gln, 59 μM Val and 49 μM Pro could account for 88.2, 98.8, 99.6, 90.6, 62.3 and 61.3% complexing of Cu at 33 μM respectively (Table 5.3). Combination of the 6 key amino acids could account for 99.6% complexing of Cu at 33 μM, which is most similar to the measured level of Cu complexing in tomato xylem sap. Simulated xylem sap without His and simulated xylem sap without NA could account for 99.6 and 99.0% Cu complexing respectively.

Table 5.2 Effect of Cu concentration in rooting media on amino acid concentrations (μM) in tomato and chicory xylem sap. Data are presented as means ± S.E. (n=3).

Amino acid	Amino acid concentration (μM) in the xylem sap from		Relative increase[1]
	0.05 mg Cu L^{-1}	20 mg Cu L^{-1}	
Tomato sap			
Gln	133±21	719±83	5.4
His	110±9	600±39	5.5
Asn	100±7	460±41	4.6
Val	25±8	59±10	2.4
NA	13±1	70±9	5.4
Pro	7±2	49±6	7.0
Chicory sap			
His	180±5	932±88	5.2
Gaba	145±4	720±92	5.0
Glu	140±8	650±39	4.6
Leu	68±4	296±27	4.4
NA	20±9	127±9	6.4
Phe	23±9	92±10	4.0

1 Relative increase is calculated as (amino acid concentrations in the xylem sap of plants exposed to 20 mg Cu L^{-1})/(amino acid concentrations in the xylem sap of control plants).

Table 5.3 Free Cu^{2+} concentrations in simulated saps and tomato xylem sap from high Cu treatment (20 mg Cu L^{-1}) at pH 5.60. Free Cu^{2+} concentration data are presented as means ± S.E. (n=3).

Titration solution	Total Cu ($\mu g\ L^{-1}$)	Free Cu^{2+} ($\mu g\ L^{-1}$)	Free Cu^{2+} as % of total Cu
460 µM Asn	2121	250 ± 50	11.8
600 µM His	2121	26 ± 1	1.2
70 µM NA	2121	9 ± 1	0.4
718 µM Gln	2121	200 ± 23	9.4
59 µM Val	2121	800 ± 136	37.7
49 µM Pro	2121	820 ± 128	38.7
Asn+His+NA+Gln+Val+Pro	2121	9 ± 1	0.4
Asn+NA+Gln+Val+Pro	2121	9 ± 1	0.4
Asn+His+Gln+Val+Pro	2121	21 ± 2	1.0
Tomato sap	2121	7 ± 1	0.3

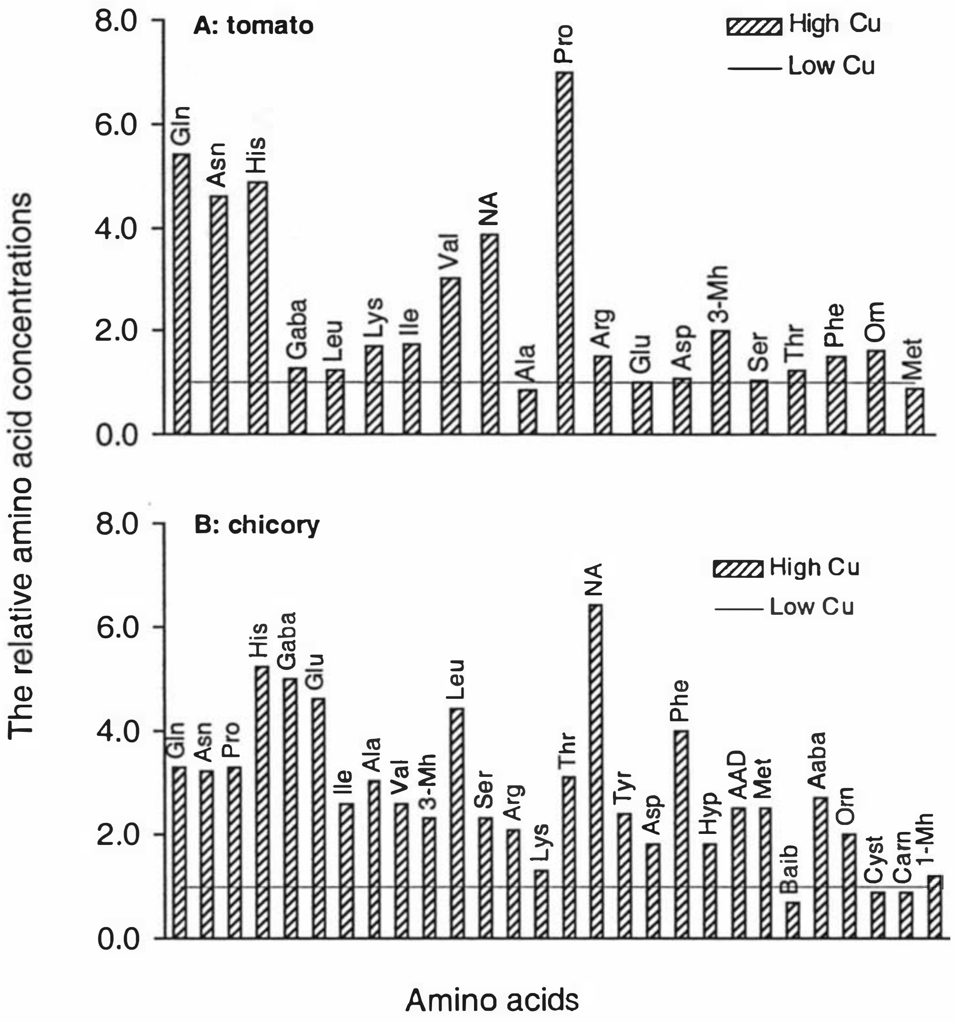

Fig. 5.1 Relative xylem amino acid concentrations in plants exposed to 20 mg Cu L^{-1} in the rooting media compared to amino acid concentrations in the xylem sap of control plants (0.05 mg Cu L^{-1}). Amino acids are listed from left to right in the order of decreasing abundance in the xylem saps. Only those amino acids present at ≥ 1 μM in any sample are shown. A: tomato xylem sap; B: chicory xylem sap.

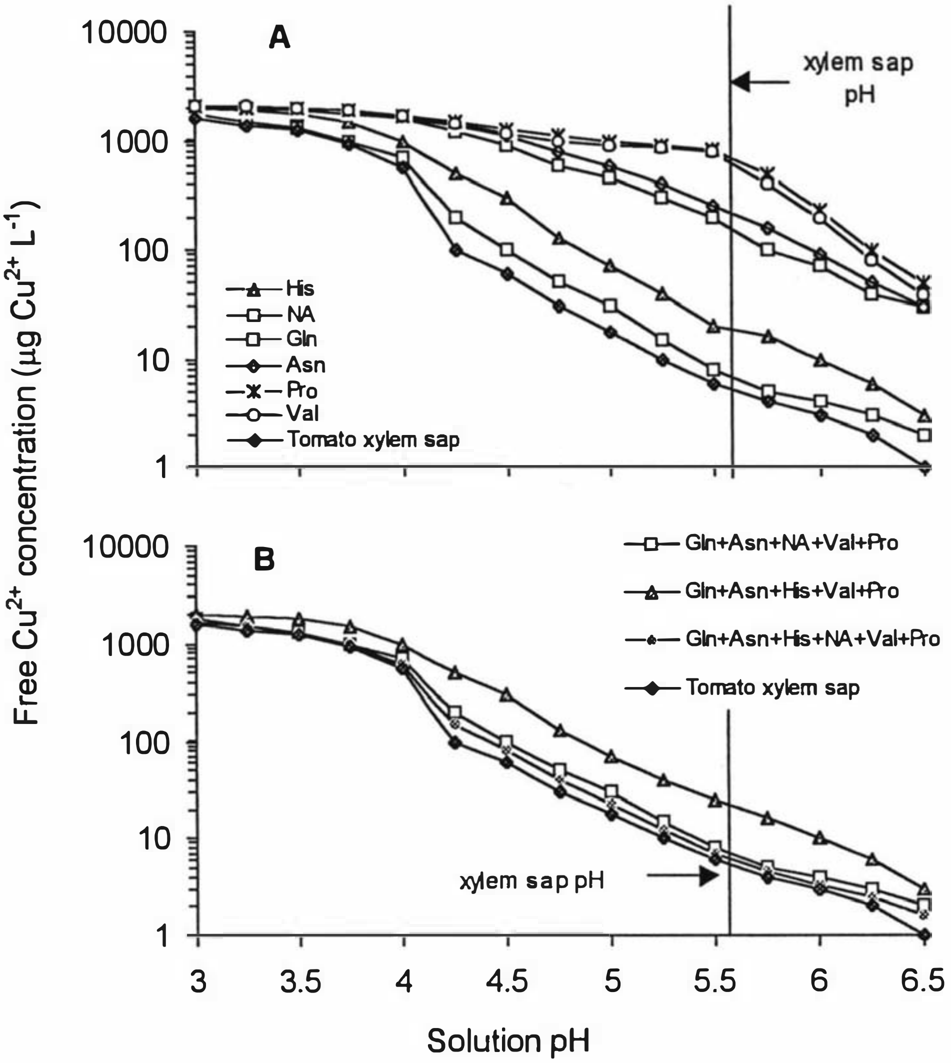

Fig. 5.2 Free Cu^{2+} concentration vs pH titration curves of simulated saps and tomato xylem sap. A: simulated saps with single amino acid; B: Simulated saps with combination of amino acids.

5.3.2.2 Chicory xylem sap and simulated saps

For a single complexing agent, the curve of 64 μM NA with 26 μM Cu was most similar, closely followed by 466 μM His, to the curve of chicory xylem sap, especially for pH values below 5 (Fig. 5.3A).

Titration experiments were conducted with the following solutions: **A**: simulated xylem sap (His + Gaba + Glu + Leu + NA + Phe); **B**: as **A** without His; **C**: as **A** without NA; **D**: chicory sap. The pattern of Cu^{2+} release from Cu-complexes of solution A is most similar to solution D (chicory xylem sap), followed by solutions B and C (Fig. 5.3B).

At pH 5.6 (xylem sap pH), 466 μM His, 360 μM Gaba, 325 μM Glu, 148 μM Leu, 64 μM NA and 46 μM Phe could account for 98.9, 64.0, 55.0, 57.3, 99.6 and 58.0% complexing of Cu at 26 μM respectively. Combination of the 6 key amino acids could account for 99.6% complexing of Cu at 26 μM, which is most similar to the degree of Cu complexing measured in chicory xylem sap. Simulated xylem sap without His and simulated xylem sap without could account for 99.6 and 98.9% Cu complexing respectively (Table 5.4).

5.3.3 Effect of competing cations on Cu complexation

The addition of 6.5 and 33 μM Zn to simulated sap NA and His solutions had no significant effect on free Cu^{2+} concentrations (Table 5.5), 99.5% of the Cu remained complexed to NA and His.

Table 5.4 Free Cu^{2+} concentrations in simulated saps and chicory xylem sap (2-fold diluted) from high Cu treatment (20 mg Cu L^{-1}) at pH 5.60. Free Cu^{2+} concentration data are present as means ± S.E. (n=3).

Titration solution	Total Cu ($\mu g\ L^{-1}$)	Free Cu^{2+} ($\mu g\ L^{-1}$)	Free Cu^{2+} as % of total Cu
466 μM His	1667	19 ± 2	1.1
360 μM Gaba	1667	600 ± 30	36.0
325 μM Glu	1667	750 ± 80	45.0
148 μM Leu	1667	712 ± 23	42.7
64 μm*M* NA	1667	7 ± 1	0.4
46 μm*M* Phe	1667	700 ± 66	41.9
His+Gaba+Glu+Leu+NA+Phe	1667	7 ± 1	0.4
Gaba+Glu+Leu+NA+Phe	1667	7 ± 1	0.4
His+Gaba+Glu+Leu+Phe	1667	18 ± 1	1.1
Chicory sap	1667	5 ± 1	0.3

Table 5.5 Effect of the presence of competing cation Zn^{2+} on free Cu^{2+} concentrations in simulated xylem saps at pH 5.60. Free Cu^{2+} concentration data are present as means ± S.E. (n=3).

Titration solution	Total Cu ($\mu g\ L^{-1}$)	Free Cu^{2+} ($\mu g\ L^{-1}$)
70 μM NA + 600 μM His	0	-
70 μM NA + 600 μM His	2121	8.7±0.6
70 μM NA + 600 μM His + 6.5μM Zn	2121	9.0±0.9
70 μM NA + 600 μM His + 33μM Zn	2121	9.2±0.8

5.3.4 Effect of Cu treatment on xylem sap His and NA concentrations

Both His and NA concentrations in chicory and tomato xylem saps increased with increasing Cu concentrations in NFT solutions (Fig.5.4). These results indicate that the synthesis of His and NA in plants was induced indirectly by increasing Cu concentrations in rooting media.

5.3.5 The relationships between Cu and His, NA concentrations in xylem saps of chicory and tomato

Regression analyses showed that total Cu concentrations in xylem saps were strongly linearly related to concentrations of His ($y = 0.2840x + 0.0026$, $r^2 = 0.98$, d.f. = 23, $p < 0.001$) and NA ($y = 0.0385x + 0.0021$, $r^2 = 0.94$, d.f. = 23, $P < 0.001$) in xylem saps of chicory and tomato (Fig.5.5).

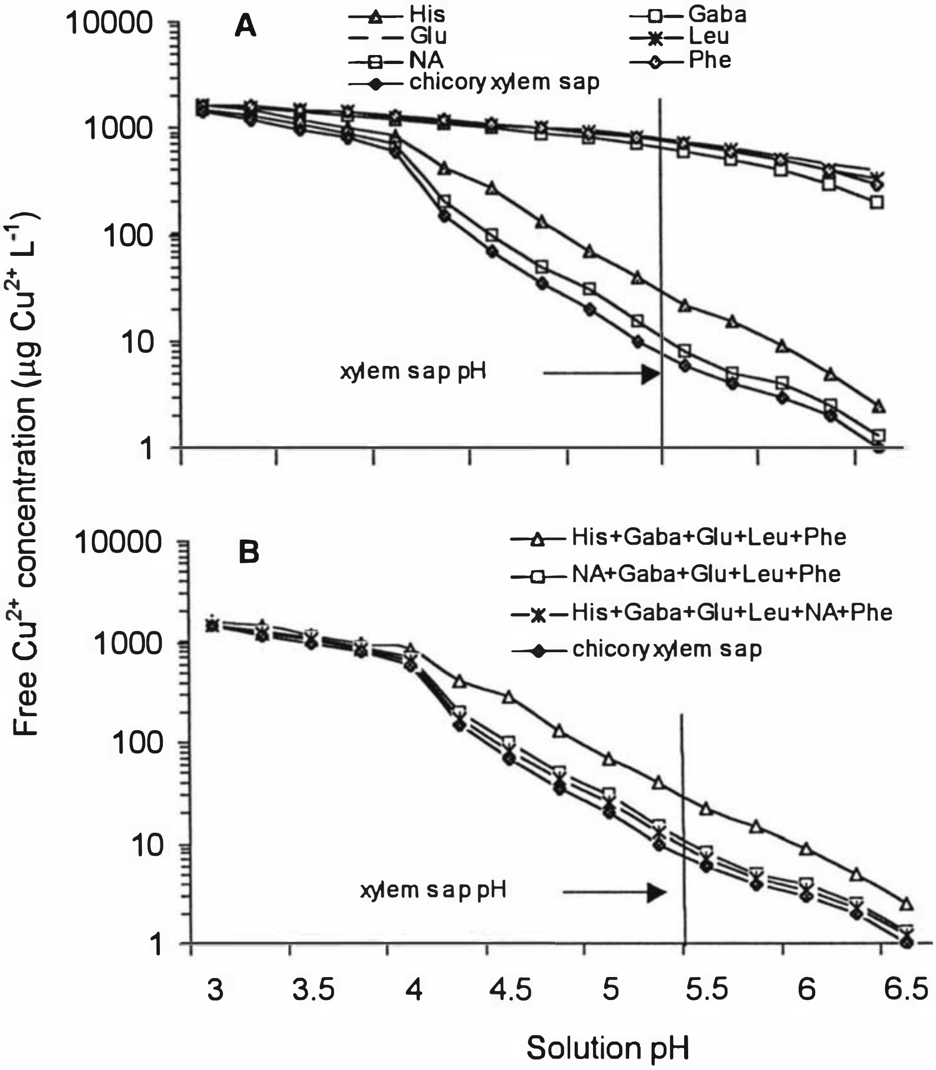

Fig. 5.3 Free Cu^{2+} concentration vs pH titration curves of simulated saps and chicory xylem sap (2-fold diluted). A: simulated saps with single amino acid; B: Simulated saps with combination of amino acids.

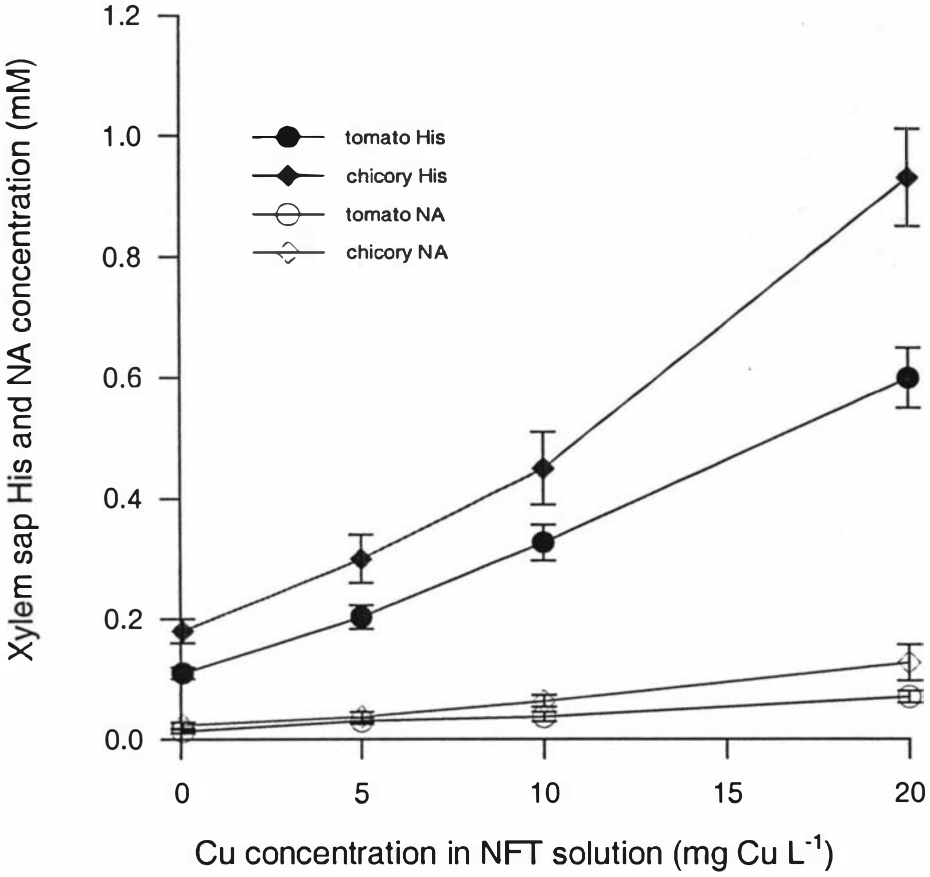

Fig. 5.4 Effect of Cu concentration in NFT solutions on xylem sap His and NA concentrations. Data shown are means of three replicates. The vertical bars indicate the S.E (n = 3).

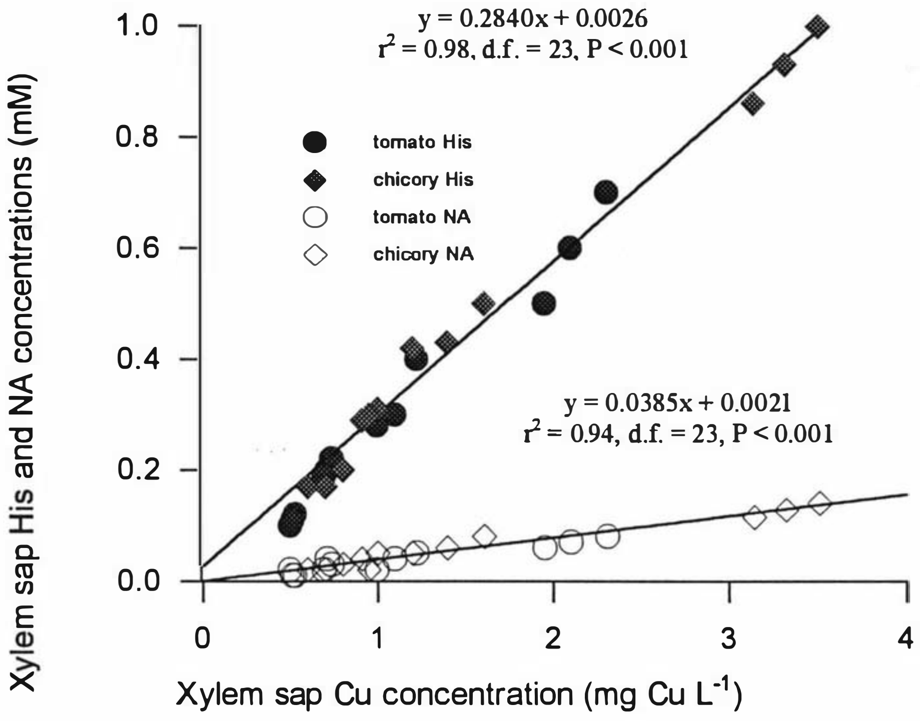

Fig. 5.5 Relationship between Cu and His and NA concentrations in xylem sap of chicory and tomato.

5.4 DISCUSSION

As reported in Chapter 4, nutrient solution Cu concentrations influenced the total Cu concentration in xylem saps of tomato and chicory plants. Values from the literature for the concentration Cu and other solutes in xylem sap are difficult to compare because they were determined under vastly different experimental conditions. Collection times, plant ages, and growth media are rarely the same. For example, Cu concentrations in xylem saps have been measured for several plant species, including tomato, soybean and sunflower, and ranged from 0.6 to 20 μM, depending on plant species, plant age, time of sampling and Cu treatments (Graham, 1979; Pich and Scholz, 1996; White et al. 1981b).

In the present study, the xylem saps were collected for only 1.5 hours after plant excision and are considered to provide information that approximates the *in vivo* system (Senden et al. 1992; White et al. 1981b). The use of a cupric-sensitive electrode to measure free Cu^{2+} concentration in tomato xylem sap has permitted quantification of the degree of chelation of Cu within the system. The results presented herein demonstrate that most of Cu was transported as complexes in the xylem sap, less than 0.3% of Cu was in the form of free Cu^{2+} in tomato and chicory sap (Table 5.3, 5.4). Graham (1979) found similar results for sunflower xylem sap.

Major amino acids in xylem exudates of tomato and chicory plants were separated by reversed phase high performance liquid chromatography (RP-HPLC) and quantified by UV detection. This method offers fast and inexpensive amino acid analysis (Fekkes, 1996; Fierabracci et al. 1991; Krämer et al. 1996; Senden et al. 1992). The major amino acids in xylem sap are usually species dependent (Pate, 1976) and their concentrations are influenced by many factors (Pate, 1976). In the present study, we found that the total amino acid concentrations in chicory xylem sap were higher than in tomato xylem sap. White and coworkers found that the total amino acid concentrations in soybean sap were higher than in tomato sap (White et al. 1981b). Asp and Glu, and particularly the amides Asn and Gln, seem to be consistently present in high concentrations (Pate, 1976).

Nicotianamine (NA), a phytosiderophore, was detected in both tomato and chicory xylem saps (Fig.5.1). The widespread occurrence of NA in higher plants has been noted by others (Budêšínský et al. 1980; Noma et al. 1971; Noma and Noguchi, 1976). It appears that the occurrence of NA in chicory has not been previously reported.

When exposed to high Cu concentrations in the rooting media, relative increases in the concentration of certain amino acids and amides are greater than others. This induced response is species dependent (Fig.5.1). Increased production of a free amino acid in plants as a specific and proportional response to Cu treatment has not to our knowledge been reported previously. Krämer et al. (1996), however, have successful detected increased free His concentrations in the xylem sap of the Ni hyperaccumulator (*Alyssum lesbiacum*) in response to Ni treatments. They subsequently identified His as the major Ni chelator.

The pH-sensitive Cu-binding 'signatures' of amino acids showing the largest induced response to increased Cu in rooting media were studied. NA probably plays a most important role in Cu binding in both chicory and tomato xylem saps (Fig. 5.2, 5.3). In the absence of NA, His would likely play a very important role in Cu binding (Table 5.3, 5.4). The roles of Gln and Asn were negligible in the presence of NA and His (Fig. 5.2). Although for chicory xylem sap, the pH-sensitive Cu binding 'signatures' study was conducted on diluted xylem sap, it is not unreasonable to suggest the same trends would exist in undiluted xylem saps.

Cations, especially heavy-metals Co, Fe, Mn, Ni and Zn, may affect Cu speciation. Nickel has the second highest stability constant with NA which is about 2.5 log units lower than Cu with NA. Competition from Ni would be negligible because Ni concentrations in xylem sap are extremely low ($<5\ \mu g\ L^{-1}$). Zinc has the third highest stability constant with NA (14.7-15.4), but it is still 3-4 log units lower than Cu with NA (18.6) (Stephan et al., 1996). Although the concentrations of Zn and other competing cations were not determined in the present study because of the limited xylem sap volume. Xylem sap Zn concentrations, in tomato plants receiving normal Zn treatments, were reported in the range from 6.5 *μM* (White et al., 1981b) to 33 *μM* (Krijger et al., 1999). The results showed that the presence of 6.5 and 33 *μM* Zn had no significant effect on Cu complexation by 70 *μM* NA and 600 *μM* His (Table 5.5). This is probably due to (1) Zn's lower stability constant with NA and His compared to Cu (May et al., 1977; Stephan et al., 1996) and (2) Zn is likely bound primarily to citric acid and malic acid (Welch, 1995; White et al., 1981a).

In a chemical speciation model, White et al. (1981a) indicated that Cu would be bound primarily to Asn, Gln and His in tomato xylem sap. Since then, NA was identified and quantified in tomato xylem sap and suggested as a possible Cu translocator in tomato xylem sap in 1994 (Pich et al. 1994; Pich and Scholz, 1996; Walker and Welch, 1986). In a recent review, Welch (1995) suggested that the metal speciation model of White and co-workers' was not a good model for xylem sap because they did not determine the concentrations of other phytosiderophores, such as NA, in their xylem sap samples. For this reason, their predicted forms of micronutrient metals in their samples may not be accurate.

Our results show that when phytosiderophores, such as NA are present, they bind micronutrient metals more strongly than the organic acids included in White and co-workers' xylem sap metal speciation model (White et al., 1981a). Our results (Fig. 5.2, 5.3) showed that the roles of Gln (at 718 μM) and Asn (at 460 μM) were negligible in the presence of 70 μM NA and 600 μM His in simulated xylem sap. NA probably plays the most important role in complexing of Cu in both chicory and tomato xylem saps.

In the present study, other phytosiderophores, such as mugineic acid, have not been analysed. Sugiura and Nomoto (1984) have found that, in rice xylem sap, mugineic acid formed a strong complex with Cu (logK=18.3). For Cu-binding, it could compete efficiently with NA. However, working with NA-less mutant and wild type tomatoes, Pich and Scholz (1996) concluded that, given the high stability constant of NA-Cu-complex (logK=18.6), NA was likely to be the dominant Cu complexing ligand. The NA-less mutants became Cu deficient.

Moreover, according to theoretical calculations made by Stephan et al. (1996) the NA-Cu-complex in the xylem at a pH of approximately 6.0 is regarded as very stable. NA is perhaps not the sole Cu translocator in tomato, other phytosiderophores (such as mugineic acid) and amino acids (such as His) may compete with NA to bind Cu.

Our results (Table 5.3, 5.4) indicate that, at normal xylem sap concentration, His alone can complex more than 98.8 % Cu in tomato xylem sap and 98.9% Cu in chicory sap at pH 5.6 (xylem sap pH). Suprisingly, Pich and Scholz (1996) found that Cu transport was inefficient in the xylem sap of NA-less mutant tomato plants. According to our results (Fig. 5.2, 5.3; Table 5.3, 5.4), Cu transport would be efficient in tomato xylem sap even in the absence of NA, provided His was present at about 600 μM. These contrasting results may arise from the fact that different tomato varieties were used as experimental material, or His concentrations in the xylem sap of NA-less mutant might have been low as well.

Additional analyses of xylem saps from chicory and tomato plants grown at all NFT solution copper concentrations showed consistent increases in NA and His as NFT solution Cu concentration increased (Fig.5.4). Interestingly in both chicory and tomato xylem sap Cu concentrations increased in similar direct proportion to increases in sap NA and His concentrations (Fig.5.5). Sap NA and His concentrations were always high enough to account for all complexed Cu in xylem sap. These results are the first reported indication that increased external Cu concentration will induce synthesis of His and NA.

5.5 CONCLUSIONS

More than 99.7% of all Cu transported in xylem sap of tomato and chicory is in a bound form. Increases in concentrations of Cu in rooting media induces selective synthesis of certain amino acid which include NA, His, Asn and Gln which have high stability constants with Cu. NA and His have the highest binding constants for Cu, and their concentration increases correlated best with increasing Cu. Copper complexing studies with xylem sap and amino acid solutions indicate that the concentrations of NA and His in chicory and tomato xylem saps can account for all the bound Cu carried in the sap. Further studies should investigate whether Cu transport from roots to shoots is greater in plants with naturally high NA or His concentrations in xylem sap. Such plants may be exploited in providing adequate Cu in forage grazed by ruminants. The role of mugineic acid in metal transport in a wider range of plants also needs further investigation.

CHAPTER 6

AN INVESTIGATION OF COPPER UPTAKE AND REDISTRIBUTION WITHIN TOMATO (*Lycopersicon esculentum* Mill. cv Rondy) PLANTS

6.1 INTRODUCTION

In Chapter 4, Cu accumulation in chicory and tomato plants was found to be significantly lower than theoretical shoot Cu accumulation (calculated from xylem sap Cu concentration and plant water use). It was suggested that some of xylem transported Cu was internally circulated between xylem and phloem (Liao et al., 2000a, Chapter 4). This speculation is reasonable, because recent work has demonstrated that Fe, Na, K, and Mg in plants are circulated between root and shoot (Stephan and Scholz, 1993; Welch, 1995).

The current knowledge of the translocation of micronutrients such as Fe, Mn, Zn and Cu in the phloem is still comparatively limited. Some early work provides evidence that heavy metals are translocated from mature organs to young actively growing tissues via the phloem (Brown et al., 1965; Butovac and Wittwer, 1957; Hocking and Pate, 1978; Nable and Loneragan, 1984; Pate and Hocking, 1978; Piper, 1942; Piper and Walkley, 1943; Vose, 1963; Williams, 1955; Wood and Womersley, 1964). The concentrations of various elements in the phloem sap have been measured in some species (Table 6.1) and from these results it is evident that micronutrient concentration in the phloem sap depends on species, organ, and the ontogenetic state of the plant (Stephan and Scholz, 1993).

Recently, it has become apparent that in *Ricinus* the redistribution of Na, K and Mg occurs continuously between the root and shoot during the lifetime of the plant (Jeschke and Pate, 1991a,b; Jeschke et al., 1991). There is also similar evidence of circulation for iron (Pierson et al., 1986; Van Der Mark et al., 1982), rubidium and strontium (Kuppelwieser and Feller, 1990). So far, most exchange and circulation experiments have been made with alkali and alkaline earth metals and up to now, few results are available for heavy metals.

Table 6.1 Concentrations of Fe, Mn, Zn and Cu in the phloem sap of different species (μM).

Species	Source of sap	Fe	Mn	Zn	Cu	Reference
Arenga saccharifera	Inflorescence	71.0	18.2	45.9	31.5	1
Yucca flaccida	Inflorescent stalk	25.1	9.1	32.1	6.3	2
Lupinus albus	Fruit bleeding	175	25.5	88.7	6.3	3
Lupinus albus	Fruit bleeding	62.7-223.8	36.4-65.5	10.7-93.3	12.6-20.5	4
Lupinus angustifolius	Fruit bleeding	125.3	10.9	84.1	3.1	5
Nicotiana glauca	Stem incision	168.3	15.8	243.2	18.9	6
Ricinus communis	Stem incision	20.0-33.0				7

References: 1—Tammes (1958), 2—Tammes and Van Die (1964), 3—Pate (1975), 4—Pate and Hocking (1978), 5—Pate (1975), 6—Hocking (1980), 7—Maas et al. (1988).

The forms of micronutrients in phloem are far from clear. Micronutrients must be present as metal complexes in phloem sap if they are to move freely in the phloem stream because of the high pH and high phosphate level (eg. 14 *mM*) of phloem sap (ie. at a pH greater than 8, these metals in their free form would precipitate as mixed oxides, hydroxides, and phosphates) (Welch, 1995). Iron was reported to co-transport with deoxy-mugineic acid (Mori et al., 1991), and nicotianamine (Scholz, 1989; Stephan and Scholz, 1993). Very little is known about the forms of Mn and Zn in phloem sap and almost nothing is known about Cu and Ni forms in phloem sap (Kochian, 1991; Tiffin, 1972). Copper is classified as having variable mobility in phloem sap (Welch, 1986; Loneragan, 1981). The alkaline pH of phloem sap and the high levels of nitrogenous compounds present indicate that Cu is most likely to be bound to organic compounds.

It has been shown that the non-proteinaceous amino acid nicotianamine (NA), which is ubiquitous in the plant kingdom (Budêšínský et al., 1980; Noma and Noguchi, 1976; Procházka and Scholz, 1984; Rudolph et al., 1985), could fulfil the role of a micronutrient (Fe, Mn, Zn, Ni and Cu) chelator within the symplast and in the phloem (Pich and Scholz, 1991; Schmidke and Stephan, 1995; Scholz et al., 1988; Schreiber, 1986; Stephan et al. 1994, 1996; Stephan and Scholz, 1993). Logarithmic complex formation constants for NA complexed with Cu, Zn, Fe, and Mn range from 18.6 to 8.8 (Anderegg and Ripperger, 1989; Beneš et al., 1983) (Table 6.2). They are considerably higher than those of most other components capable of complexing Cu present in the phloem sap. However to date no one has successfully detected or separated the complexes between NA and micronutrients under physiological conditions.

Table 6.2 Stability constants ($\log K_{MeNA}^{-}$) of nicotianamine (NA) with various divalent transition metal ions (from Stephan et al., 1996).

Mn(II)	Fe(II)	Co(II)	Zn(II)	Ni(II)	Cu(II)	References
8.8	12.1	14.8	14.7	16.1	18.6	Beneš et al. (1983)
	12.8		15.4			Anderegg and Ripperger, (1989)

Hocking (1980) has shown that the concentration of complexed Cu in the phloem sap can exceed that of xylem sap by a factor of 10. It is expected that transport of Cu, mainly complexed with NA or histidine (His), in both xylem and phloem influences the distribution of Cu in chicory and tomato plants (Liao et al., 2000a,b, Chapters 4 and 5). Few studies have been conducted to study the xylem-phloem circulation of micronutrients. A successful example is the use of the split-root technique to investigate the movement of Cd and water in wheat plants. Kirkham (1980) found that Cd was taken up by roots exposed to Cd, transported to the shoots, and then transported down the plant to roots not exposed to Cd. It is unknown how relative concentrations of Cu chelators in xylem and phloem, and the respective phloem and xylem flow rates, influence Cu distribution in plants.

Water transpiration rates have been shown to have a large effect on macronutrient translocation under conditions of high macronutrient medium supply (Marschner, 1986). There is no reason to believe that this relationship should not also hold for micronutrient translocation rates in xylem sap (Welch, 1995).

6.1.1 Hypothesis

Copper concentrations in plant shoots are influenced by the variation in rates of phloem and xylem Cu transport.

Such a hypothesis may offer an explanation of why slow growing plants in low fertility and hot environments, where xylem transport rates are expected to be high relative to phloem flow rates, may show Cu hyperaccumulation (such as *H. katangense* growing over Cu ore deposits in Zaïre). When grown rapidly under high-fertility greenhouse conditions, however, less Cu may accumulate in shoots, as relative phloem transport may be higher.

6.1.2 Objective

The objective of this study was twofold. Firstly, to test the simple hypothesis that some of the Cu transported to shoots by xylem sap is transformed to phloem, and recirculated to roots via phloem mass flow. Secondly, to investigate the distribution of Cu in the plant due to changes in xylem and phloem flow rates caused by increased evapotranspiration and more or less photosynthate production.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design

To test the hypothesis that there is significant Cu transport in phloem, tomato plants with split-root systems were grown in hydroponic culture in which one half of the root system was exposed to high Cu concentrations (Fig. 6.1). Redistribution of Cu to the non-Cu-treated half of the root system would indicate phloem transport.

To examine the influence of relative changes in phloem and xylem Cu transport rates on shoot Cu uptake, differences in:

- relative humidity,

- light intensity
- nutrient solution concentration (solution fertility)

were imposed on replicates of the tomato plants having split-root systems (Fig. 6.1). These treatments were expected to influence the water use of the tomato plants and therefore the relative rate of xylem and phloem transport.

6.2.2 Plant culture and experimental treatments

Tomato (*Lycopersicon esculentum* Mill. Rondy) seeds were germinated in 1:1 bark and pumice medium. After two weeks, seedlings of even size were carefully washed with deionised water, the roots carefully divided into two equal parts, and the plants transformed into a split-root system (Fig. 6.1).

The main treatments imposed on the plants were:

Relatively humidity (RH)

1) Low RH (45±5%) — was achieved by pumping the air through 2 kg silica gel and then into the plant growth box (Fig. 6.1). The silica gel was replaced every day.
2) High RH (90±5%) — was achieved by pumping the air through distilled water and then into the plant growth box.

Light intensity

1) Low light intensity — was obtained by growing plants under 50% light cut-off standard shade cloth.
2) High light intensity — no shading was applied (Fig. 6.1).

Nutrient concentration

1) High nutrient concentration — a half-strength Hoagland nutrient solution (68 mg L^{-1} KH_2PO_4, 253 mg L^{-1} KNO_3, 590 mg L^{-1} $Ca(NO_3)_2.4H_2O$, 123 mg L^{-1} $MgSO_4.7H_2O$, 11 mg L^{-1} FeEDTA, 613 μg L^{-1} H_3BO_3, 1690 μg L^{-1} MnSO4.H2O, 546 μg L^{-1} $ZnSO_4.7H_2O$, 13 μg L^{-1} $NiCl_2$, 123 μg L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$, 195 μg L^{-1} $CuSO_4.5H_2O$, pH 5.5.
2) Low nutrient concentration — 10 times dilution of high nutrient concentration solution, except Cu (195 μg L^{-1} $CuSO_4.5H_2O$).

Copper treatment

1) *0/0* Cu treatment — both halves of the split-root system grown in nutrient solutions with 50 μg L^{-1} background Cu.
2) *0/10* Cu treatment — one half of the split-root system grown in nutrient solutions with 50 μg L^{-1} background Cu, the other half of the split-root system was exposed to 10 mg Cu L^{-1}.

The codes of the treatments were shown in Table 6.3.

Table 6.3 Treatments

Relatively Humidity	Light intensity	Nutrient concentration	Cu treatment	Code
Low RH (**H_0**)	Shading (**L_0**)	High (**N_1**)	*0/0*	**$H_0L_0N_1$*0/0***
	No shading (**L_1**)	High (**N_1**)		**$H_0L_1N_1$*0/0***
	No shading (**L_1**)	Low (**N_0**)		**$H_0L_1N_0$*0/0***
	Shading (**L_0**)	High (**N_1**)	*0/10*	**$H_0L_0N_1$*0/10***
	No shading (**L_1**)	High (**N_1**)		**$H_0L_1N_1$*0/10***
	No shading (**L_1**)	Low (**N_0**)		**$H_0L_1N_0$*0/10***
High RH (**H_1**)	Shading (**L_0**)	High (**N_1**)	*0/0*	**$H_1L_0N_1$*0/0***
	No shading (**L_1**)	High (**N_1**)		**$H_1L_1N_1$*0/0***
	No shading (**L_1**)	Low (**N_0**)		**$H_1L_1N_0$*0/0***
	Shading (**L_0**)	High (**N_1**)	*0/10*	**$H_1L_0N_1$*0/10***
	No shading (**L_1**)	High (**N_1**)		**$H_1L_1N_1$*0/10***
	No shading (**L_1**)	Low (**N_0**)		**$H_1L_1N_0$*0/10***

Fig. 6.1 The split-root technique.

There were three replicates for each treatment within the low or high RH growth box, but no external replication of the humidity treatments. Each replicate comprised three plants in each container with each container having one litre of nutrient solution. The nutrient solutions were continually aerated using air bulbs (Fig. 6.1), and were renewed every two days in the first week and then every day in the second week. Nutrient solutions from each container were sampled for Cu concentration. Water loss from each container was recorded prior to renewing the solutions. During the experimental period, the glasshouse temperatures were controlled at 25±5°C, with supplementary lighting provided by sodium-vapour lamps to give an 18-h photoperiod.

In the initial stages of the experiment all plant root systems were exposed to 50 µg Cu L^{-1} (ie. *0/0*). Plants were grown for 4 days before the experimental treatments were applied (ie. *0/0* or *0/10*). At day 4, selected plants were sampled to record initial plant dry weight.

To avoid cross contamination with Cu the point at which the two halves of the root system separated was raised to 6 cm above the solutions and kept dry. Care was taken to avoid the Cu contamination of nutrient solutions during the experimental period.

6.2.3 Plant harvest

The Cu treatments were imposed for a period of 14 days. On the 10th day of the Cu treatment, young mature leaves and old leaves (two per plant) from both sides of the plants were sampled. At the final harvest, individual plants were harvested in three parts: shoots, roots (two parts). Shoots and roots were washed and dried (See Section 2.2.1.1 for methods), the dry weight of each plant part was recorded and the total final plant dry weight of individual plants was calculated. Plant growth was expressed as dry weight gain (final weight - average weight of initial seedlings).

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6.2.4 Analysis

6.2.4.1 Nutrient solutions

Copper concentrations in nutrient solutions were measured using FAAS (see Section 2.3.1.2.1 for details).

6.2.4.2 Plants

Copper concentrations in nitric acid digests of leaves, shoots and roots were measured using FAAS (see Section 2.2.1.2 for details).

6.2.5 Data analysis

Data were treated as two separated complete randomised designs (CBD) for the low and high relative humidity box, respectively. Multiple comparisons were performed for the treatments within each box. Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using the SAS GLM procedure (SAS Institute, 1990). The ANOVA analysis of root Cu concentration and accumulation was performed on the logarithmic transformed data. T-tests were performed for the paired data from the low and high relative humidity boxes. Significantly different means (LSD tests, probability level *P*) are labelled with different letters in the data presentation tables.

6.3 RESULTS

6.3.1 Plant growth

6.3.1.1 Total plant dry weight

Exposure of half of the roots to 10 mg Cu L^{-1} significantly decreased plant growth of all treatments for both low and high RH compared to the corresponding plants grown in *0/0* Cu treatments (Table 6.4). Under both low and high RH, shading of tomato plants significantly decreased plant dry weight gain in both the

0/0 and *0/10* Cu treatments. The low solution nutrient concentrations ($H_0L_1N_0$ and $H_1L_1N_0$) significantly decreased plant dry weight gain in *0/0* Cu treatment but no such impact was observed for the *0/10* Cu treatment (Table 6.4).

The results of t-tests showed that plants grew significantly better in the high humidity box than in the low humidity box, except between the treatments $H_0L_0N_1$*0/10* and $H_1L_0N_1$*0/10* (Table 6.4).

Table 6.4 Effects of relative humidity (RH), light intensity, solution nutrient concentration and Cu treatments on plant shoot dry weight gain. Significant differences (*P*<0.05) between treatment means (in rows) are labelled with different letters. The standard errors of means are shown in parentheses. T-tests were performed for paired data from low and high humidity boxes (in columns), significantly different means were shown with *P* values and NS for no significant difference. Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

Relative	------------*0/0*-------------			---------*0/10*-----------		
Humidity	**L_0N_1**	**L_1N_1**	**L_1N_0**	**L_0N_1**	**L_1N_1**	**L_1N_0**
Low RH (**H_0**)	2.7 c	5.9 a	3.3 b	2.0 d	2.9 bc	2.5 cd
	(0.2)	(0.3)	(0.1)	(0.3)	(0.1)	(0.4)
High RH (**H_1**)	3.6 c	7.5 a	5.4 b	2.4 d	3.9 c	3.5 c
	(0.2)	(0.4)	(0.2)	(0.2)	(0.2)	(0.5)
Significance	0.05	0.05	0.01	NS	0.05	0.05

6.3.1.2 Root dry weight

Exposure of one half of the root system to 10 mg Cu L^{-1} resulted in a significant decrease in the growth of both halves of the root system, compared to plants whose root systems were exposed to 50 Cu μg L^{-1} only. The $H_1L_0N_1$ and $H_0L_0N_1$ treatments were the exceptions (Fig. 6.2). When one half of the root system was exposed to 10 mg Cu L^{-1}, neither shading nor low fertility significantly altered the dry weight of roots except between $H_0L_0N_1$ and $H_0L_1N_1$ and $H_0L_1N_0$

treatments (Fig. 6.2). The dominant constraint on plant growth of Cu toxicity masked the effect of shading and low solution nutrient concentrations.

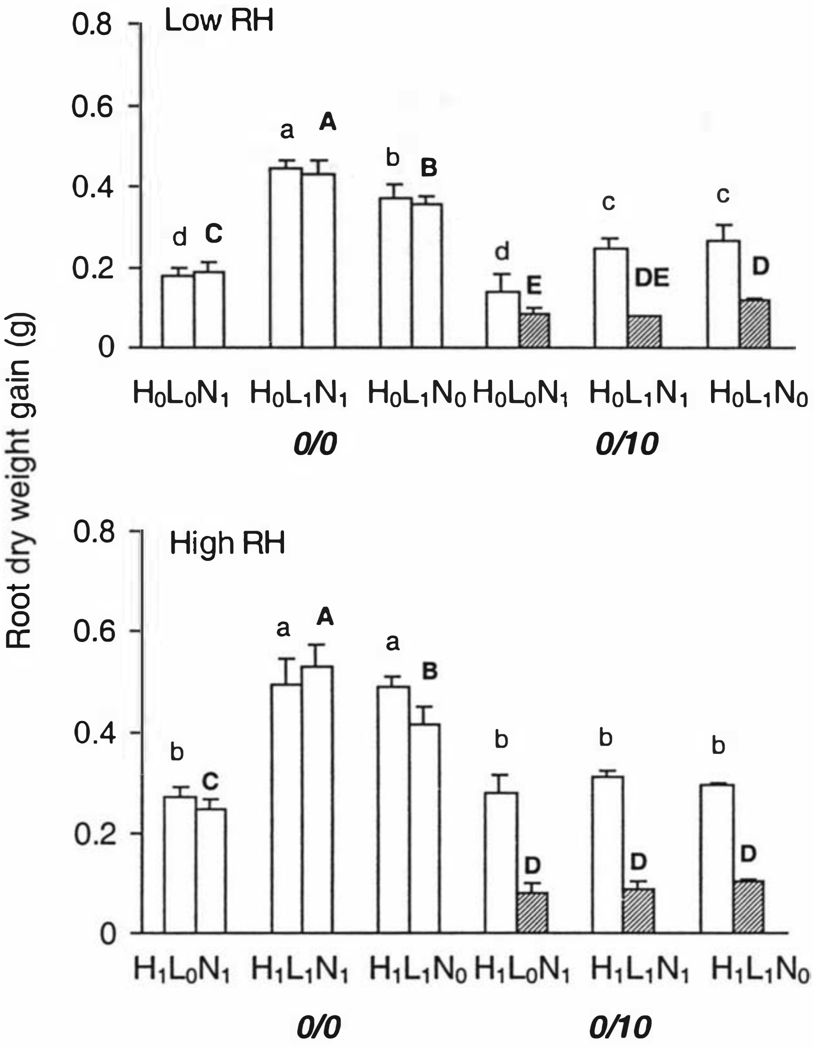

Fig. 6.2 Tomato root growth in split-root systems exposed to different concentrations of 50 μg Cu L^{-1} (clear columns) and 10 mg L^{-1} Cu (shaded columns). The vertical bar on the top of each column is the standard error. Significant differences (*P*<0.05) between treatment means are labelled with different letters (A, B, C, D, E and a, b, c, d for right-hand side and left-hand side root, respectively). Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

6.3.2 Water use

Irrespective of exposure one half of the root system to Cu or not, plants grown under high RH used significantly (t-test) less water to produce dry matter compared to the corresponding treatments under low RH (Table 6.5).

Under low RH, the combination of high light intensity and low solution nutrient concentrations resulted in significantly higher plant water use, whereas high light intensity and high solution nutrient concentrations produced the lowest values, which differed little with Cu treatment ($H_0L_1N_1$*0/0* and $H_0L_1N_1$*0/10*) (Table 6.5).

Under high RH, the treatment effects were similar but smaller. Cu treated plants did not show a significant reduction in plant water use when shaded (Table 6.5). The WUE of plants grown in *0/10* Cu treatment was not significantly different from the corresponding *0/0* Cu treatments when RH was high ($H_1L_1N_1$*0/0* and $H_1L_1N_1$0/*10*) (Table 6.5).

Table 6. 5 Effects of relative humidity (RH), light intensity, solution nutrient concentration and Cu treatments on tomato plant water use (mL g^{-1} DM). Significant differences (*P*<0.05) between treatment means (in rows) are labelled with different letters. The standard errors of means are shown in parentheses. T-tests were performed for paired data from low and high humidity boxes (in columns), significantly different means were shown with *P* values. Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

Relative	------------*0/0*-------------			---------*0/10*-----------		
Humidity	L_0N_1	L_1N_1	L_1N_0	L_0N_1	L_1N_1	L_1N_0
Low RH (H_0)	212 b	170 d	263 a	190 c	166 d	228 b
	(15)	(4)	(8)	(5)	(8)	(12)
High RH (H_1)	183 a	159 bc	183 a	164 abc	150 c	178 ab
	(17)	(1)	(15)	(8)	(9)	(8)
Significance	0.01	0.05	0.05	0.05	0.05	0.05

6.3.3 Copper concentration in young and old leaves

Lateral position on the plant made no significant differences to leaf Cu concentrations in both the *0/0* and *0/10* Cu treatments (Fig. 6.3). Whereas leaf age had no effect on leaf Cu concentration on *0/0* Cu treated plants, young leaves had significantly higher Cu concentrations in the *0/10* Cu treatments. High light intensity and high solution nutrient concentrations produced the lowest young leaf Cu concentrations (Fig. 6.3).

6.3.4 Shoot Cu concentration and Cu accumulation

Humidity change failed to have a significant effect on shoot Cu concentrations for all treatments (Table 6.6).

Exposure of one half of the root system to 10 mg L^{-1} Cu (*0/10* Cu treatment) resulted in significant higher shoot Cu concentrations compared to the *0/0* Cu treatments (Table 6.6). Under both high and low RH, shading had no significant effect on shoot Cu concentrations of tomato plants grown in *0/0* or *0/10* Cu treatment at high nutrient concentrations ($H_0L_0N_1$ vs $H_0L_1N_1$, $H_1L_0N_1$ vs $H_1L_1N_1$). Lower solution nutrient concentrations resulted in significantly higher shoot Cu concentration when plants were grown in *0/10* Cu treatments. Humidity failed to have a significant effect on shoot Cu concentrations for all treatments (Table 6.6).

It was noted earlier that *0/10* mg L^{-1} Cu treatment had a marked effect on plant dry matter production. Therefore, when considering Cu transport rates, the amounts of total Cu accumulated in a plant part may be a more useful indicator of Cu transport than the Cu concentration. Exposure of one half of the root system to 10 mg L^{-1} Cu resulted in a significantly higher shoot Cu accumulation compared to the *0/0* Cu treatments except when plant growth was limited by shading ($H_1L_0N_1$*0/0* vs $H_1L_0N_1$*0/10*) (Table 6.6). Under both high and low RH, neither shading nor low solution nutrient concentrations had significant effect on shoot Cu accumulation by tomato plants grown in *0/0* or *0/10* Cu treatments compared to those with no shading or high solution nutrient concentration treatments, except in the case of $H_1L_1N_1$*0/10* and $H_1L_0N_1$*0/10* (Table 6.6).

These results indicated that plants exposed to higher solution Cu concentrations and lower solution nutrient concentration had significantly higher shoot Cu concentrations. This was a function of the difference in nutrient limited plant growth because the product of dry matter, shoot Cu concentration and shoot Cu accumulation did not differ for the same treatments.

	$H_0L_0N_1$		$H_0L_1N_1$		$H_0L_1N_0$	
Young leaves	13.3	14.6	9.5	10.0	14.5	13.6
Old leaves	13.4	12.9	9.8	8.9	13.6	12.9
Nutrient solution	***0***	***0***	***0***	***0***	***0***	***0***
Young leaves	42.0	43.8	33.6	35.1	47.5	49.7
Old leaves	21.0	22.9	21.9	21.2	24.9	21.2
Nutrient solution	***0***	***10***	***0***	***10***	***0***	***10***

	$H_1L_0N_1$		$H_1L_1N_1$		$H_1L_1N_0$	
Young leaves	15.0	13.2	12.1	10.6	14.3	13.2
Old leaves	13.0	14.1	9.9	10.8	12.8	13.9
Nutrient solution	***0***	***0***	***0***	***0***	***0***	***0***
Young leaves	41.7	38.3	39.6	40.1	48.7	45.0
Old leaves	20.4	23.3	19.6	18.5	25.7	23.4
Nutrient solution	***0***	***10***	***0***	***10***	***0***	***10***

Fig. 6.3 Copper concentrations (mg Cu kg^{-1} DW) of leaves sampled from both sides of tomato plants grown in *0/0* and *0/10* Cu treatment solutions. Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

Table 6. 6 Effects of relative humidity (RH), light intensity, solution nutrient concentration and Cu treatments on shoot Cu concentrations and accumulation of tomato plants. Significant differences (*P*<0.05) between treatment means (in rows) are labelled with different letters. The standard errors of means are shown in parentheses. Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

Shoot Cu concentration

	------------*0/0*-------------			---------*0/10*-----------		
	L_0N_1	**L_1N_1**	**L_1N_0**	**L_0N_1**	**L_1N_1**	**L_1N_0**
Low RH (**H_0**)	13.5 c	8.5 c	14.0 c	31.1 ab	25.3 b	35.1 a
	(0.5)	(1.7)	(2.8)	(2.8)	(6.0)	(8.3)
High RH (**H_1**)	13.5 c	9.6 c	12.3 c	27.1 ab	23.6 b	28.7 a
	(0.9)	(1.2)	(1.3)	(1.8)	(4.5)	(2.9)
Significance	NS	NS	NS	NS	NS	NS

Shoot Cu accumulation

	------------*0/0*-------------			---------*0/10*-----------		
	L_0N_1	**L_1N_1**	**L_1N_0**	**L_0N_1**	**L_1N_1**	**L_1N_0**
Low RH (**H_0**)	31.1 c	42.9 bc	36.7 bc	56.2 ab	64.6 a	72.6 a
	(2.2)	(8.8)	(8.0)	(3[illegible])	(18.3)	(11.3)
High RH (**H_1**)	42.0 b	61.6 b	55.6 b	54.3 b	83.6 a	90.5 a
	(2.6)	(5.1)	(5.3)	(8.1)	(19.2)	(21.7)
Significance	NS	NS	NS	NS	NS	NS

NS — no significant difference.

6.3.5 Root Cu concentration

Irrespective of RH, exposure of one half of the root system to 10 mg L^{-1} Cu resulted in significantly higher Cu concentrations in both halves of the root system compared to those of the corresponding *0/0* Cu treatments (Fig. 6.4).

Irrespective of RH, shading caused no changes to root Cu concentrations in *0/0* Cu solutions. Low solution nutrient concentration resulted in higher root Cu concentrations, especially under high RH condition.

With exposure of half of the root system to 10 mg L^{-1} Cu under both low and high RH, neither light intensity nor solution nutrient concentration had significant effect on the Cu concentrations of both halves roots, except $H_1L_1N_1$ vs. $H_1L_1N_0$ (Fig. 6.4).

6.3.6 Root Cu accumulation

Irrespective of RH, exposure of half of the root system to 10 mg L^{-1} Cu resulted in a significantly higher Cu accumulation in both half roots compared to the corresponding *0/0* Cu solutions (Fig. 6.5).

Irrespective of RH, shading significantly decreased Cu accumulation in both halves of root systems bathing in *0/0* Cu solutions, whereas solution nutrient concentration generally had no significant effect on root Cu accumulation (Fig. 6.5). This suggests that Cu translocation from one half root system to another half root system may be influenced by the photosynthate production.

When grown in *0/10* Cu solutions under low RH, shading increased the Cu accumulated in roots exposed to 10 mg Cu L^{-1} and significantly decreased the amount of Cu translocated to and accumulated in the half exposed to low Cu (Fig. 6.5). Low solution nutrient concentration increased the amount accumulated in the half root system exposed to high Cu (Fig. 6.5). This mostly resulted from root dry matter differences (Fig. 6.3). This did not occur at high RH. When grown in *0/10* Cu solution under high RH, neither shading nor solution nutrient concentration had significant effect on Cu accumulation in both halves of roots (Fig. 6.5).

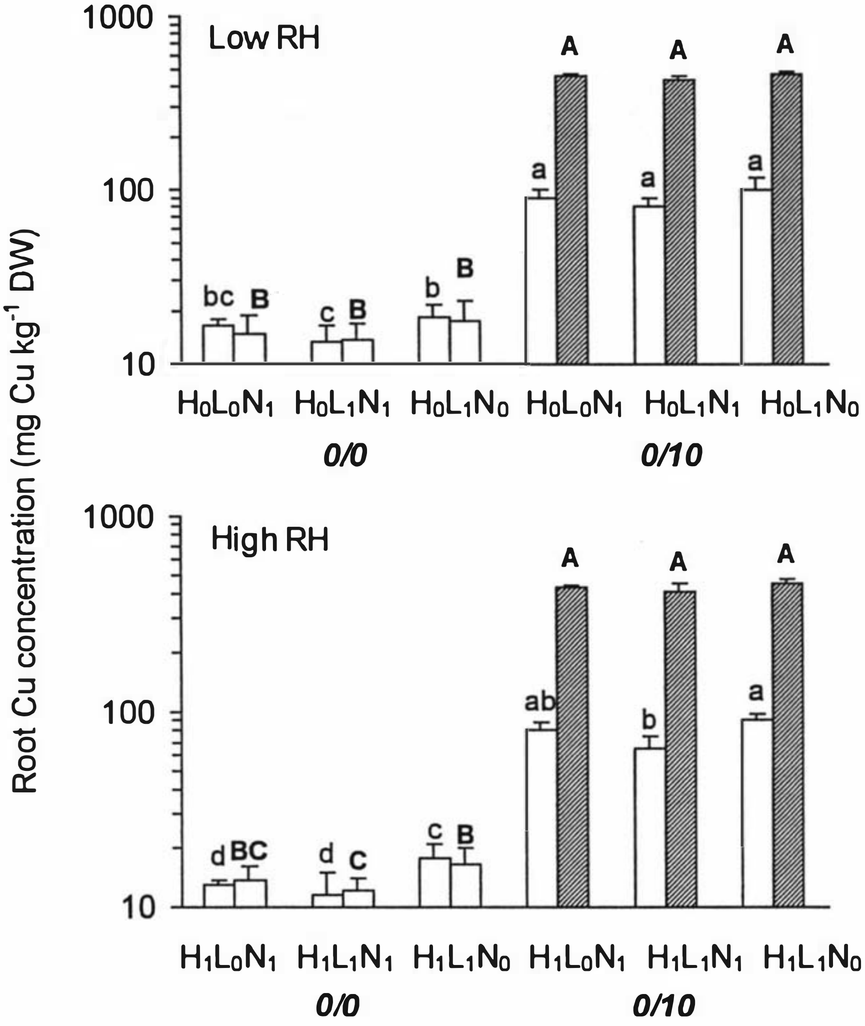

Fig. 6.4 Tomato root Cu concentrations in split-root systems exposed to different concentrations of 50 μg Cu L^{-1} (clear columns) and 10 mg L^{-1} Cu (shaded columns). The ANOVA was performed on logarithmic transformed data. The vertical bar on the top of each column is the standard error. Significant differences (*P*<0.05) between treatment means are labelled with different letters (A, B, C, D, E and a, b, c, d for right-hand side and left-hand side root, respectively). Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

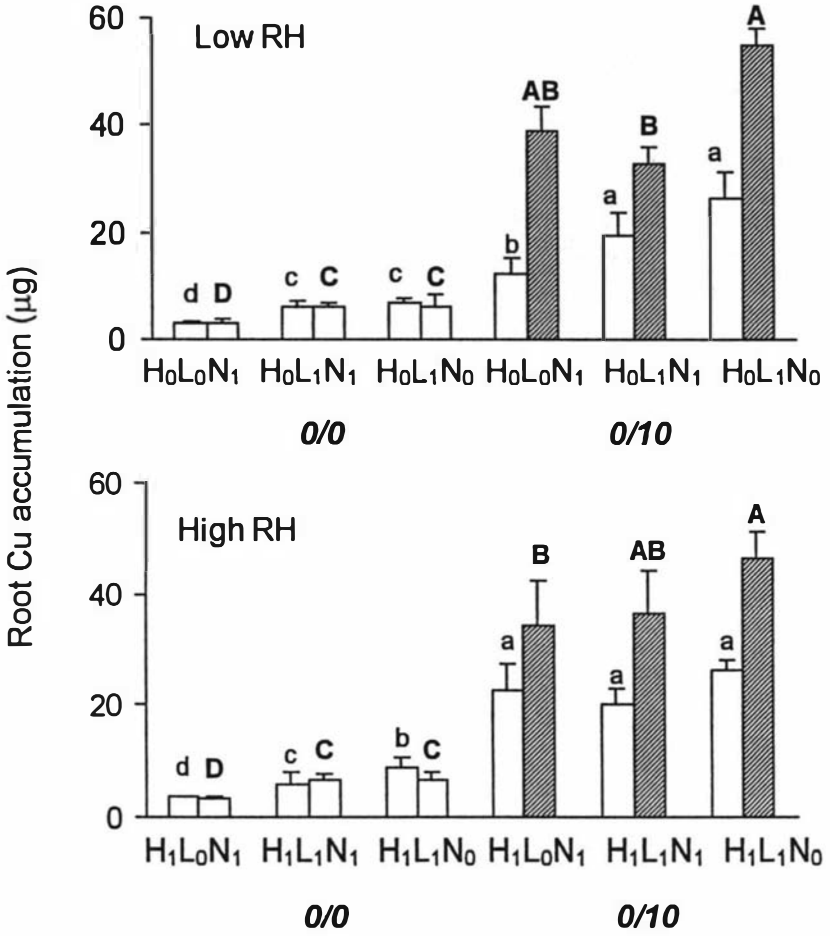

Fig. 6.5 Tomato root Cu accumulations in split-root systems exposed to different concentrations of 50 µg Cu L^{-1} (clear columns) and 10 mg L^{-1} Cu (shaded columns). The ANOVA was performed on the logarithmic transformed data. The vertical bar on the top of each column is the standard error. Significant differences ($P<0.05$) between treatment means are labelled with different letters (A, B, C, D, E and a, b, c, d for right-hand side and left-hand side root, respectively). Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

6.3.7 Copper distribution in plants with half roots exposed to high Cu concentration

Under low RH and high RH, 9-20 and 14-19 μg Cu per plant was translocated and accumulated in half the root system not exposed to high Cu (10 mg Cu L^{-1}) during the experimental period (Table 6.7). These amounts of Cu accounted for 16-27% of shoots Cu uptake and 8-13% of total plant Cu uptake under low RH. The corresponding values under high RH were 17-35 and 10-17% respectively (Table 6.7).

Regular analysis of the nutrient solutions bathing roots not exposed to high Cu showed small increases in Cu concentration (10-15 μg Cu L^{-1}) (detailed data not shown), assumed to result from Cu exudation from roots. No major change in Cu concentration arose from nutrient solution exchange between half root systems was detected.

The amounts of Cu exuded from roots to the *0* Cu (50 μg Cu L^{-1}) nutrient solutions during the experimental period, expressed as Cu increase in solution (ΔCu1, μg) were 13-27 and 13-19 μg under low RH and high RH respectively (Table 6.7).

The sum of the Cu increase in solution and the amount of Cu translocated to and accumulated in half the root systems not exposed to high Cu accounted for 42-63 and 40-59% of shoot Cu (Table 6.7)

Table 6.7 Increase in Cu in solution (ΔCu1, μg), and root tissue (ΔCu2, μg) of the half roots not exposed to high Cu and their proportions to Cu accumulation in shoots (%). Three-letter code referring to level of RH, light intensity and nutrient concentration are listed in Table 6.3.

Treatment	ΔCu1*	ΔCu2**	% of ΔCu2 as shoot Cu	% of ΔCu2 as total plant Cu	% of (ΔCu1 +ΔCu2) as shoot Cu
$H_0L_0N_1$*0/10*	27	9	16	8	63
$H_0L_1N_1$*0/10*	13	14	22	12	42
$H_0L_1N_0$*0/10*	16	20	27	13	48
$H_1L_0N_1$*0/10*	13	19	35	17	59
$H_1L_1N_1$*0/10*	19	14	17	10	40
$H_1L_1N_0$*0/10*	17	19	21	12	40

*ΔCu1 is the Cu increase in the solution at each change of nutrient solutions, calculated as: Σ(solution Cu concentration difference × Vol_{final}).

**ΔCu2 is calculated as: Cu accumulation in the half roots not exposed to high Cu in *0/10* Cu treatment minus the mean of Cu accumulation in both half of roots in *0/0* Cu treatment.

6.3.8 Relationship between shoot Cu concentration, plant water use (mL g^{-1} DM) and plant size

A positive correlation was found between shoot Cu concentration and plant water use (Fig. 6.6). Shoot Cu concentrations were not significantly related to plant size.

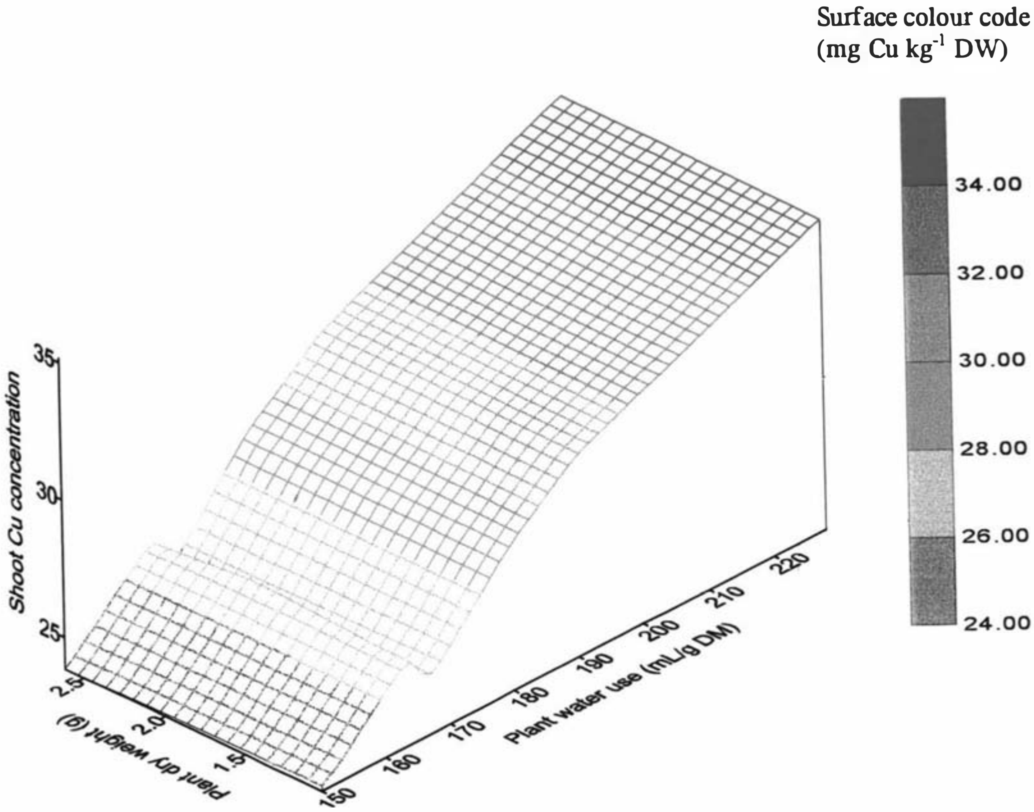

Fig. 6.6 Relationship between plant water use (mL g^{-1} DM) and shoot Cu concentrations (mg Cu kg^{-1} DM) of tomato plants grown in *0/10* Cu solutions.

6.4 DISCUSSION

6.4.1 Effect of RH, light intensity and Cu treatments on plant growth and water use

Growth inhibition and reduction of biomass production are general responses of higher plants to heavy metal toxicity (Baker and Walker, 1995; Woolhouse and Walker, 1981). Without Cu addition, both shading and low nutrient concentration treatments significantly decreased plant growth, but Cu toxicity was the dominant growth constraint when half of the root system was exposed to a nutrient solution containing 10 mg Cu L^{-1} (Table 6.4). This indicated that a Cu concentration of 10 mg Cu L^{-1} was phytotoxic in static hydroponic culture, although this Cu concentration was not phytotoxic in the earlier NFT culture (Chapters 4, 5).

Plants grew significantly better under high relative humidity than in low relative humidity (Table 6.4). Other workers have shown similar effects of RH on plant growth of various species. For example, sugar beet (Ford and Thorne, 1974), cucumber (Bakker et al., 1987), tomato (Acock et al., 1976; Bakker, 1990; Lipton, 1970; Swalls and O'Leary 1976). This effect is probably caused by high RH increasing the stomatal conductance, and therefore the net photosynthetic rates (Bunce, 1984; Grange and Hand, 1987).

Plants, grown under high RH conditions, had higher water use efficiency (WUE, g DM L^{-1}) than those grown under low RH conditions (Table 6.5). This is because of the higher net photosynthetic rate under high RH conditions (Bunce, 1984). As far as solution nutrient concentrations is concerned, the results reported here are similar to those of Oertli et al. (1990), who found that tomato plant WUE increased with the increase of solution fertility.

6.4.2 Evidence of Cu redistribution within plants

Copper was taken up by roots exposed to Cu and then transported to shoots. Irrespective of RH, exposure of one half of the root system to 10 mg Cu L^{-1} (*0/10* Cu treatment) resulted in marked increases in shoot Cu concentration compared to the *0/0* Cu treatments (Table 6.6). The same trend was found for shoot Cu accumulation (Table 6.6). Marked increases in Cu concentrations of roots not exposed to high Cu when half of the root system was exposed to 10 mg Cu L^{-1} provides evidence for xylem/phloem interchange of Cu. It is evident that, under the experimental conditions reported here, that Cu was transported uniformly to the leaves. Bilateral division of the roots did not lead to non-uniform Cu distribution within each plant (Fig. 6.3) because Cu concentrations for the same age leaves were similar. If the Cu, taken up by roots exposed to Cu, was transported to some specific parts of shoots via the xylem bundles, connecting the roots and these specific parts of shoots, significant differences in Cu concentrations of leaves sampled from both sides of the individual plants would have been observed.

The amounts of Cu transported to, and accumulated in, the half roots not exposed to high Cu, were equivalent to 16-27 and 17-35 % of shoot Cu uptake under low and high RH respectively (Table 6.7). When Cu exuded to nutrient solution is added the amounts of translocated Cu becomes equivalent to 40-63% of shoot Cu.

It is concluded that Cu was taken up by roots exposed to Cu, transported to the shoots via xylem sap uniformly, and then transported down the plant to roots not exposed to Cu via the phloem. Kirkham (1980) found similar results in a study of movement of Cd in split-root wheat plants.

As discussed in Chapter 4, if all xylem transported Cu were accumulated in shoots, the theoretical shoot Cu accumulation would be much higher than actual shoot Cu accumulation. In this present study, the water use efficiency of tomato plants was in the range 150 to 263 mL g^{-1} DW (Table 6.5). If the mean of 200 mL

g^{-1} DW is used to calculated the theoretical shoot Cu accumulation (Volume of water used × xylem sap Cu concentration of tomato plants), the theoretical shoot Cu accumulation would be about 4 times higher than the actual shoot Cu accumulation, i.e. actual shoot Cu accumulation would account for 1/4 of the theoretical shoot Cu accumulation.

The amount of Cu transported to and accumulated in the half roots not exposed to high Cu was equivalent to about 25% of measured shoot Cu accumulation. This root Cu could not be derived from contanimation of the 0 Cu nutrient solution which remained at <65 μg Cu L^{-1}. Solutions of <65 μg Cu L^{-1} will not allow root Cu concentration (Fig.6.4) rise to 70-80 mg Cu kg^{-1} DM measured (see Table 4.2 in Chapter 4). Therefore, the 10-15 μg Cu L^{-1} increase in the 0 Cu solution concentration resulted from Cu exudation from roots. This amount of Cu increase in the solution without Cu supplement was approximately equivalent to another 25% of measured shoot Cu accumulation. It could be assumed that a similar amount of Cu was transported back and accumulated in the half roots exposed to high Cu, and that a similar amount was released to nutrient solution with 10 mg Cu L^{-1} addition. So the total amount of Cu transported down to both half roots accounted for one eighth of the theoretical shoot Cu accumulation. The amount of Cu exuded into nutrient solutions accounted for another one eighth. It is proposed that the rest of xylem-transported Cu equal to half of the theoretical shoot Cu accumulation would have been recirculated between xylem and phloem fluids. That is although it is measured in the xylem exudate it does not contribute to over all shoot Cu accumulation.

Herbik et al. (1996) reported good evidence of Cu recirculation via the phloem by feeding tomato plants with Cu via cut surface of the shoot axis. They found that a tomato mutant incapable of phloem transport of heavy metals did accumulate Cu in their leaves, while the wild type did not. Other workers have reported evidence of circulation for iron (Pierson et al., 1986; Van der Mark et al., 1982), rubidium and strontium (Kuppelwieser and Feller, 1990).

The following Cu circulation model is suggested for the study conducted in this chapter. One quarter of the xylem-transported Cu is net sequestered into shoot;

three quarters of xylem-transported Cu is transformed to the phloem, and then transported down to roots, where one quarter of xylem-transported Cu was sequestered into roots and/or released into solutions; the remaining half of xylem-transported Cu is transformed into the xylem again, and then circulated within the plant (Fig. 6.7).

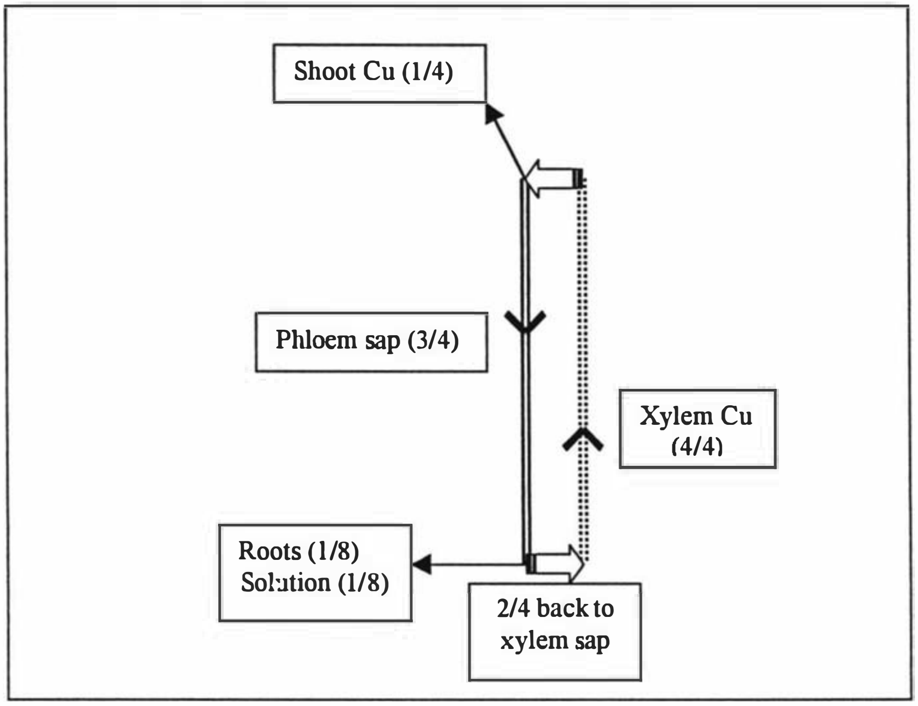

Fig. 6. 7 Possible model of Cu redistribution within the tomato plant.

6.5 CONCLUSIONS

Accumulation of Cu in shoots cannot be explained simply by measuring Cu concentration and mass flow of water in the xylem. Copper was taken up by roots exposed to Cu, transported to the shoots uniformly, and then some of this Cu was sequestered into leaves, the remaining Cu was transported down the plant to the roots (both halves), where some of the Cu was sequestered into roots and/or released into solution. The remaining Cu was circulated back into the xylem.

In this experiment, the dominant growth constraining effect of Cu toxicity masked the effects of relative humidity, light intensity, and solution nutrient concentrations on Cu treated plant growth. Thus subtle changes in xylem and phloem flow rates could not be effected in Cu treated plants, and their effects on Cu redistribution within plants was not elucidated. Nevertheless, there is evidence that Cu redistribution within tomato plants relates to the rates of photosynthate production for plants grown in *0/0* Cu solutions under both high and low RH, and plants in *0/10* Cu solution under low RH. It is suggested that a relatively low solution Cu concentration (for example 5 mg Cu L^{-1} or less) should be used to investigate Cu movement in tomato plants under various conditions (various light intensity, solution fertility, RH etc.), thus could lead to fuller understanding of the mechanisms controlling Cu movement via xylem sap and phloem sap.

It is unclear whether the transfer of Cu from xylem to phloem occurs in stem or shoot. More detailed xylem and phloem analysis of different plant parts would be required to solve this uncertainty.

CHAPTER 7

EFFECTS OF AMINO ACIDS AND CASEIN ON COPPER UPTAKE FROM SOIL BY CHICORY (*Cichorium intybus* L. cv. Grasslands Puna)

Publication arising from chapter:

Liao, M.T., Hedley, M.J., Woolley, D.J., Brooks, R.R., Nichols, M.A. 1999 Effect of amino acids and casein on Cu uptake by chicory from soil. *Proceedings of New Zealand Grasslands Association*, 61: 181-184.

7.1 INTRODUCTION

Copper deficiency in grazing animals is widespread in New Zealand (Korte et al., 1996), costing the farming industry several million dollars in animal remedies each year. In New Zealand, Cu deficiency in ruminants can occur as a result of ingesting pasture containing less than 3 to 4 mg Cu kg^{-1} DM and from molybdenum/sulphur-induced Cu deficiency (Grace, 1983). Recently, Grace et al. (1998) found a good correlation between herbage Cu concentration and liver Cu concentration of grazing Romney sheep. Only trace amounts of Cu are required for plants and animals, but the contents of forage plants may sometimes not provide adequate Cu intake for the optimum performance of ruminant animals (Jarvis, 1978). Overcoming the problem with Cu fertilisers has met with mixed success, with generally low uptake of Cu by pasture plants. It has been reported that increased pasture Cu concentrations in response to fertiliser application are short-lived necessitating frequent Cu applications. An important contribution to the prevention of Cu deficiency in animals would be to increase the Cu concentrations of forage plants. This can be achieved by modest increases in Cu uptake by major pasture grasses or by changing the botanical composition of the forage crop to include species with greater Cu content.

A wide range of Cu sources has been evaluated for their effectiveness as fertilisers when applied to soils or foliage (Gilkes, 1981). Bluestone ($CuSO_4.5H_2O$, 25% Cu), oxidised Cu ore (18% Cu) and pyrite roaster residues

(2.2% Cu) were equally effective sources for oats. But the most commonly used compound appears to be $CuSO_4.5H_2O$ which farmers used for oats in Western Australia as long ago as 1879 (Gilkes, 1981).

There are some contrasting reports on the effectiveness of $CuSO_4.5H_2O$ topdressing and the residual effects. Some reports indicate that topdressing is not effective in raising the Cu concentration of pasture (Anonymous, 1975; Archer, 1971; Mitchell et al., 1957), yet others show that it is effective (Cunningham, 1946; Grace et al., 1998) and has a considerable residual effect (Cunningham, 1946; Hogan, et al., 1964; Reith, 1975; Reuter, et al., 1977; Reuther et al., 1968). The effectiveness of pasture $CuSO_4.5H_2O$ topdressing depends on the nature of the pasture and on soil properties (Sherrell and Rawnsley, 1982). Plants differ in their ability to absorb Cu from the soil, so pastures dominated by species with a high capacity to absorb Cu will respond better to topdressing than those which consist mainly of species with low absorption capacity (Adams and Elphick, 1956; Andrew and Throne, 1962). The residual effects of topdressing also differ between soils. In practice, pasture topdressing with $CuSO_4.5H_2O$ has been the major measure to prevent primary Cu deficiency (Grace et al., 1998; Cunningham, 1946).

Efforts have been made to improve the efficiency of micronutrient fertilisers, but not much progress has been achieved. Most of the studies have focussed on the development of metal chelates for foliar application (Hsu 1986a, b; Hsu et al. 1982, 1986; Jeppsen, 1991). Jeppsen (1991) found that the metal uptake by corn, tomatoes, apples, potatoes and wheat was improved by foliar supplementation of amino acid chelates of various metals (Fe, Zn, Mn, Ca etc.). But little or no information is available for Cu, especially for soil application.

From the results of mathematical models, field experiments and studies with synthetic chelates, Bineev *et al.* (1985) confirmed that free amino acids in soils form chelates with Cu and thereby facilitate the migration of the trace element in the soil-plant system. More recently, it was found that free nicotianamine (NA) and histidine (His) are the major Cu complexing ligands in tomato and chicory xylem sap (Liao et al. 1999b, 2000b; Pich and Scholz 1996). Chicory (*Cichorium* *intybus* L. cv. Grasslands Puna), a forage plant, is recognised as having higher Cu concentration in shoots than many other pasture species, and is a valuable source of forage Cu (Thomas and Thompson, 1948; Thomas et al., 1952).

This paper tests the hypothesis that Cu uptake in chicory (*Cichorium intybus* L. cv. Grasslands Puna) could be increased by addition of amino acids to Cu fertilisers.

7.2 MATERIALS AND METHODS

7.2.1 Soil preparation

A sample of Manawatu silt loam (29 g kg^{-1} OM, 6.0 pH, 52 $\mu g\ g^{-1}$ Olsen P, 17 cmol(+) kg^{-1} CEC, 2 $\mu g\ g^{-1}$ EDTA extractable Cu) was obtained from a 0-15 cm soil depth under permanent pasture.

The soil was air-dried and sieved (<5 mm). Subsamples of 500 g of soil were weighed into plastic bags. Cu was applied either as $Cu(OH)_2$ or $CuSO_4.5H_2O$ at 50 mg or 100 mg Cu kg^{-1} air-dry soil. Histidine (His), methionine (Met), aspartic acid (Asp) and casein were used as additives. The amount of amino acids (His, Met, Asp) and casein applied were determined by the amount needed to supply 18 mg organic-N/pot. $(NH_4)_2SO_4$ was supplied to make up the total N application to 50 mg per pot. The Cu fertilisers, amino acids, casein, and $(NH_4)_2SO_4$ were mixed with the dry soil in plastic bags before placing the soil in the pots.

7.2.2 Plant culture and harvest

Even-sized chicory cv. Grassland Puna seedlings were transplanted into the pots. Distilled water was supplied to maintain 80% of pot water capacity. After 10 days, 200 mL of trace element free complete nutrient solution (800 mg NH_4NO_3, 150 mg L^{-1} KH_2PO_4, 150 mg L^{-1} K_2HPO_4, 100 mg L^{-1} K_2SO_4, 100 mg L^{-1} $MgCl_2.6H_2O$, 25 mg $CaCO_3$, pH 5.5) was added weekly to each pot. During the experimental period, the temperature in the glasshouse was partly controlled (heated at 13 °C, and ventilated at 25 °C). Plants were harvested at 40, 70 and 90 days after transplanting. Harvested plant materials were rinsed with distilled water, and oven dried at 60°C for 48 hours. Plant dry weight (DW) was recorded.

7.2.3 Sample analysis

Copper concentrations in nitric acid digests of plant shoots were measured using FAAS (see Section 2.2.1.2 for details).

7.2.4 Data analysis

Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using SAS GLM procedure (SAS Institute, 1990).

7.3 RESULTS AND DISCUSSION

7.3.1 Plant growth

Irrespective of the forms and rates of Cu applied, the addition of amino acids and casein had no significant effects on chicory shoot dry weight (Fig. 7.1). No obvious Cu toxicity symptoms were observed in any of the treatments during the experimental period. No Cu deficiency symptoms were observed for the control plants grown in Manawatu silt loam.

7.3.2 Shoot Cu concentration

When Cu was supplied as $Cu(OH)_2$ at 50 mg Cu kg^{-1} soil, there were no significant increases in shoot Cu concentrations above the control (Fig. 7.2). At 100 mg Cu kg^{-1} soil addition, $Cu(OH)_2$ alone significantly increased shoot Cu concentrations above the concentrations measured in the control. At harvest 3, all amino acid and protein additives significantly increased shoot Cu concentrations compared to control plants and $Cu(OH)_2$ alone (Fig. 7.2), but there were no significant differences among additives.

Application of $CuSO_4.5H_2O$ significantly increased shoot Cu concentrations compared to control plants. The casein + $CuSO_4.5H_2O$ treatment resulted in significantly higher shoot Cu concentrations than the treatment without additive ($CuSO_4.5H_2O$ alone) at harvest 3 at 50 mg Cu addition and at harvest 2 and harvest 3 at 100 mg Cu addition (Fig. 7.2).

Over three harvests, the chicory shoot Cu concentrations ranged from 9 to 29 mg Cu kg^{-1} DW (Fig. 7.2).

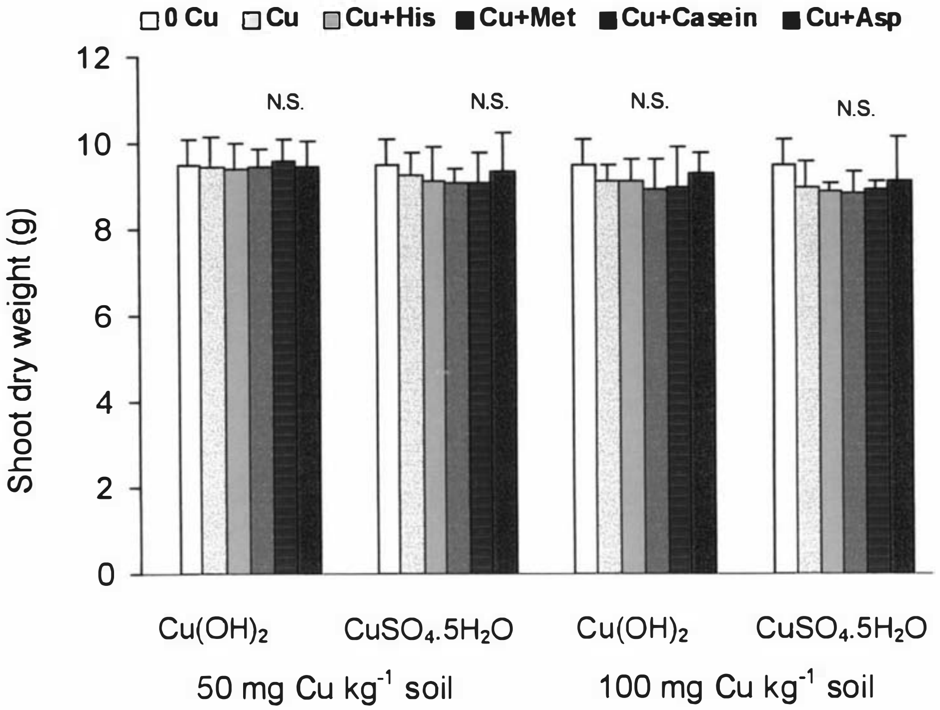

Fig. 7.1 Effect of Cu sources, rate of application and additives on cumulative shoot dry weight over 3 harvests of chicory plants grown in pots of Manawatu silt loam. Vertical error bars are the standard error of means. NS means no significant difference.

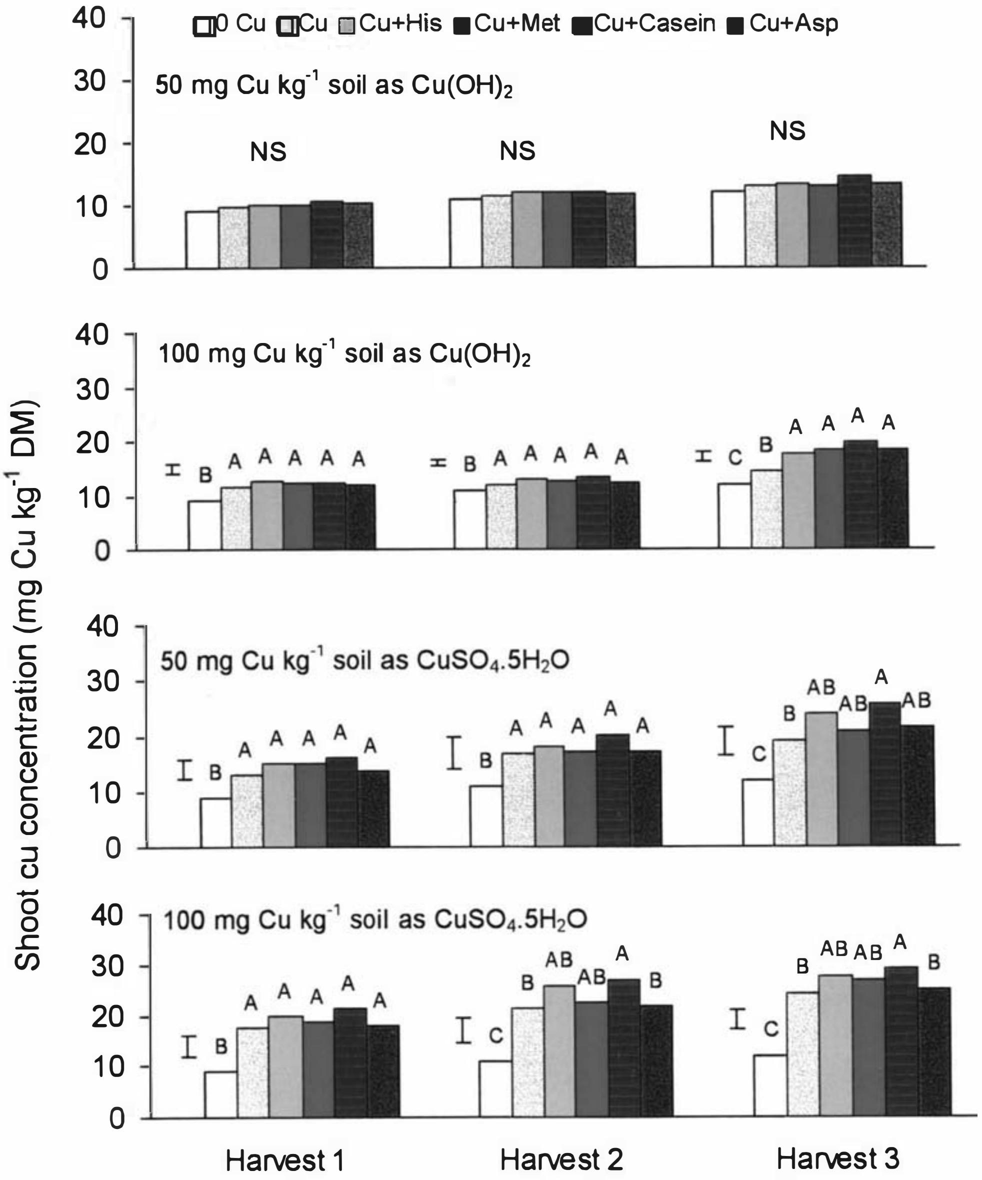

Fig. 7.2 Effect of Cu sources, rate of application on shoot Cu concentration of chicory plants grown in pots of Manawatu silt loam. NS indicates no significant difference. The vertical error bars are the $LSD_{0.05}$ values calculated from ANOVA analysis. Significant differences ($P<0.05$) between treatment means at a single harvest are signified by different letters.

7.3.3 Cumulative Cu uptake

Shoot Cu accumulation significantly increased (*P* values in Table 7.1) with increased Cu application rate (Table 7.1). This result agrees with that of other workers (Beck 1962; Grace et al. 1998; Jarvis 1978, 1980; McLaren and Williams, 1981). Applications of $CuSO_4.5H_2O$ fertiliser resulted in significantly higher shoot Cu accumulation than $Cu(OH)_2$ (Table 7.1), reflecting the lower solubility of $Cu(OH)_2$.

When Cu was supplied as $Cu(OH)_2$, additives had no significant effect on cumulative shoot Cu uptake by chicory plants at the rate of 50 mg Cu kg^{-1} soil as $Cu(OH)_2$ (Table 7.2). At the rate of 100 mg Cu kg^{-1} soil as $Cu(OH)_2$, all additives, except Met, resulted in significantly higher total shoot Cu accumulation than $Cu(OH)_2$ alone (Table 7.2).

At the rate of 50 mg Cu kg^{-1} soil, the total Cu uptake from casein + $CuSO_4.5H_2O$ was significantly higher than from the Met + $CuSO_4.5H_2O$ and $CuSO_4.5H_2O$ alone treatments (Table 7.2). At the rate of 100 mg Cu kg^{-1} soil, the addition of His and casein resulted in significantly higher total shoot Cu accumulation than Met + $CuSO_4.5H_2O$, Asp + $CuSO_4.5H_2O$ and $CuSO_4.5H_2O$ alone. The total shoot Cu accumulation of the casein + $CuSO_4.5H_2O$ treatment was significantly higher than the His + $CuSO_4.5H_2O$ treatment (Table 7.2).

Table 7.1 Effects of Cu fertiliser source and application rate on accumulated shoot dry weight, and shoot Cu accumulation of chicory plants grown in pots of Manawatu silt loam. Data are means of three replicates.

Cu Fertiliser	Shoot Cu uptake / pot (mg) Harvest 1	Harvest 2	Harvest 3	Total shoot dry weight per pot (g)	Total shoot Cu uptake per pot (mg)
Cu form					
$Cu(OH)_2$	0.041	0.039	0.036	9.27	0.115
$CuSO_4.5H_2O$	0.065	0.063	0.053	9.07	0.181
P	0.05	0.01	0.05	NS	0.01
Cu rate (mg Cu kg^{-1} soil)					
0	0.035 a	0.040 a	0.026 a	9.47	0.102 a
50	0.049 b	0.045 a	0.039 b	9.32	0.132 b
100	0.054 c	0.056 b	0.048 c	9.03	0.158 c
P	0.01	0.05	0.01	NS	0.01

Within a harvest, results of LSD tests are indicated either as no significant (NS) or at the stated probability level (*P*) if significant.

Table 7.2 The effect of Cu source, application rate and additives on accumulated shoot Cu accumulation of chicory plants grown in pots of Manawatu silt loam. Data shown are means of three replicates. Significantly different means are labelled with different letters. NS means no significant difference.

----------Treatment--------- Cu source	additive	Accumulated Cu uptake (mg)	Efficacy of additive (%)
50 mg Cu as $Cu(OH)_2$ kg^{-1} soil	None	0.105	
	His	0.108	2.86 b
	Met	0.109	3.81 b
	Asp	0.109	3.81 b
	Casein	0.115	9.52 a
	P	NS	0.01
100 mg Cu as $Cu(OH)_2$ kg^{-1} soil	None	0.113 b	
	His	0.123 a	8.85 c
	Met	0.120 ab	6.19 d
	Asp	0.127 a	12.39 b
	Casein	0.130 a	15.04 a
	P	0.01	0.01
50 mg Cu kg^{-1} soil as $CuSO_4.5H_2O$	None	0.147 b	
	His	0.163 ab	10.88 b
	Met	0.153 b	4.08 c
	Asp	0.157 ab	6.80 c
	Casein	0.177 a	20.41 a
	P	0.01	0.01
100 mg Cu kg^{-1} soil as $CuSO_4.5H_2O$	None	0.185 c	
	His	0.212 b	14.60 b
	Met	0.193 c	4.32 c
	Asp	0.191 c	3.24 c
	Casein	0.224 a	21.08 a
	P	0.01	0.01

7.3.4 Efficacy of additives

In order to compare the relative efficacy of additives on increasing chicory shoot Cu uptake, an index for the effectiveness of additives on chicory Cu uptake, ie. the efficacy of additives (E_i) was calculated for each fertiliser form and rate as

$$E_i = \frac{(C_a - C_0)}{C_0} \times 100 \qquad \text{(Eqn. 7.1)}$$

where C_a is the cumulative shoot Cu uptake from a Cu fertilised treatment with additive; C_0 is the cumulative shoot Cu uptake from a Cu fertilised treatment without additive.

When Cu was supplied at 50 mg Cu as $Cu(OH)_2$, only casein significantly increased plant Cu uptake. All additives significantly increased plant Cu uptake at 100 mg Cu as $Cu(OH)_2$ (Table 7.2). When Cu was supplied as $CuSO_4.5H_2O$, all additives significantly increased plant Cu uptake at both Cu application rates. In general, casein is more effective. Bineev et al. (1985) found that applying Met and Gly to a grey forest soil increased rye Cu uptake. They found that the total content of amino acids in soil increased as a result of enhanced microbial activity. Free amino acids in soil are capable of forming chelates with Cu. These are likely to facilitate the migration of the trace element in the soil-plant system (Bineev et al., 1985) (See Chapter 9 for more detailed discussion of Cu-SOM complexes in soil soils).

7.3.5 Recovery of fertiliser Cu

An index for the apparent percentage recovery of applied fertiliser Cu (R_i) was calculated for each fertiliser form and rate as

$$R_i = \frac{(C_t - C_c)}{C_a} \times 100 \qquad \text{(Eqn. 7.2)}$$

where C_t is the cumulative shoot Cu uptake from a Cu fertilised treatment; C_c is the cumulative shoot Cu uptake from the control treatment; C_a is the rate Cu applied per pot.

The results showed that the apparent recovery of Cu is very low, with ranges of 0.18-0.32% for $CuSO_4.5H_2O$ and 0.01-0.06% for $Cu(OH)_2$ (Fig. 7.3). The apparent recovery of Cu from the $CuSO_4.5H_2O$ fertiliser was significantly higher compared to $Cu(OH)_2$ fertiliser ($P<0.01$, Fig. 7.3). This is due to the lower rate of dissolution of the $Cu(OH)_2$-based fertiliser.

When Cu was supplied as $Cu(OH)_2$, all additives significantly increased the apparent recovery of Cu from $Cu(OH)_2$ fertilised soil (Fig. 7.3). Among the four additives, casein was the most effective.

At the rate of 50 mg Cu kg^{-1} soil, all additives, except Met, significantly increased the apparent recovery of Cu from $CuSO_4.5H_2O$ fertilised soil (Fig. 7.3). Casein resulted in significant higher apparent recovery of Cu compared to the other additives. At the rate of 100 mg Cu kg^{-1} soil, addition of casein and His significantly increased the apparent recovery of Cu, the difference between these two additives were not significant (Fig. 7.3).

7.4 CONCLUSIONS AND IMPLICATIONS

The $Cu(OH)_2$ fertiliser was less effective than $CuSO_4.5H_2O$ fertiliser in supplying Cu to chicory. This is due to the low solubility of $Cu(OH)_2$ fertiliser. Addition of amino acids and casein to both $Cu(OH)_2$ and $CuSO_4.5H_2O$ fertilisers cause only small increases in Cu uptake by chicory plants. The results indicate that amino acid or protein-based Cu fertilisers offer little increased agronomic value over the commercially used $CuSO_4.5H_2O$ fertiliser. Casein-coated $CuSO_4.5H_2O$ has a low corrosive potential and this may warrant further investigation.

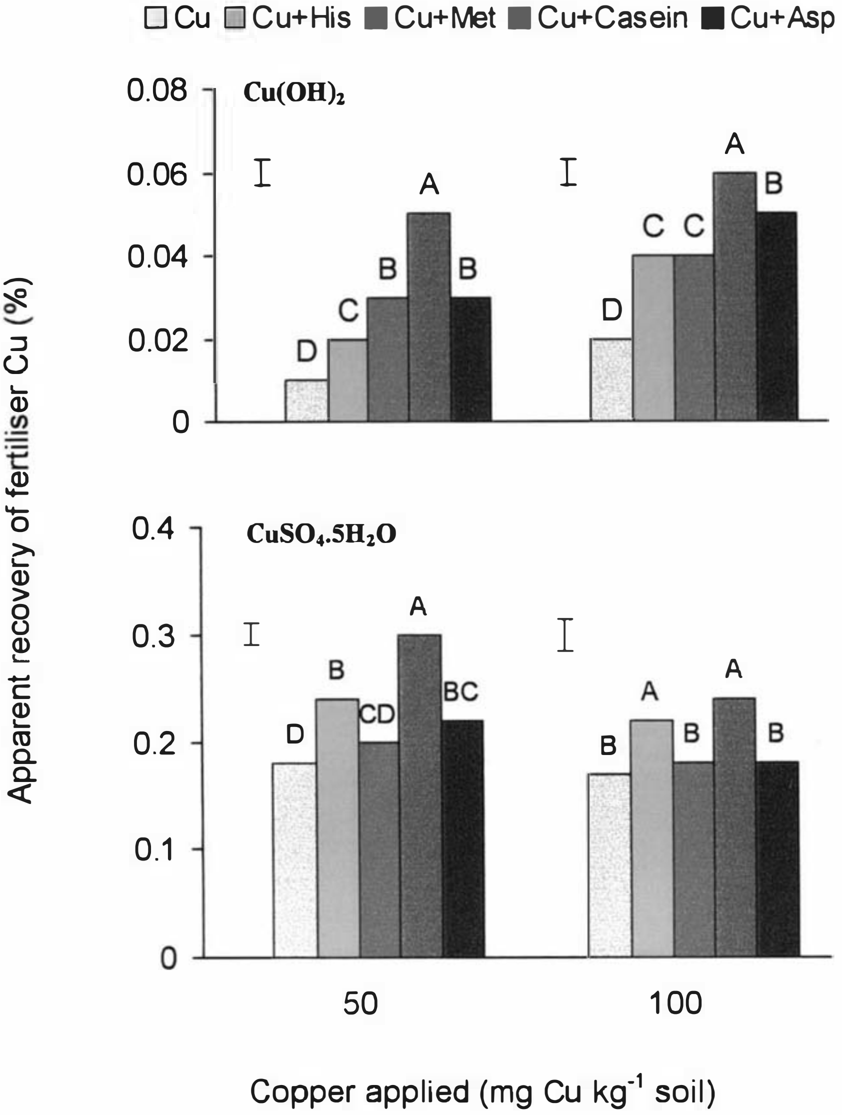

Fig. 7.3 Effect of additives on apparent Cu recovery of Cu from fertilisers, over 3 harvests, of chicory grown in pots of Manawatu silt loam. The vertical error bars are the $LSD_{0.05}$ values calculated from ANOVA analysis. Significant differences ($P<0.05$) between treatment means at a single harvest are signified by different letters.

CHAPTER 8

AGRONOMIC EFFECTIVENESS OF SELECTED FERTILISERS IN SUPPLYING COPPER TO PERENNIAL RYEGRASS (*Lolium perenne* cv. Nui)

Publication arising from chapter:

Liao, M.T., Hedley, M.J., Woolley, D.J., Longanthan, P., Brooks, R.R., Nichols, M.A. 2000 Agronomic effectiveness of selected fertilisers in supplying copper to perennial ryegrass (*Lolium perenne* cv. Nui). *Nutrient Cycling in Agroecosystems* (in preparation)

8.1 INTRODUCTION

It was reported in Chapter 7 that the initial uptake of Cu from $CuSO_4$ fertilised soil by chicory plants can be increased by 10-21% by addition of an amino acid (histidine) and casein (Liao et al., 1999a, Chapter 7). The study reported in this Chapter builds on this work by granulating the fertilisers (as in commercial production) and evaluating their performance with the most common pasture species perennial ryegrass (*Lolium perenne* cv. Nui).

The currently used $CuSO_4$ fertiliser is highly corrosive to fertiliser spreader equipment. It was believed that coating of $CuSO_4$ with organic materials should reduce the corrosiveness of this fertiliser, and produce a granular Cu fertiliser that could be mixed easily and uniformly with physical blends of phosphatic fertilisers (Loganathan et al., 1998). A Ca-caseinate-$CuSO_4$ granule fertiliser has been developed (Loganathan et al. 1998) with the aim of both increasing Cu uptake by pastures and reducing the corrosiveness of the fertiliser to topdressing plane airframes.

Loganathan et al. (1998) compared the corrosiveness of Ca-caseinate-$CuSO_4$ with that of $Cu(OH)_2$-based fertiliser (provided by Ravensdown Fertiliser Coop Ltd) and the currently used $CuSO_4.5H_2O$ fertiliser. Three methods to assess corrosiveness at different moisture regimes were used. (1) weight changes when dry Al foil and fertilisers are kept in contact with each other under high humidity conditions (85-95% R.H.) for 10 days; (2) electric current and potential generated when Cu and Al rods are immersed in moist fertilisers; (3) rate of dissolution of fertilisers in water. They concluded that the Ca-caseinate-CuSO4 was the least corrosive and the $Cu(OH)_2$-based fertiliser is the most corrosive (Loganathan et al. 1998; 1999a, b).

The objective of the present study was to compare the agronomic effectiveness of the granular Ca-caseinate-$CuSO_4$ with that of $Cu(OH)_2$-based fertiliser (provided by Ravensdown Fertiliser Coop Ltd) and the currently used $CuSO_4.5H_2O$ fertiliser in supplying Cu to perennial ryegrass (*Lolium perenne* cv. Nui).

8.2 MATERIALS AND METHODS

8.2.1 Soil preparation

Samples of Ashhurst stony silt loam (Typic Orthic Brown Soil, Ashhurst) and Wairoa pumice soil (Pumice Soil, Wairoa) were obtained from a 0-15 cm soil depth under permanent pastures. Both soils had received very little Cu fertiliser in the past. The soils were air-dried and sieved (<5 mm). The basic characteristics of the two soils are shown in Table 8.1.

8.2.2 Experimental design and treatment

A 4×4×2 factorial design was used. The experimental treatments were:

- Copper fertilisers: Ca-caseinate-$CuSO_4$ (1.4-2 mm), $Cu(OH)_2$-based product (1.4-2 mm), $CuSO_4.5H_2O$ (1.4-2 mm), and $CuSO_4.5H_2O$ (<0.5 mm);
- Copper rates: 0 (control), 12.5, 25 and 50 kg Cu ha^{-1};
- Soils: Ashhurst stony silt loam, Wairoa pumice soil.

There were 3 replicates for each treatment.

8.2.3 Plant culture and harvest

Subsamples of 300 g air-dried soil were weighed into pots. Fertilisers were then evenly applied at the soil surface. The fertilisers were covered with a further 100 g soil. About 50 seeds of perennial ryegrass (*Lolium perenne* cv. Nui) were spread on the soil surface in each pot and the seeds were allowed to germinate. The moisture content of the soil was maintained at 80% of "pot field capacity". The germinated seedlings were thinned to 15 plants per pot. The pots were kept inside a glasshouse where the temperature in the glasshouse was partly controlled (heated at 13 °C and ventilated at 25 °C), and the pots arranged randomly according to the experimental design. A complete nutrient solution free of micronutrients (800 mg NH_4NO_3, 150 mg L^{-1} KH_2PO_4, 150 mg L^{-1} K_2HPO_4, 100 mg L^{-1} K_2SO_4, 100 mg L^{-1} $MgCl_2.6H_2O$, 25 mg $CaCO_3$) was added twice a week. Thirty days after thinning of seedlings, the plants were cut 2 cm above the soil surface, dried for 48 hours at 60°C in oven and weighed. A total of 6 harvests at 30-day intervals were made.

Table 8.1 Basic characteristics of soils used for study.

Soil	pH	Olsen P (mg kg^{-1})	CEC cmol(+) kg^{-1}	C	N	EDTA-extractable Cu (mg kg^{-1} soil)
				%		
Ashhurst stony silt loam	5.4	25.7	15	4.1	0.36	2.04
Wairoa pumice soil	5.4	31.7	25	8.2	0.71	1.57

8.2.4 Recovery of fertiliser granules from soil

At the final harvest, one set of pots (one replicate) was sliced horizontally through the fertilised zone and any visible fertiliser granules were recovered as complete as possible to determine the changes of size and Cu content. The dry weights of the granules were recorded.

The remaining replicates of each treatment were used for soil solution studies (see Chapter 9).

8.2.5 Analysis

8.2.5.1 Plants

Copper concentrations in nitric acid digests of plant shoots were measured using FAAS (see Section 2.2.1.2 for details).

8.2.5.2 Fertiliser

Each fertiliser granule recovered from the pots (see Section 8.2.4 for detail) was weighed and dissolved in 5 mL 2*M* HCl overnight, Cu concentration of the solution was determined using FAAS (see Section 2.2.1.2 for details).

8.2.6 Data analysis

Significant differences between treatment means were evaluated using analysis of variance (ANOVA), performed by using SAS GLM procedure (SAS Institute, 1990).

8.3 RESULTS AND DISCUSSION

8.3.1 Cu fertiliser solubility

After more than 7 months' plant growth in the pots, there were no visible fertiliser granules found in the pots fertilised with Ca-caseinate-$CuSO_4$ (1.4-2 mm) and $CuSO_4.5H_2O$ (both 1.4-2 mm and <0.5 mm) fertilisers. However, in pots fertilised with the $Cu(OH)_2$-based product, visible granules were found in both soils (Fig. 8.1).

The Cu contents and size (weight) of granules of the $Cu(OH)_2$-based product before and after the experimental period are presented in Table 8.2. The granule weight decreased by 33.6% (from 6.85 mg initially to 4.55 mg finally). There was no significant difference in Cu concentrations (25% and 26% respectively). These results were consistent with those of Loganathan et al. (1998; 1999a, b), and confirmed the low solubility of $Cu(OH)_2$-based product. It is interesting to see that the Cu content of the $Cu(OH)_2$-based fertiliser granules slightly increased from 25% to 26% through the experimental period. Only about 30% of the Cu dissolved from the granules (Table 8.2). This result suggests that the rate of Cu dissolution from the $Cu(OH)_2$-based product is less than the rate of weight loss. Loganathan et al. (1999a) found a similar result in a Cu dissolution experiment.

Table 8.2 The differences in fertiliser granule weight and Cu content before and after the experimental period. Data are mean ± S.E. (n=10).

Cu fertilisers	Granule weight (mg)		Cu content (%)	
	original	final	original	final
$Cu(OH)_2$-base product (1.4-2 mm)	6.85 ± 0.56	4.55 ± 1.23	25.05 ± 0.54	26.36 ± 1.09
Ca-caseinate-$CuSO_4$ (1.4-2mm)	6.43 ± 0.22	/	21.18 ± 0.43	/
$CuSO_45H_2O$ (1.4-2 mm)	6.81 ± 0.19	/	25.03 ± 0.10	/
$CuSO_45H_2O$ (<0.5 mm)	/	/	25.09 ± 0.15	/

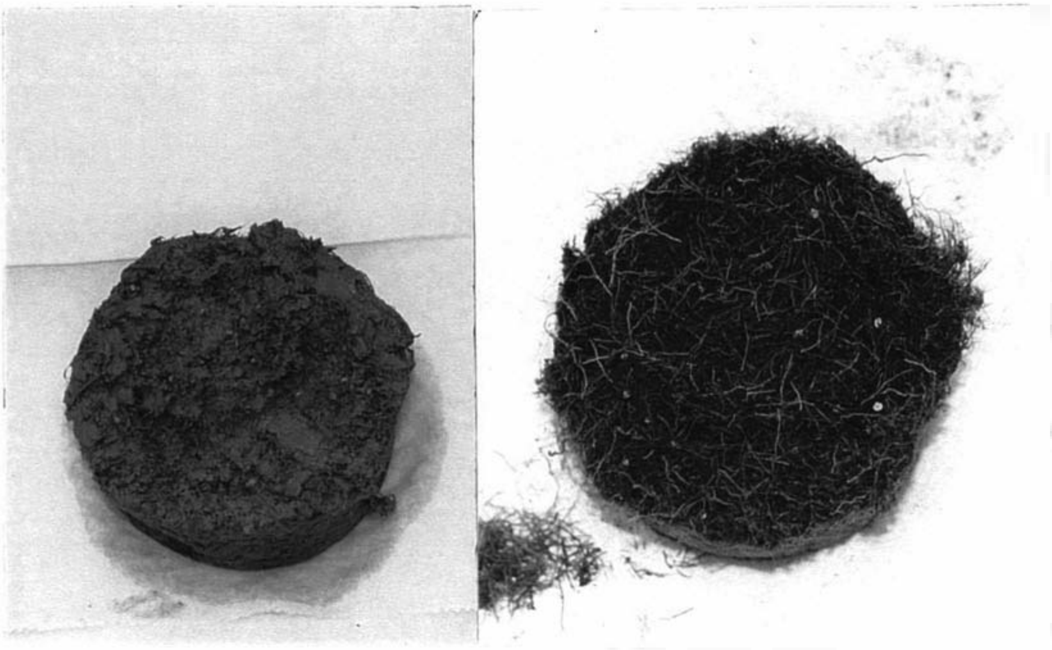

Fig. 8.1 Visible fertiliser granules of the $Cu(OH)_2$-based product in the fertilised zone of Ashhurst stony silt loam (left) and Wairoa pumice soil (right) after more than 7 months plant growth in the pots.

8.3.2 Ryegrass dry matter production

No obvious Cu toxicity symptoms were observed in any of the treatments during the experimental period. No Cu deficiency symptoms were observed for the control plants grown in both Ashhurst stony silt loam and Wairoa pumice soil through the experimental period.

Neither the Cu fertiliser source, nor the applied Cu rate significantly affected the dry matter production of ryegrass grown in pots of Ashhurst stony silt loam or Wairoa pumice soil at each of 6 harvests (data not shown). However, the cumulative yields of the 6 harvests were significantly altered by Cu fertiliser source, Cu rate and soil type (Table 8.3). Ca-caseinate-$CuSO_4$ resulted in a significant higher cumulative yield compared to $Cu(OH)_2$-based product and $CuSO_45H_2O$ (<0.5 mm). Application of Cu significantly increased the cumulative yield compared to control, but there were no significant differences between 12.5, 25 and 50 kg Cu ha^{-1} treatments. Ryegrass grown in pots of Ashhurst stony silt loam produced significantly higher cumulative yield than grown in pots of Wairoa pumice soil (Table 8.3).

Table 8.3 The effect of Cu fertiliser source, application rate, and soil type on the cumulative yield and the cumulative Cu uptake of ryegrass over 6 harvests. Data shown are means of three replicates. Significantly different means are labelled with different letters.

	Cumulative yield per pot (g)	Cumulative Cu uptake per pot (µg)	Apparent recovery of fertiliser Cu (%)
Cu fertiliser			
$CuSO_4.5H_2O$ (1.4-2 mm)	20.29 ab	147 b	0.47 a
$Cu(OH)_2$-based product (1.4-2 mm)	19.99 b	124 c	0.17 c
Ca-caseinate-$CuSO_4$ (1.4-2 mm)	20.69 a	152 a	0.53 a
$CuSO_4.5H_2O$ (<0.5mm)	20.16 b	143 b	0.40 b
P	0.05	0.01	0.01
Cu application rate (kg Cu ha^{-1})			
0	19.49 b	111 d	-
12.5	20.34 a	135 c	0.49 a
25	20.57 a	150 b	0.39 b
50	20.72 a	170 a	0.30 c
P	0.01	0.01	0.01
Soil type			
Ashhurst stony silt loam	21.36	182	0.51
Wairoa pumice soil	19.20	101	0.28
P	0.01	0.01	0.01

8.3.3 Shoot Cu concentration

Applications of $CuSO_4$-based fertilisers resulted in significant higher shoot Cu concentrations over 6 harvests compared to $Cu(OH)_2$-based fertiliser. The $Cu(OH)_2$-based Cu fertiliser resulted in the lowest shoot Cu concentration. There were no significant differences between the three $CuSO_4$-based fertilisers, although Ca-caseinate-$CuSO_4$ generally resulted in slightly higher shoot Cu concentrations (Table 8.4). In the first two harvests, application of $CuSO_4.5H_2O$ (1.4-2 mm) significantly increased ryegrass shoot Cu concentrations compared to $CuSO_4.5H_2O$ (<0.5 mm) (Table 8.4). This indicates the effectiveness of granulation. At a given applied Cu rate, Ca-caseinate-$CuSO_4$ granules resulted in the highest shoot Cu concentration in both soils followed by $CuSO_45H_2O$ granules (Fig. 8.2, 8.3).

Ryegrass shoot Cu concentrations significantly increased with increased Cu application rate (Table 8.4), and the increases varied with Cu fertiliser source, soil type and time (Fig. 8.2, 8.3). Other workers have reported similar results for ryegrass (Jarvis, 1978, 1980; Jarvis and Robson, 1982; Khan et al., 1998).

The shoot Cu concentration of ryegrass plants grown in pots of Ashhurst stony silt loam was always significantly higher than those grown in pots of Wairoa pumice soil were. The Wairoa pumice soil has higher organic matter content (Table 1) and more Cu-OM complexes that are not available to plants may form. Khan et al. (1998) found that ryegrass grown in the soil with a lower organic matter content (Manawatu silt loam) contained higher Cu concentration than those grown in soils with high organic matter content, and contributed this to the rapid immobilisation of Cu into the organic fraction. Other researchers (McBride et al., 1998; McGrath et al., 1988; Minnich et al., 1987; Salam and Helmke, 1998) have found similar effects of increasing soil organic matter content reducing Cu availability in terms of Cu^{2+} activity.

In general, shoot Cu concentrations increased with time during the first two or three harvests, and thereafter decreased with time (Table 8.4). $Cu(OH)_2$-based fertiliser did not effect a sustained slow release of Cu that could have maintained herbage Cu at a high concentration. The higher shoot Cu concentration at harvest 2 than at harvest 1 may be because at harvest 1, the root systems did not fully develop. Decreased shoot Cu concentrations as a function of plant age are consistent the findings of other workers (Gladstones et al., 1975; Gomide et al., 1969; Jarvis and Whitehead, 1983; Thomas et al., 1952). There was no significant difference in shoot Cu concentrations between the $CuSO_4$ and Ca-caseinate-$CuSO_4$ treatments.

8.3.4 Cumulative shoot Cu uptake

Application of $CuSO_4$-based fertilisers resulted in significantly higher cumulative shoot Cu uptake compared to $Cu(OH)_2$-based fertiliser. Among the three $CuSO_4$-based fertilisers, Ca-caseinate-$CuSO_4$ was significantly more effectiveness than the others were (Table 8.3). Liao et al. (1999a) found a similar result for chicory plants grown in Manawatu silt loam. The lower Cu uptake from finely divided $CuSO_4$ fertiliser may be due to higher Cu fixation in the soils.

The cumulative ryegrass shoot Cu uptake significantly increased with the increases of Cu applications (Tables 8.3, 8.5; Fig. 8.4).

The shoot Cu concentration of ryegrass plants grown in Ashhurst stony silt loam was always significantly higher than those grown in Wairoa pumice soil were. This is consistent with shoot Cu concentrations (see Section 8.3.3).

At a given Cu application rate, the Ca-caseinate-$CuSO_4$ product resulted in the highest cumulative Cu uptake, and $Cu(OH)_2$-based Cu fertilisers resulted in a significant lower cumulative Cu uptake than did the other three Cu fertilisers (Fig. 8.4). When grown in Ashhurst stony silt loam, Ca-caseinate-$CuSO_4$ fertiliser resulted in a significantly higher Cu uptake than $CuSO_4.5H_2O$ fertilisers (powder) did (Fig. 8.4).

Table 8.4 The effect of Cu fertiliser source, application rate, and soil type on shoot Cu concentrations of ryegrass at each harvest over 6 harvests. Data shown are means of three replicates. Significantly different means are labelled with different letters.

	Shoot Cu concentration (mg Cu kg^{-1} DW)					
	Harvest					
	1	2	3	4	5	6
Cu fertiliser						
$CuSO_4.5H_2O$ (1.4-2 mm)	7.4 a	7.8 a	7.5 a	7.4 a	6.3 a	6.0 a
$Cu(OH)_2$-based product (1.4-2 mm)	5.1 c	6.5 c	6.6 b	6.3 b	5.6 b	5.7 b
Ca-caseinate-$CuSO_4$ (1.4-2 mm)	7.7 a	7.7 ab	7.5 a	7.5 a	6.3 a	6.1 a
$CuSO_4.5H_2O$ (<0.5mm)	6.9 b	7.5 b	7.3 a	7.3 a	6.2 a	6.0 a
P	0.01	0.01	0.01	0.01	0.01	0.01
Cu application rate (kg Cu ha^{-1})						
0	4.3 d	5.9 d	5.9 d	5.8 d	5.4 d	5.5 c
12.5	6.3 c	7.2 c	6.8 c	7.0 c	5.7 c	5.6 c
25	7.2 b	7.8 b	7.6 b	7.5 b	6.2 b	6.0 b
50	9.3 a	8.5 a	8.6 a	8.0 a	7.1 a	6.7 a
P	0.01	0.01	0.01	0.01	0.01	0.01
Soil type						
Ashhurst stony silt loam	8.8	9.4	9.0	8.7	7.1	7.1
Wairoa pumice soil	4.7	5.3	5.5	5.4	5.1	4.8
P	0.01	0.01	0.01	0.01	0.01	0.01

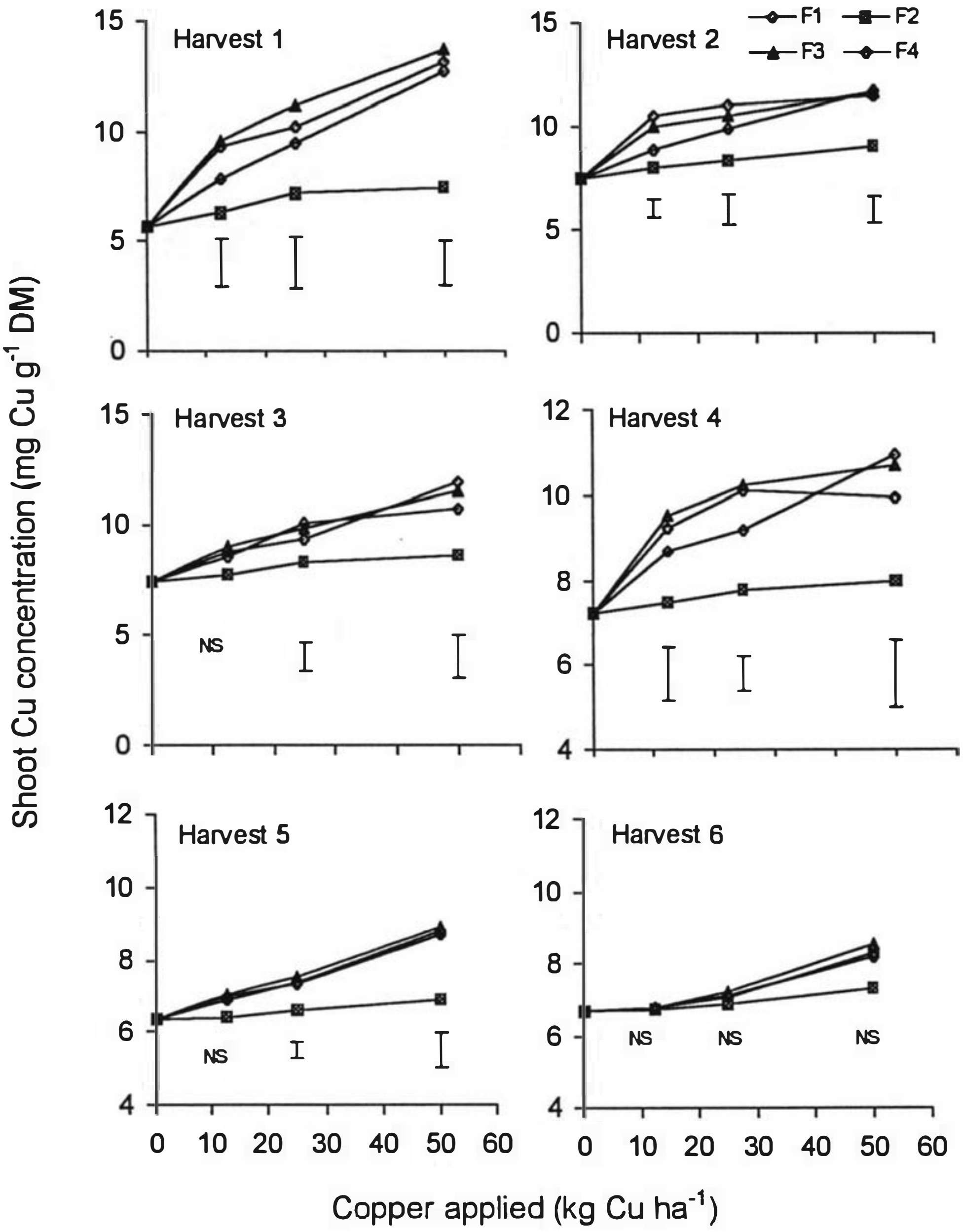

Fig. 8.2 Effect of Cu source and application rate on shoot Cu concentration of ryegrass grown in pots of Ashhurst stony silt loam over 6 harvests. F1: $CuSO_4.5H_2O$ chips (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ powder (<0.5 mm). Vertical error bars represent LSD at P = 0.05 and NS not significant.

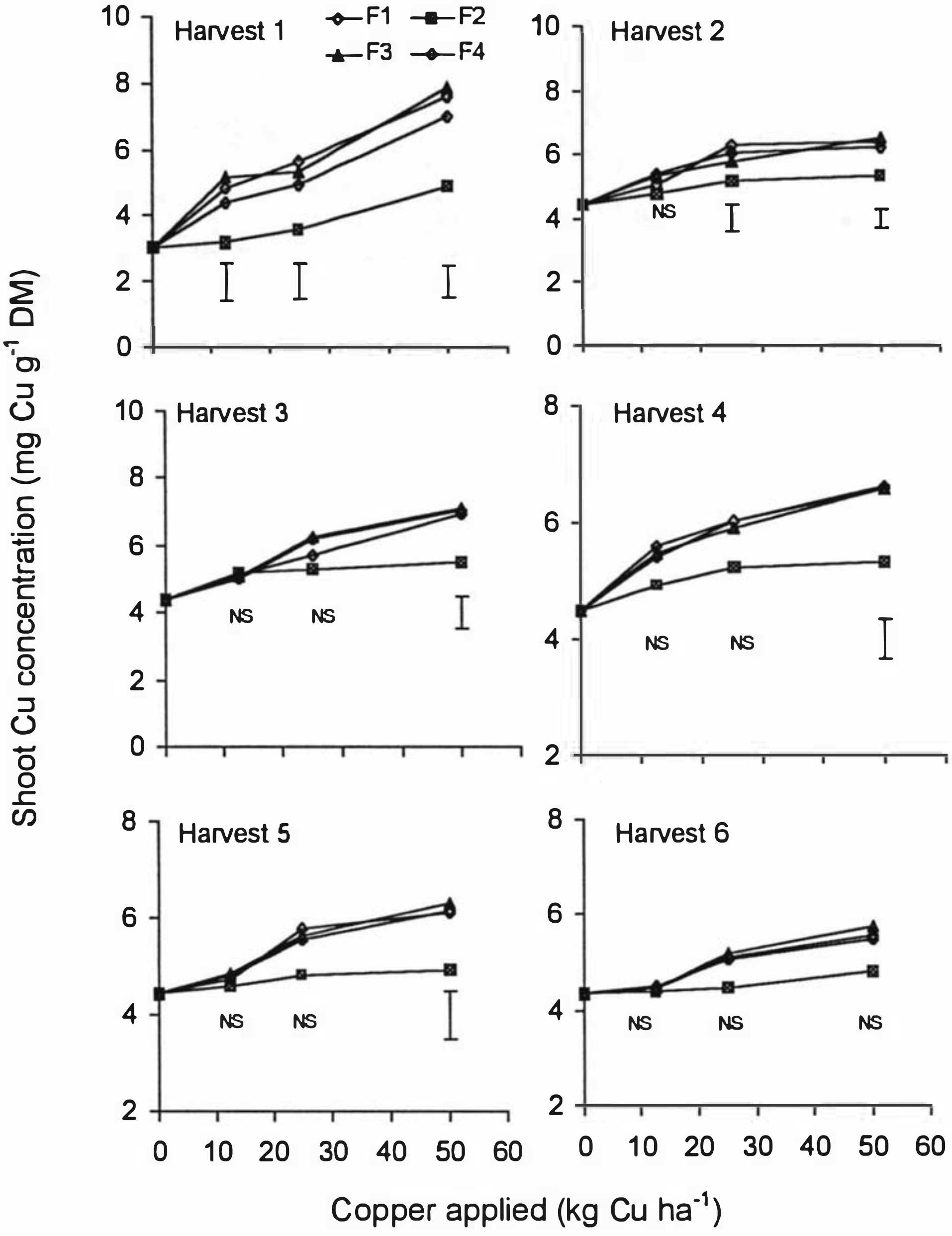

Fig. 8.3 Effect of Cu sources and rates on shoot Cu concentration of ryegrass grown in pots of Wairoa pumice soil over 6 harvests. F1: $CuSO_4.5H_2O$ chips (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ powder (<0.5 mm). Vertical error bars represent LSD at P = 0.05 and NS not significant.

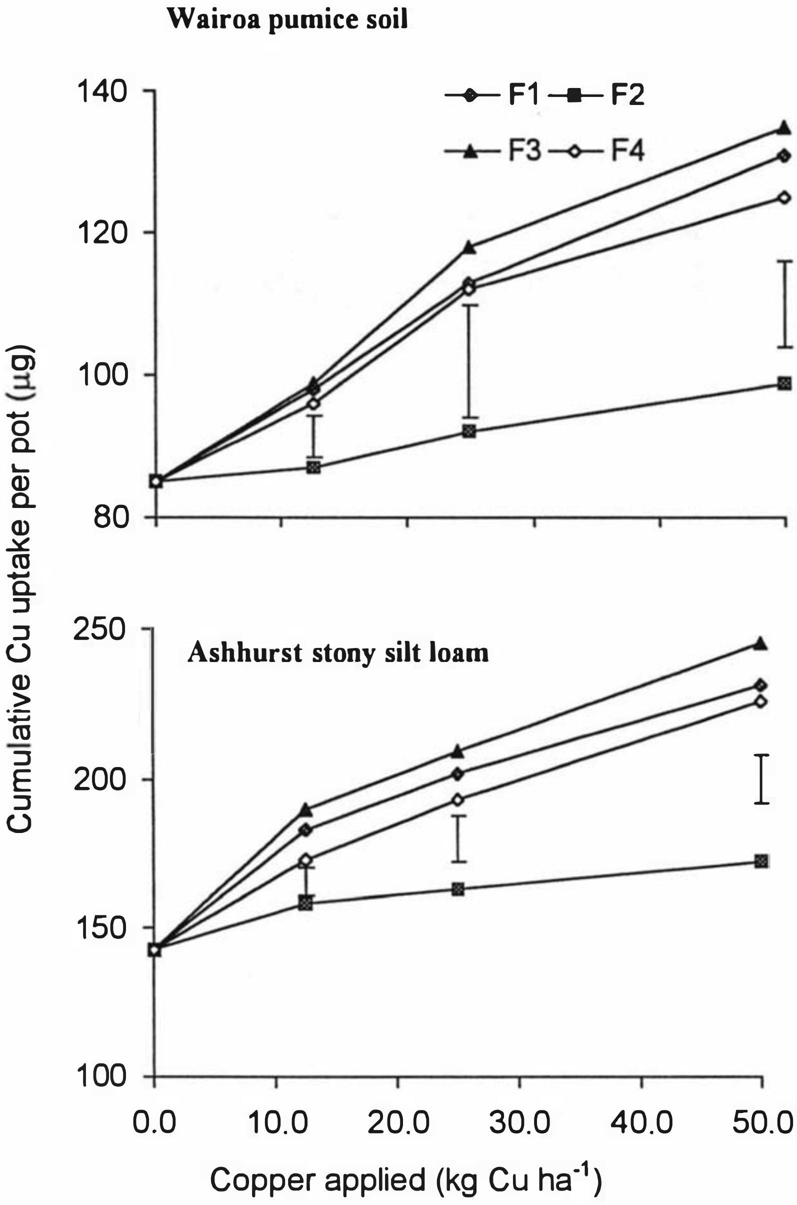

Fig. 8.4 Effect of Cu source and application rate on cumulative shoot Cu uptake, over 6 harvests, of ryegrass grown in pots of Wairoa pumice soil and Ashhurst stony silt loam. F1: $CuSO_4.5H_2O$ chips (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ powder (<0.5 mm). Vertical error bars represent LSD at P = 0.05.

8.3.5 Recovery of fertiliser Cu

An index for the apparent recovery of applied fertiliser Cu (R_i) was calculated for each fertiliser form and application rate (see Eqn. 7.2 in Section 7.3.5 for details).

The apparent recovery of Cu from the $CuSO_4$-based fertilisers was significantly higher compared to the $Cu(OH)_2$-based product (Table 8.3; Fig. 8.5). This is due to the lower rate of dissolution of the $Cu(OH)_2$-based fertiliser. In general, there were no significant differences among $CuSO_4.5H_2O$ (both granules and powder) and Ca-caseinate-$CuSO_4.5H_2O$ granule fertiliser except when applied at 12.5 kg Cu ha^{-1} to Ashhurst stony silt loam (Fig. 8.5).

The apparent recovery of fertiliser Cu decreased with increasing Cu application when ryegrass was grown in both Ashhurst and Wairoa pumice soils (Table 8.6; Fig. 8.5).

The apparent recovery of fertiliser Cu from the Ashhurst stony silt loam was significantly higher than that from the Wairoa pumice soil (Table 8.3).

The results showed that the percentage Cu recovery is very low, with ranges of 0.15 - 0.94% in Ashhurst stony silt loam and 0.10- 0.41% in Wairoa pumice soil. The lower recovery in Wairoa pumice soil is probably due to the higher organic matter content of this soil (8.2% organic carbon in Wairoa pumice soil compared to 4.1% organic carbon in Ashhurst stony silt loam, Table 8.1).

In order to compare the effectiveness of casein on the apparent recovery of fertiliser of chicory grown in pots of Manawatu silt loam over three harvests (see section 7.3.5 for details) and of ryegrass grown in Ashhurst stony silt loam and Wairoa pumice soil (this Chapter). The data were recalculated and represented in Table 8.5. At the same Cu application rate and during the same experimental period, the apparent recovery of $Cu(OH)_2$-based fertilisers and $CuSO_4.5H_2O$ alone by chicory grown in pots of Manawatu silt loam was lower than that of ryegrass grown in the Ashhurst stony silt loam. This is likely to be caused by the higher Cu uptake of chicory from the control treatment, and/or the effectiveness of granulating. The Ca-caseinate-$CuSO_4$ recovery was similar for both Manawatu and Ashhurst soils. Ryegrass recovery of Cu from Wairoa pumice soil fertilised with Ca-caseinate-$CuSO_4$ granule was lower than the other two soils.

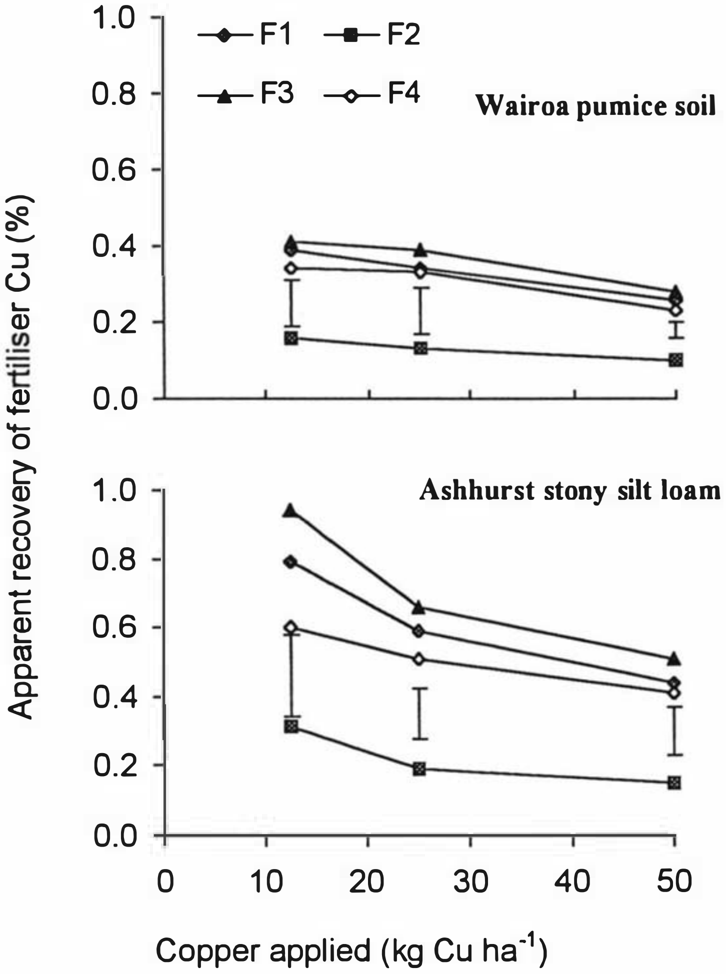

Fig. 8.5 Effect of Cu sources and rates on the apparent recovery of Cu from fertilisers, over 6 harvests, of ryegrass grown in pots of Ashhurst stony silt loam and Wairoa pumice soil. F1: $CuSO_4.5H_2O$ chips (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ powder (<0.5 mm). Vertical error bars represent LSD at P = 0.05.

Table 8. 5 Effect of plant species, soil type, Cu source and application rate on the apparent recovery of fertiliser Cu over the first 3 harvests (90 days after transplanting (chicory) and/or seedling thinning (ryegrass)).

	Soil type	Cu rate	Cu source	Apparent recovery of fertiliser Cu (%)
Chicory (Chapter 7)	Manawatu silt loam	50 mg Cu kg^{-1} soil	$Cu(OH)_2$	0.01
			$Cu(OH)_2$+casein	0.06
			$CuSO_4.5H_2O$	0.18
			$CuSO_4.5H_2O$+casein	0.32
Ryegrass	Ashhurst stony silt loam	50 kg Cu ha^{-1} (≈50 mg Cu kg^{-1} soil)*	$Cu(OH)_2$-based product (1.4-2mm)	0.11
			$CuSO_4.5H_2O$ (1.4-2mm)	0.32
			$CuSO_4.5H_2O$ (<0.5mm)	0.27
			Ca-caseinate-$CuSO_4$	0.34
	Wairoa pumice soil		$Cu(OH)_2$-based product (1.4-2mm)	0.06
			$CuSO_4.5H_2O$ (1.4-2mm)	0.17
			$CuSO_4.5H_2O$ (<0.5mm)	0.14
			Ca-caseinate-$CuSO_4$	0.18

* Assuming the bulk density of top 10 cm soil is 1000 kg m^{-3}

8.4 CONCLUSIONS AND FUTURE STUDIES

Granulated $Cu(OH)_2$-based fertiliser was less effective than $CuSO_4$-based fertilisers in supplying Cu to ryegrass. This is due to the low solubility of the $Cu(OH)_2$-based fertiliser. Addition of casein to granulated $CuSO_4$ can slightly improve Cu availability to ryegrass, but the Cu recovery by plants remains soil type dependent. Increased Cu uptake by ryegrass fertilised with Ca-caseinate-$CuSO_4$ fertiliser is likely caused by: (1) lower dissolution rate compared to $CuSO_4.5H_2O$ fertiliser; (2) decomposition of caseinate stimulating soil microbial activity, and then facilitating Cu-SOM moving to root surface. Chapter 9 investigates the Cu speciation in soil solutions, the relationship between plant Cu uptake and Cu species in soil solutions.

The combination of low corrosiveness and slightly improved agronomic effectiveness of the caseinate-$CuSO_4$ product suggests that this material have the potential to use in Cu topdressing of pastures. It is recommended that field trials be carried out to determine the true agronomic value of this product.

CHAPTER 9

LINKING PLANT COPPER UPTAKE AND SOIL SOLUTION COPPER POOLS IN COPPER-FERTILISED SOILS

Publications arising from chapter:

Liao, M.T., Hedley, M.J., Woolley, D.J., Longanthan, P., Brooks, R.R., Nichols, M.A. 2000 Knowledge to explain the "unexplainable" Cu uptake by plants from soils. In *Soil research--a knowledge industry for land-based exporters.* (Eds L D Currie, M J Hedley, D J Horne and Loganathan P). Occasional Report No. 13. Fertiliser and Lime Research Centre, Massey University, Palmerston North (Accepted).

Liao, M.T., Hedley, M.J., Woolley, D.J., Brooks, R.R., Nichols, M.A. 2000 The dependence of plant copper uptake on soil solution copper pools in copper-fertilized soils. Pp 99-104 *In* Proceedings of Soils 2000 conference of ASSSI (WA), Muresk Institute of Agriculture, Northam. WA. 11-13 July 2000.

9.1 INTRODUCTION

In Chapter 8, it was found that at a given Cu application rate, the Cu concentration of ryegrass plants grown in Ashhurst stony silt loam (Typic Orthic Brown Soil, Ashhurst) was nearly double of those grown in Wairoa pumice soil (Pumice Soil, Wairoa). In this chapter, the Cu speciation in soil solutions and the linkage between plant tissue concentrations and soil solution Cu pools are studied to develop a fuller understanding of the mechanisms of plant uptake Cu from soils.

Micronutrient metals predominately occur as insoluble forms in the solid phase of soil fractions. These insoluble forms are not directly accessible to plant roots for absorption (Lindsay, 1991; Shuman, 1991). The total soluble concentration of micronutrient ions, ion pairs, metal-chelates, and metal-complexes in most soil solutions is very low and in most soils, too low to supply adequate soluble quantities of certain micronutrients to meet the metabolic requirements of plants, especially for those plants growing on alkaline soils (Welch, 1995). The total soluble soil solution concentration range and the critical soluble micronutrient species activity range in soil solution for optimum plant growth in typical soils are listed in Table 9.1.

The mechanisms of Cu absorption by plant roots from soil are far from clear at the present. In soil systems, equilibrium exists between metals associated with solid phases and soluble metal species in the soil solution (Checkai et al., 1987). The knowledge of the concentration and form of Cu in the soil solution and the factors that control these is essential for understanding the mechanisms of its uptake by plants. In soil solution, Cu is almost entirely complexed either by root exudates and organic matter of soil origin (Geering and Hodgson, 1969; Hodgson et al., 1966; Römkens, 1998; Sanders, 1982). In a soil Cu fractionation study, McLaren and Crawford (1973) suggested that Cu concentration in soil solution is controlled by equilibria involving specifically adsorbed Cu and that most of the available Cu reserves are organically bound. There is considerable evidence that Cu is not absorbed as a chelate whether the ligands be EDTA or soil organic matter. Cu is absorbed more rapidly from Cu^{2+} solutions than from Cu EDTA or Cu DTPA (DeKock and Mitchell, 1957; Dragun et al., 1976), and high molecular weight humic materials from soil also decreased the rate of absorption of Cu (Graham, 1981). Furthermore, there is evidence that normally the ligand is lost prior to absorption (Graham, 1981).

Table 9.1 Major available soluble species, total soil solution concentrations of micronutrients (Fe, Mn, Zn and Cu) in soils (from Welch, 1995).

Micronutrient	Major available soluble species	Soil solution range
Fe	Fe^{2+}, ion pairs, and chelates, various Fe(II,III)-complexes	10^{-6} to 10^{-10} M
Mn	Mn^{2+}, various Mn(II,III,IV)-complexes, ion pairs, and chelates	10^{-4} to 10^{-9} M
Zn	Zn^{2+}, various Zn(II)-complexes, ion pairs, and chelates	10^{-6} to 10^{-9} M
Cu	Cu^{2+}, various Cu(I,II)-complexes, ion pairs, and chelates	10^{-6} to 10^{-9} M

Determination of the free metal ion concentration is of fundamental importance because these species enter into all equilibria between different forms of the metals in solution (Sanders, 1982). Soil solution chemistry plays an important role in predicting the bioavailability of nutrients to plants, especially when solution concentrations are expressed in terms of single ion activities (Adams, 1971; Sparks, 1984). To determine the activities of ionic species in soil solution, an extract of the soil solution must be obtained that accurately reflects the true soil solution. A rapid centrifugation method for obtaining soil solution has been developed by Elkhatib et al. (1987) which offers a reliable, rapid, contamination-free, and inexpensive method for obtaining soil solution for chemical characterisation.

Research which links ion activities to plant responses has a long history (Allen, et al., 1980; Checkai, et al., 1987; Linehan and Shepherd, 1979; Sparks, 1984). There is considerable evidence, primarily from solution culture experiments, that the plant uptake or toxicity of heavy metals show a marked dependence on the chemical speciation of the metal in solution (Parker et al., 1995), and higher-plant responses generally correlate best with the activity of free, uncomplexed metal ions in solution (Parker and Pedler, 1997). The knowledge of plant metal uptake and its speciation in soil solution is relative limited. Chaney et al. (1972) observed an obligatory reduction of ferric chelates in iron uptake of soybeans. Aluminium concentrations in coffee leaves have been shown to correlate with soil Al^{3+} activity (Pavan et al., 1982). Similarly, Cd^{2+} activity has been found to correlate with Cd uptake in soil-grown Swiss chard (Bingham et al., 1983, 1984). However, McLaughlin et al. (1997) found that potato tuber Cd concentrations were not related to soil solution Cd^{2+} activities or activities in diluted salt extracts of soil, but were related to the degrees of chloro-complexation of Cd in solution. Dragun et al. (1976) attempted to investigate the correlation between Cu uptake by maize and Cu^{2+} activity in a soil solution, but failed to draw any conclusions because of too much variability in their data.

The first comprehensive study on the relationship between Cu^{2+} activity and plant Cu uptake was reported by Minnich et al. (1987), who conducted a glasshouse pot trial in a Mardin silt loam amended with $CuSO_4$ (75-300 mg Cu kg^{-1}) or with sludge (0-120 g kg^{-1}). They found a good correlation between soil solution Cu^{2+} activity and Cu accumulation in young snapbean plants (a linear relation found for Cu salt treatments, and an exponential relation found for sludge treatments). But only a moderately correlation was found for total soluble Cu in Cu salt treatments, and no relation was found in sludge treatments. Recently, Some ecotoxicological studies also revealed that metal speciation in the solution phase is the one of the key factors that regulates metal uptake by plants as well as toxicity for soil and aquatic organisms (Marinussen, 1997; Renner, 1997). Most notably, Sauvé et al. (1996) investigated the tissue Cu concentrations of radish, lettuce and ryegrass and the Cu pools in soils (total, soluble and free ion Cu) in a greenhouse pot trial with urban contaminated soils. They concluded that the free metal concentration in the soil solution is a better indicator of plant metal bioavailability than either total or soluble metal. Furthermore, they hypothesized that metal bioavailability is a function of the concentration of free metal (e.g. Cu^{2+}).

Soil solution ion activity is only one of many factors that influence plant ion uptake from soils (Minnich et al., 1987). Khasawneh (1971) suggested that plant ion uptake is affected by ion intensity (ion concentration or ion activity), replenishment (the capacity of the soil to supply an ion), and relative intensity (the physiological consequences of variations in ionic composition). Although scientists often try to devise ways to study each of these factors separately, plants grown in soil necessarily reflect the combination of these factors. It is hypothesised that the soil's capacity to maintain or buffer the Cu^{2+} activity in soil solution is one of the factors limiting plant Cu uptake.

The objectives of this study were: (1) to identify the relationship between plant Cu uptake and the soil solution Cu pools: soluble Cu pool and the free Cu^{2+} pool; (2) to test the hypothesis that Cu bioavailability in soils is controlled by the free Cu^{2+} activity in the soil solution and the soil's capacity to maintain or buffer that Cu^{2+} activity.

9.2 MATERIALS AND METHODS

9.2.1 Origin of soils

After the plant growth experiment described in Chapter 8, the soils from duplicate pots of each Cu fertiliser treatment were used for soil solution studies.

9.2.2 Extraction of soil solution

Soil solutions from Cu fertilised and unfertilised soils were extracted from moist soils (80% pot field capacity) by centrifugation at 10,000 rpm (12,000 RCF) in a refrigerated centrifuge at 5°C (Elkhatib et al. 1987).

9.2.3 Chemical characteristics of soil solutions

Soil solution pH and electrical conductivity (EC) were measured immediately using a portion of the soil solution, and the remainder of the solution was filtered through 0.22-μm millipore filters. The ionic strength of the soil solution was calculated as described by Lindsay (1979):

$$I\,(\mathrm{mol\,L^{-1}}) = 0.013EC\,(\mathrm{ds\,m^{-1}}) \qquad \text{(Eqn. 9.1)}$$

where I is solution ionic strength and EC is the solution electrical conductivity.

Total dissolved Cu concentrations in the soil solutions were measured using GFAAS (see Section 2.2.1.3). The free Cu^{2+} concentrations were measured using an Orion 9629BN Ionplus^{TM} Cupric Electrode. The concentrations in solution were transformed into activities using the Davies equation (Stumm and Morgan, 1981):

$$\mathrm{Log} f_i = - CI \qquad \text{(Eqn. 9.2)}$$

where f_i is activity coefficient; the C value differs for zero, single and double charged ion pairs; I is ionic strength.

9.2.4 Free Cu^{2+}/total solution Cu equilibrium in soil solutions

Copper binding with organic matter is facilitated through carboxylic groups. The stability of Cu-complexes containing carboxylic acid functional groups is normally sensitive to solution pH. The proportion of total soluble Cu present as Cu^{2+} ions as solution pH varies provides a 'signature' for the Cu-organic complex system. The free Cu^{2+} concentrations in soil solutions at varying solution pH values were measured (see Section 5.2.3 for Methods).

9.2.5 Data analysis

The significance of differences between treatment means of total solution Cu concentration and the percentages of free Cu^{2+} as total solution Cu was tested using SAS GLM procedure (SAS Institute, 1990), and the means were separated by LSD. Regression analysis was performed to investigate the relationships between soil Cu pools and plant Cu uptake (shoot Cu concentration, and shoot Cu accumulation).

9.3 RESULTS AND DISCUSSION

9.3.1 Soil solution pH and total dissolved Cu concentrations

The pH of soil solutions from both Wairoa pumice soil and Ashhurst stony silt loam was in a narrow range 5.2-5.63 and 5.3-5.67 respectively (Fig. 9.1). The ranges of total soil solution Cu from Ashhurst stony silt loam and Wairoa pumice soil were 83-310 and 89-390 μg Cu L^{-1} respectively (Fig. 9.1, Table 9.1).

Table 9.2 Effect of the Cu source and application rate on the total soil solution Cu and the percentage of free Cu^{2+} as the total soil solution Cu concentration in the Ashhurst stony silt loam and Wairoa pumice soil. Data shown are means ± S.E. (n=2).

———Treatment——-		Total solution Cu ($\mu g\ L^{-1}$)		Free Cu^{2+} (%)	
Fertiliser	Level ($kg\ Cu\ ha^{-1}$)	Ashhurst	Wairoa	Ashhurst	Wairoa
$CuSO_4.5H_2O$ (1.4-2 mm)	Control	83±2	89±3	0.88	0.72
	12.5	89±3	94±3	1.60	0.45
	25	105±4	115±8	1.16	0.56
	50	241±15	275±12	0.35	0.27
$Cu(OH)_2$-based product (1.4-2 mm)	Control	84±3	89±3	0.88	0.72
	12.5	86±8	90±6	1.07	0.85
	25	89±3	95±9	0.89	0.51
	50	115±11	130±9	0.84	0.35
Ca-caseinate-$CuSO_4$ (1.4-2mm)	Control	83±2	89±3	0.88	0.72
	12.5	91±5	115±7	1.06	0.38
	25	129±8	150±8	0.75	0.48
	50	310±16	390±13	0.36	0.16
$CuSO_4.5H_2O$ (< 0.5 mm)	Control	83±2	89±3	0.88	0.72
	12.5	91±3	100±6	1.08	0.74
	25	124±7	150±6	0.77	0.55
	50	235±10	325±13	0.37	0.25

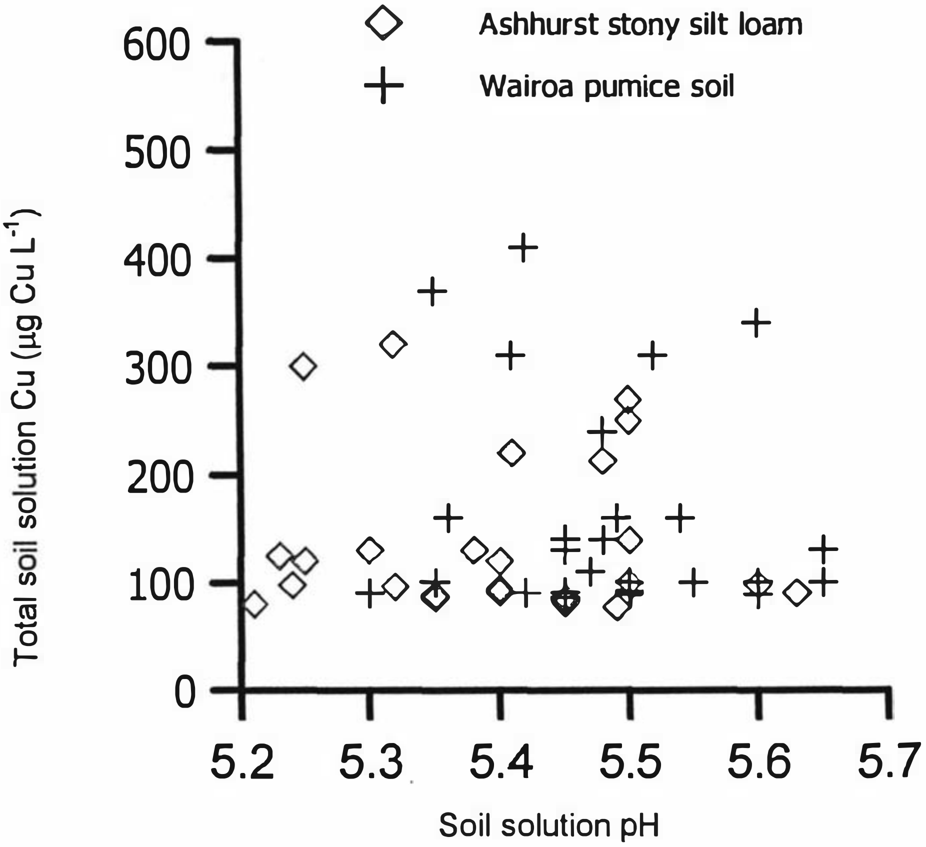

Fig. 9.1 The lack of a relationship between soil solution pH and total soil solution Cu concentration in soil solutions extracted from pots of Ashhurst stony silt loam and Wairoa pumice soil fertilised with different sources and rates of Cu fertilisers.

9.3.2 Soil solution free Cu^{2+} concentrations

Free Cu^{2+} comprised a very small part of the total dissolved Cu. On average, free Cu^{2+} comprised 0.35-1.60 and 0.16-0.85% of total dissolved Cu in the soil solution from Ashhurst stony silt loam and Wairoa pumice soil, respectively (Table 9.1). The proportions of Cu present in displaced soil solutions as cupric ion are commonly extremely small. The proportions present as inorganic complex ions, calculated from knowledge of their formation constants (Stiff, 1971), and of the concentrations of inorganic ions, are also small. Nearly all the Cu in these solutions is therefore present in organic complexes, as proposed by Hodgson and coworkers (1965, 1966). Even in the field, organic complexes are the predominant form of Cu in the soil solution (Sanders, 1982). At pH levels higher than 5, usually more than 99% of the total dissolved Cu concentration is bound to dissolved organic carbon (DOC) (Römkens, 1998, Temminghoff, 1998; Temminghoff et al., 1997). In soil solutions, up to 98% of the Cu^{2+} is complexed to the low molecular-weight organic compounds (Hodgson et al., 1966; Loganathan and Hedley, 1997).

9.3.3 Relationship between soil solution pH, total dissolved Cu and free Cu^{2+} concentrations

The total dissolved Cu concentration in the extracted soil solutions was unrelated to the pH of soil solutions from both the Ashhurst stony silt loam and Wairoa pumice soil (Fig. 9.1).

Within each soil, the pCu^{2+} was strongly related to soil solution pH for both Wairoa pumice soil ($y = 1.749x - 1.515$, $r^2 = 0.771$, $n = 26$, $P < 0.001$) and Ashhurst stony silt loam ($y = 0.788x + 3.683$, $r^2 = 0.949$, $n = 26$, $P < 0.001$) (Fig. 9.2). The pCu^{2+} is defined as the negative log(10) of the active molar concentration of Cu^{2+} ions, i.e., the active concentration of free cupric ions were calculated using the Davies theory activity coefficient.

The concentrations of soluble Cu and its free ionic activity in soil solution have been repeatedly reported to be affected by soil pH (Harsh and Doner, 1984;

Jeffery and Uren, 1983; Kuo and Jellum, 1980; Ma and Lindsay, 1995). Generally, soil researchers agree that concentrations of dissolved Cu and its free ionic activity in soil solution decrease with the increase in soil pH (El-Falaky et al., 1991; Römkens et al., 1999; Salam and Helmke, 1998;). An increase in dissolved organic matter and pH usually reduces the ion activity in solution due to complexation. Especially metals like Cu and Pb are known to form very stable metal-organic complexes (Stevenson, 1994).

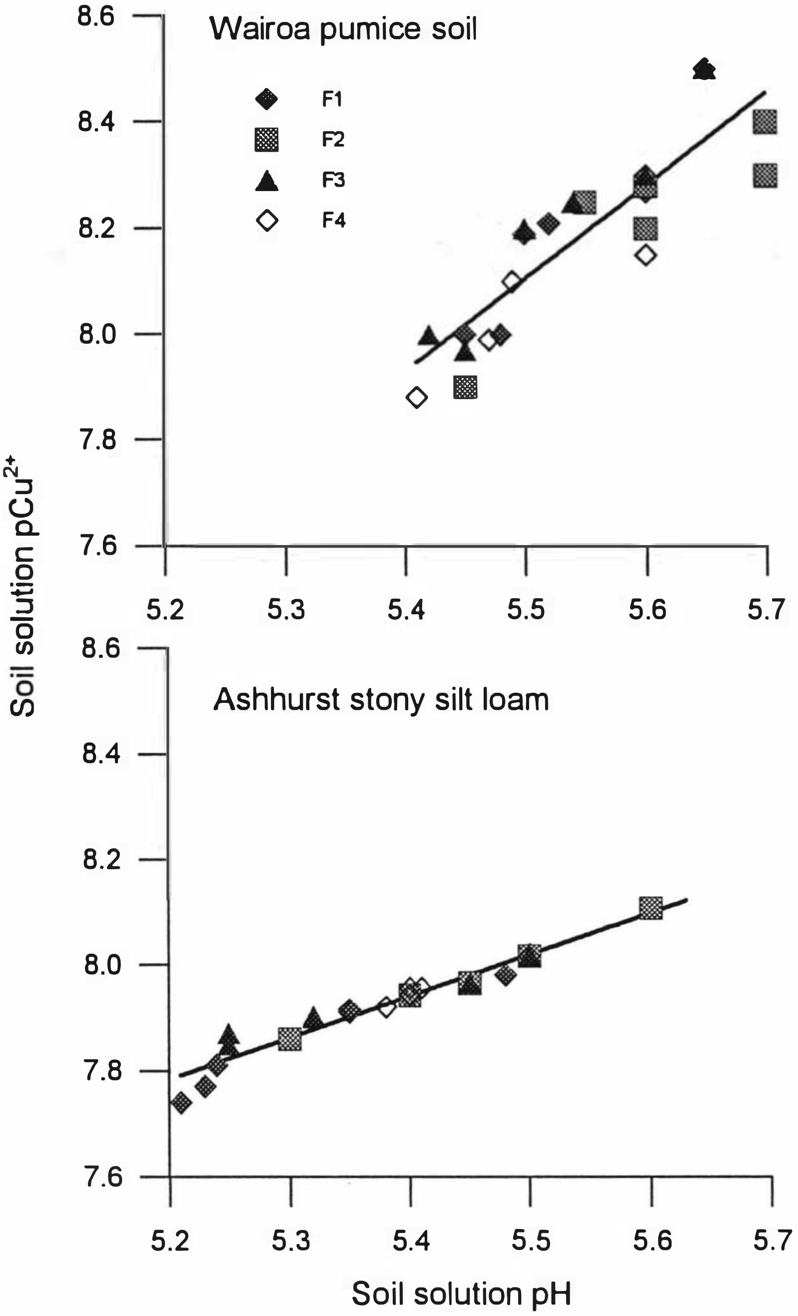

Fig. 9.2 Relationship between soil solution pH and soil solution pCu^{2+}. F1: $CuSO_4.5H_2O$ (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ (<0.5 mm).

9.3.4 Soil solution Cu activity (pCu^{2+}), total dissolved soil solution Cu concentration and shoot Cu concentration, and total shoot Cu uptake at the last harvest

Copper is generally considered to be taken up by plant roots in the form of free Cu^{2+} and therefore one would expect a good relationship between Cu^{2+}concentration in soil solution (expressed as pCu^{2+}) and Cu concentration in plants. The results from our pot trial however showed that there was no such relationship for either the Wairoa pumice soil or the Ashhurst stony silt loam (Fig. 9.3). Instead, significant linear relationships were found between total soil solution Cu and shoot Cu concentration (Wairoa pumice soil: $y = 0.0053x + 3.963$, $r^2 = 0.590$, $n = 26$, $P < 0.001$; Ashhurst stony silt loam: $y = 0.00875x + 6.108$, $r^2 = 0.734$, $n = 26$, $P < 0.001$) (Fig. 9.4), and total shoot Cu uptake at last harvest (Wairoa pumice soil: $y = 0.0124x + 8.060$, $r^2 = 0.695$, $n = 26$, $P < 0.001$; Ashhurst stony silt loam: $y = 0.0168x + 12.860$, $r^2 = 0.453$, $n = 26$, $P < 0.001$) within each soil (Fig. 9.5). A single relationship, however, cannot be derived for both soils (Fig. 9.6). These results indicated that the plant Cu uptake patterns from Wairoa pumice soil and Ashhurst stony silt loam are different. McLaren et al. (1984) found that the overall correlations between extractable soil Cu and Zn levels and the concentrations of these elements in lucerne grown on 8 soils series, in the Canterbury Plains of New Zealand, were poor. However, there were significant correlations between extractable Cu and Zn levels in individual soil series and the mean Cu and Zn concentrations of lucerne growing on the same series. Loganathan et al. (1998), measuring the total dissolved Cu concentrations of 17 soils collected from both north and south-island of New Zealand, found that the Cu concentrations of pastures grown on these soils were not related to total dissolved Cu concentrations in soil solutions extracted from the moist field soils.

These results (Fig. 9.3, 9.4, 9.5, 9.6) contrast with those of Minnich et al. (1987), who conducted a glasshouse pot trail in Mardin silt loam amended with $CuSO_4$ at 75-300 mg Cu kg^{-1} soil or sludge at 0-120 g kg^{-1} to show that young snapbean plant shoot Cu concentration and total shoot Cu uptake generally correlate best with the activity of free, uncomplexed metal ions in solution, and are largely indifferent to the presence of soluble metal-ligand complexes. Recently, Sauvé et al. (1996) found that the tissue Cu concentrations of radish, lettuce and ryegrass grown in urban contaminated soils in a glasshouse pot trial were linearly related to the cupric ion activity and the total soil Cu, but cupric ion activity yielded a higher level of statistical significance. No relation between total soil solution Cu and plant tissue Cu concentration was found. They concluded that free metal in the soil solution is a better indicator of plant metal bioavailability than either total or soluble metal. It was hypothesised that copper bioavailability in soils is controlled by the free Cu^{2+} activity in the soil solution and the soil's capacity to maintain or buffer that Cu^{2+} activity (Sauvé et al., 1996). Total dissolved Cu in soil solution mainly consists of Cu bound to dissolved organic matter and roots cannot directly take up these large molecules.

Some recent studies using solutions with carefully controlled activities of Cd^{2+} and $CdCl_n^{2-n}$ have suggested that the free ion is more available than the Cl-complexed Cd, but that the latter is not altogether "unavailable", i.e., it too contributes to plant Cd uptake (Smolders and McLaughlin, 1996a, b). Studies on phytoremediation that found that metal ligands, such as EDTA, could markedly increase plant metal uptake from soil (Bennett, 1998; McGrath, 1998; Robinson, 1997). One explanation for these observations is that breaks in the endodermal barrier at root apices and at the sites of lateral root initiation might permit passive, convective uptake of intact metal-ligand complexes in the transpiration flow of any water that is taken up apoplastically (Bell et al., 1991; Smolders and McLaughlin, 1996a, b).

Alternatively, at constant Cu^{2+} activity (but variable total Cu), Checkai et al. (1987) attributed the enhanced Cu uptake due to chelates to enhance diffusion of Cu across the undisturbed layer of water surrounding the roots, rather than to uptake of some Cu as the intact Cu chelate. Therefore, the importance of maintaining high total concentrations near the plant root surfaces should not be disregarded because both ionic and complexed species contribute to diffusive transport, which may be the major factor limiting the uptake rate in natural soil systems (Checkai, et al., 1987). McLaren et al (1990) indicated that availability of soil Cu to plants was determined by concentration and forms of Cu present in soil solution, and the ability of the soil to replenish the solution from Cu retained in the solid phase. Khasawneh (1971) also suggested that the capacity of soil to supply an ion (replenishment) is one of many factors that influence plant ion uptake. The positive relationships, reported in this thesis (Fig. 9.4 and 9.5), between the total soil solution Cu concentrations and ryegrass shoot Cu uptake are consistent with the agreement that diffusive flux of soluble Cu to roots is the limiting factor for Cu uptake in the Wairoa pumice soil and Ashhurst stony silt loam.

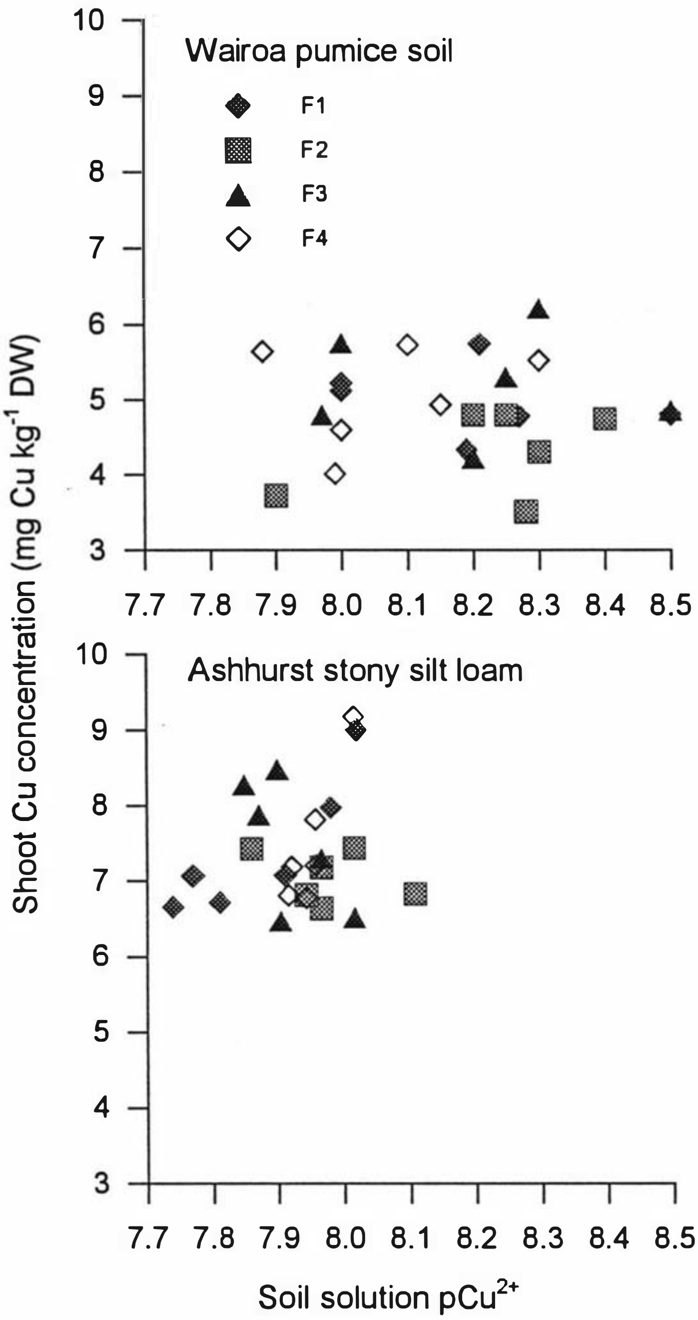

Fig. 9.3 The lack of a relationship between ryegrass shoot Cu concentrations at 6th harvest and soil solution pCu^{2+}. F1: $CuSO_4.5H_2O$ (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ (<0.5 mm).

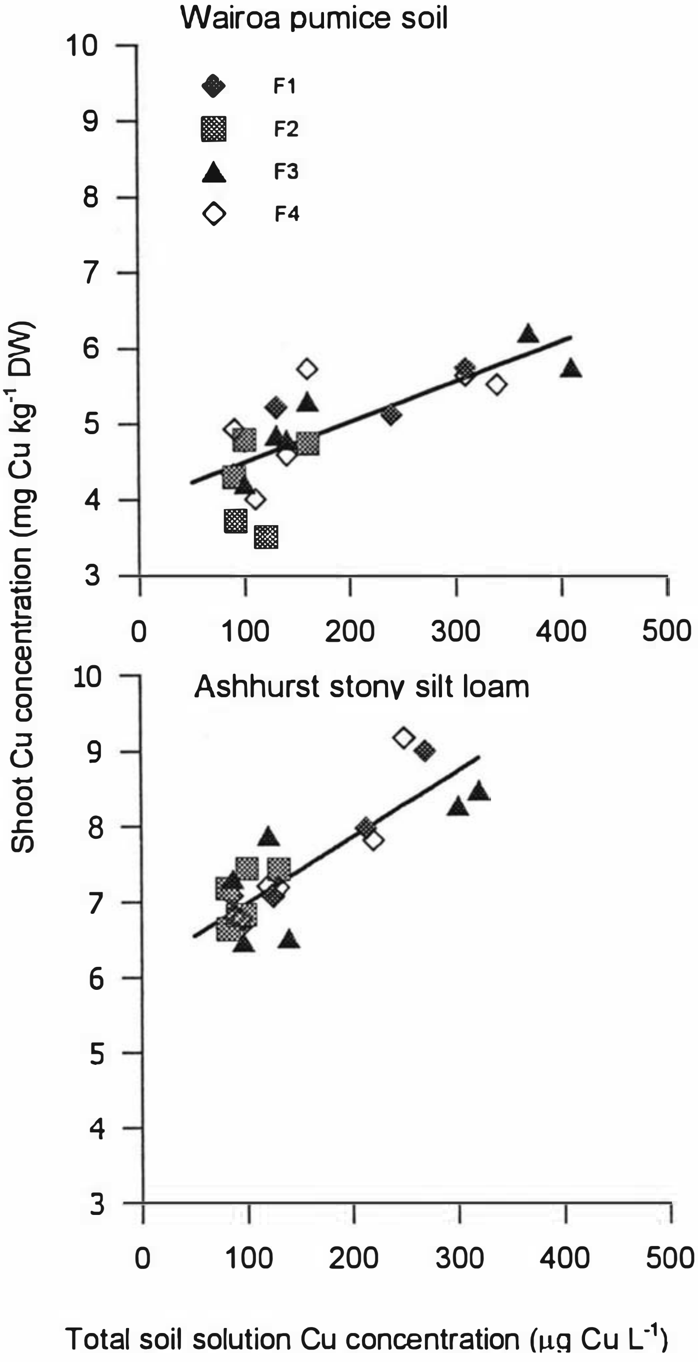

Fig. 9.4 Relationship between ryegrass shoot Cu concentrations at 6th harvest and total soil solution Cu concentration. F1: $CuSO_4.5H_2O$ (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ (<0.5 mm).

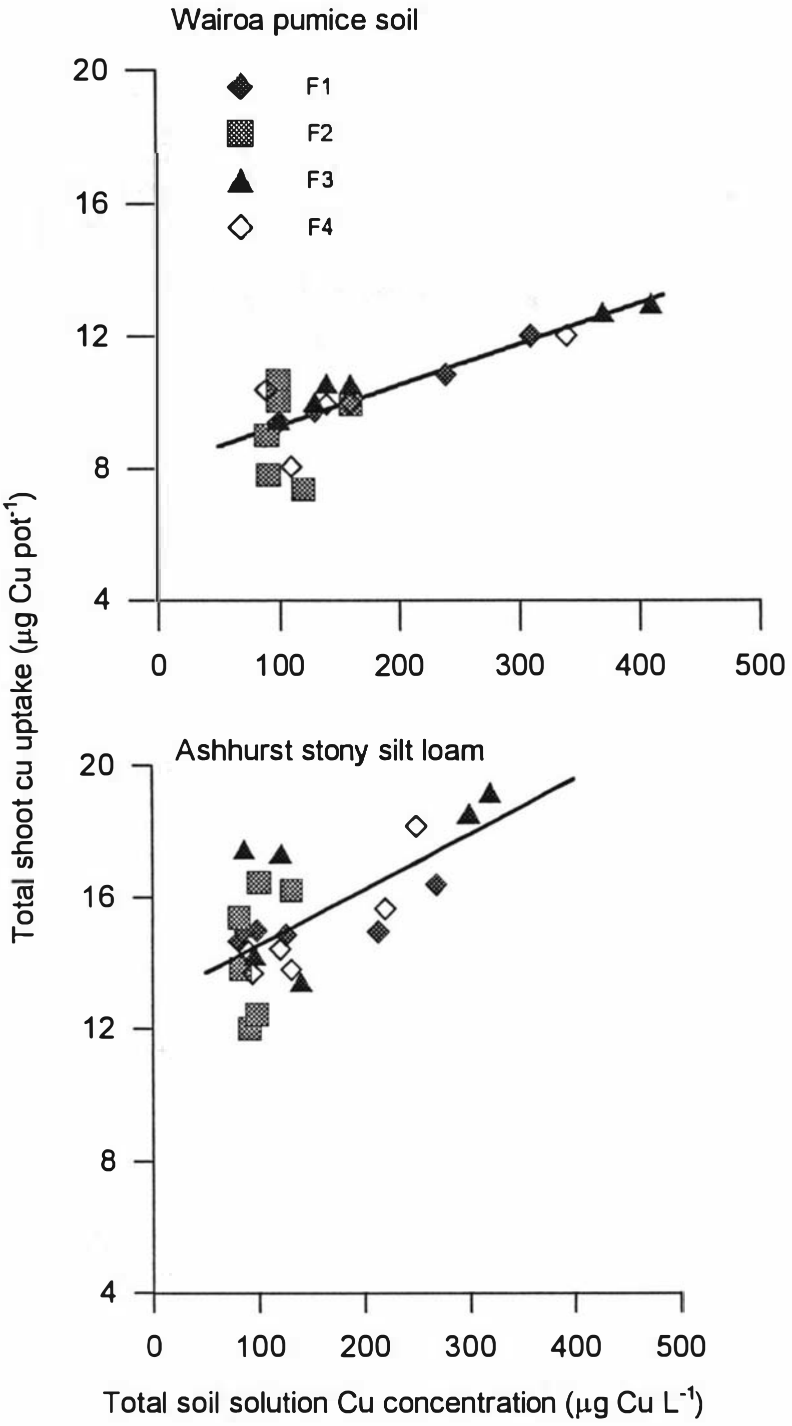

Fig. 9. 5 Relationship between shoot Cu uptake at 6th harvest and total soil solution Cu concentrations. F1: $CuSO_4.5H_2O$ (1.4-2 mm); F2: $Cu(OH)_2$-based product (1.4-2 mm); F3: Ca-caseinate-$CuSO_4.5H_2O$ (1.4-2 mm); F4: $CuSO_4.5H_2O$ (<0.5 mm).

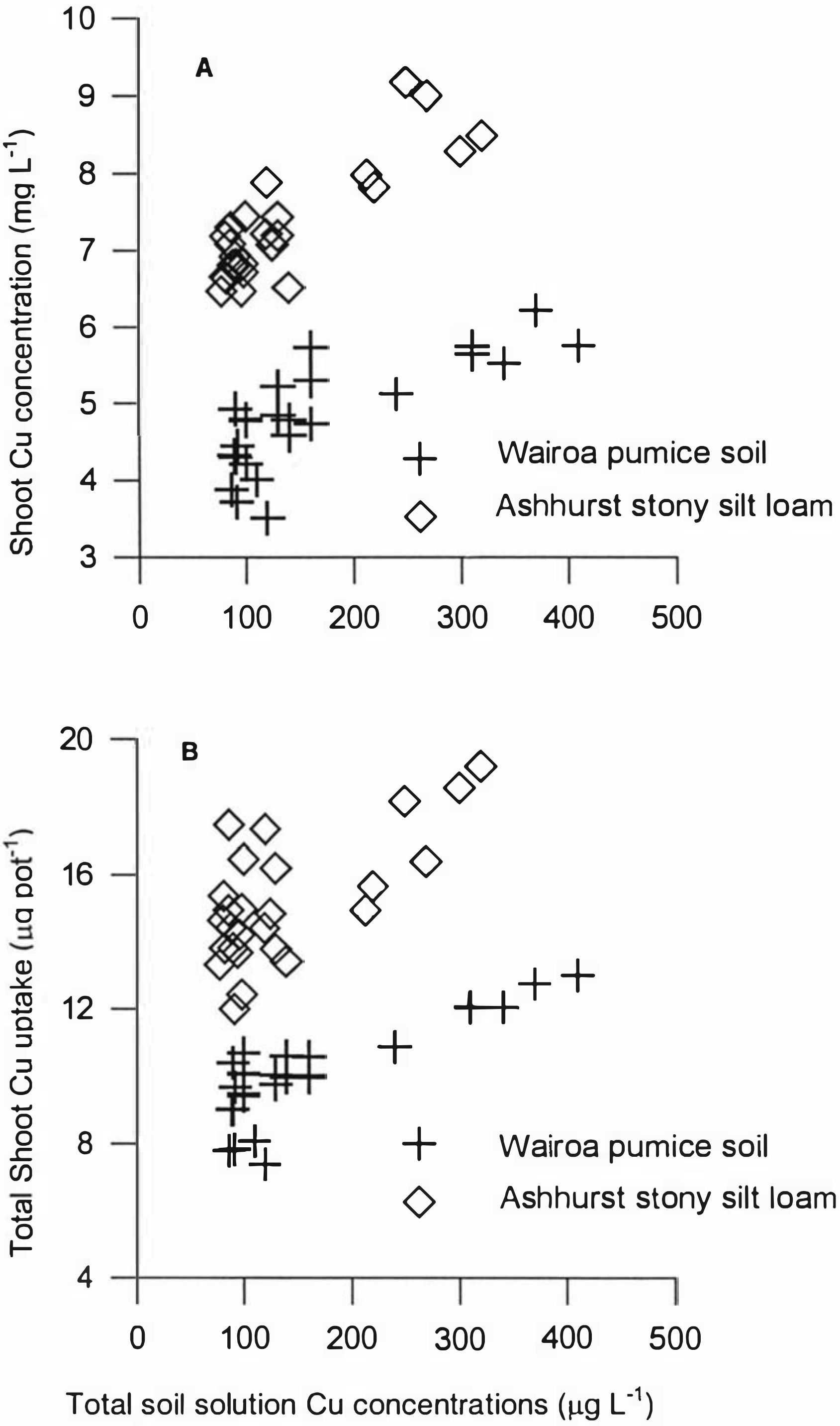

Fig. 9.6 The lack of a relationship between the total dissolved soil solution Cu concentrations and shoot Cu concentrations (A), and total shoot Cu uptake (B) at the 6th harvest in both Ashhurst stony silt loam and Wairoa pumice soil.

9.3.5 Stability of Cu-organic complexes in soil solution

In order to explain different Cu uptake by plants in the Wairoa pumice soil and Ashhurst stony silt loam, it is proposed that when organically bound Cu in soil solution moves to the vicinity of root surface it dissociates to release Cu^{2+} for plant uptake. It is also necessary to explain how Cu bound strongly to soluble soil organic complexes is released and moves into the plant to be bound by the amino acids in the xylem (Liao et al., 2000b, Chapter 5).

Many studies have shown that soil pH near the root surface is much lower than in the bulk soil (Bernal et al., 1994; Gahoonia and Nielsen, 1992; McGrath et al., 1997; Ruiz and Arvieu, 1990; Sarong et al., 1989; Wang and Zabowski, 1998; Youssef and Chino, 1989, 1991; Zoysa et al., 1999). At these low pH values, many Cu-bound organic molecules are less stable than at bulk soil pH and therefore they ionise to release Cu^{2+} (see Chapter 5). This was demonstrated in this present experiment where the Cu^{2+} concentration increased when the pH of soil solutions from Ashhurst stony silt loam and Wairoa pumice soil was reduced by the addition of acid (Fig. 9.7, 9.8).

The extent of the increase in Cu^{2+} concentrations per unit decrease in pH depends on the concentration and type of organic molecules in the soil solution (Jeffery and Uren, 1983; Liu et al., 1994; McBride, et al., 1998; McGrath et al., 1988; Salam and Helmke, 1998). In the Wairoa pumice soil which has twice as much organic carbon as the Ashhurst stony silt loam (Liao et al., 2000c, Chapter 8), the increase in Cu^{2+} concentration per unit pH decrease was approximately 30% of that in the Ashhurst stony silt loam (Fig. 9.7, 9.8). This, and the higher soil solution pH (Fig. 9.1) of Wairoa soil, are expected to explain the lower Cu uptake by plants in this soil compared to that in the Ashhurst stony silt loam. In the Ashhurst stony silt loam a slight reduction in root surface pH below 5.2 will release 3 times more Cu^{2+} that can move into the root, than in the Wairoa pumice soil.

It should be noted that the relationship between soil solution pCu^{2+} and soil solution pH of freshly extracted soil solutions show a greater increase in free

Cu^{2+} for the Wairoa pumice soil (Fig. 9.2). It is estimated that a pH decrease of 0.1 unit from pH 5.5 to 5.4 would increase the soil solution free Cu^{2+} concentrations in the Wairoa pumice soil and Ashhurst stony silt loam by 0.34 and 0.13 $\mu g\ Cu^{2+}\ L^{-1}$, respectively. The difference between these results (Fig. 9.2) and the titration of the soil solutions from two treatments (Fig. 9.7, 9.8) is currently unexplainable. The difference may result from the influence of soil solid phase, which is absent for the soil solution titrations.

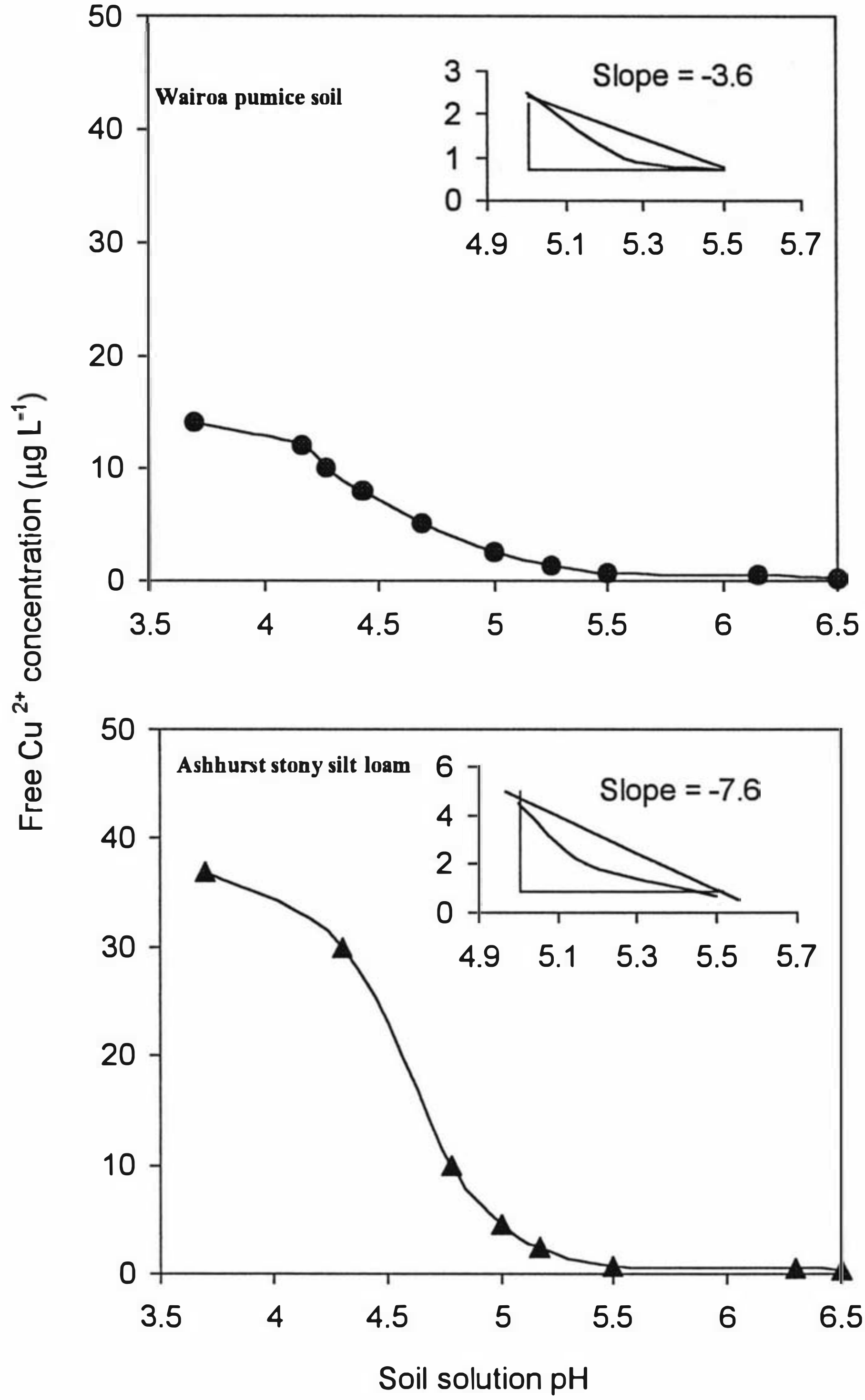

Fig. 9.7 The relationship between free Cu^{2+} concentration and pH of soil solutions from pots treated with 50 kg Cu ha^{-1} as $CuSO_4.5H_2O$ (1.4-2 mm).

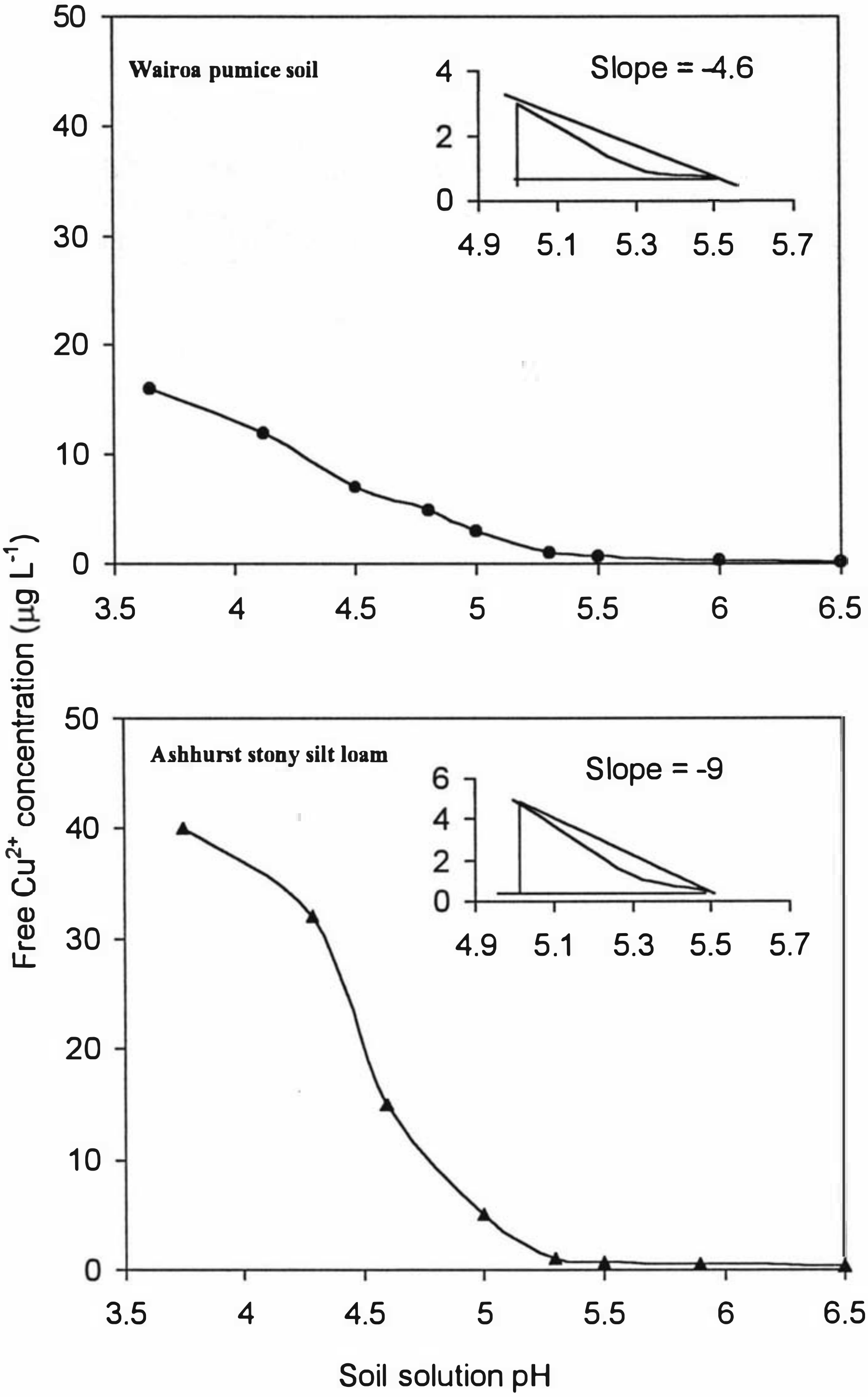

Fig. 9.8 The relationship between free Cu^{2+} concentration and pH of soil solutions from pots treated with 50 kg Cu ha^{-1} as Ca-caseinate-$CuSO_4$ (1.4-2 mm).

9.3.6 Conceptual model of plant Cu uptake from soils

From the results discussed above, and the results presented in Chapter 5, a simple model for plant Cu uptake by plants can be conceived for the Ashhurst stony silt loam. (1) Cu in soil solution, where most of Cu is bound to soil organic matter, moves to rhizosphere soils with mass flow or diffusion. (2) When this Cu moves near to the root surface, particularly near the elongation zone or zone of root hair proliferation where the pH is likely lower than the bulk soil, the Cu-SOM ionises to release free Cu^{2+} ions. (3) To achieve passive uptake of free Cu^{2+} ions, the pH in the rhizosphere of the Ashhurst stony silt loam has to decrease to < pH 5.1 and the plant root xylem be at > pH 5.6 (Fig. 9.9). In this way root surface Cu^{2+} concentrations of 2.5-3 $\mu g\ Cu^{2+}\ L^{-1}$ may allow diffusion of free Cu^{2+} to xylem sap free Cu^{2+} concentrations of 1.6-3.2 $\mu g\ Cu^{2+}\ L^{-1}$. (4) Plant roots take up the free Cu^{2+} on the root surface, and then loading into xylem, where pH is in the range 5.5 to 6.5, Cu forms strong complexes primary with NA and His, and transport to shoots via xylem sap (Fig. 9.9).

Passive uptake of free Cu^{2+} ions in the Wairoa pumice soil is less likely to occur because a rhizosphere pH of <5.0 would be required.

If pH does not change across the rhizosphere/root as described above, then the conditions represented in Fig. 9.9 would suggest that root surface, active carrier binding sites for Cu must have association constants for Cu greater than the soluble soil organic matter at the same pH. Root-exuded mucilage is unlikely to present a barrier to this process since reported association constants for mulcilage-Cu are only 3.4-5.4 (LogKa) (Mench et al., 1987, 1988; Morel et al., 1986), compared to soil organic matter of 7.8-11.3 (Stevenson and Fitch) and xylem amino acids (His and NA) of 17.5-18.6 (May et al., 1977; Stephan et al., 1996)

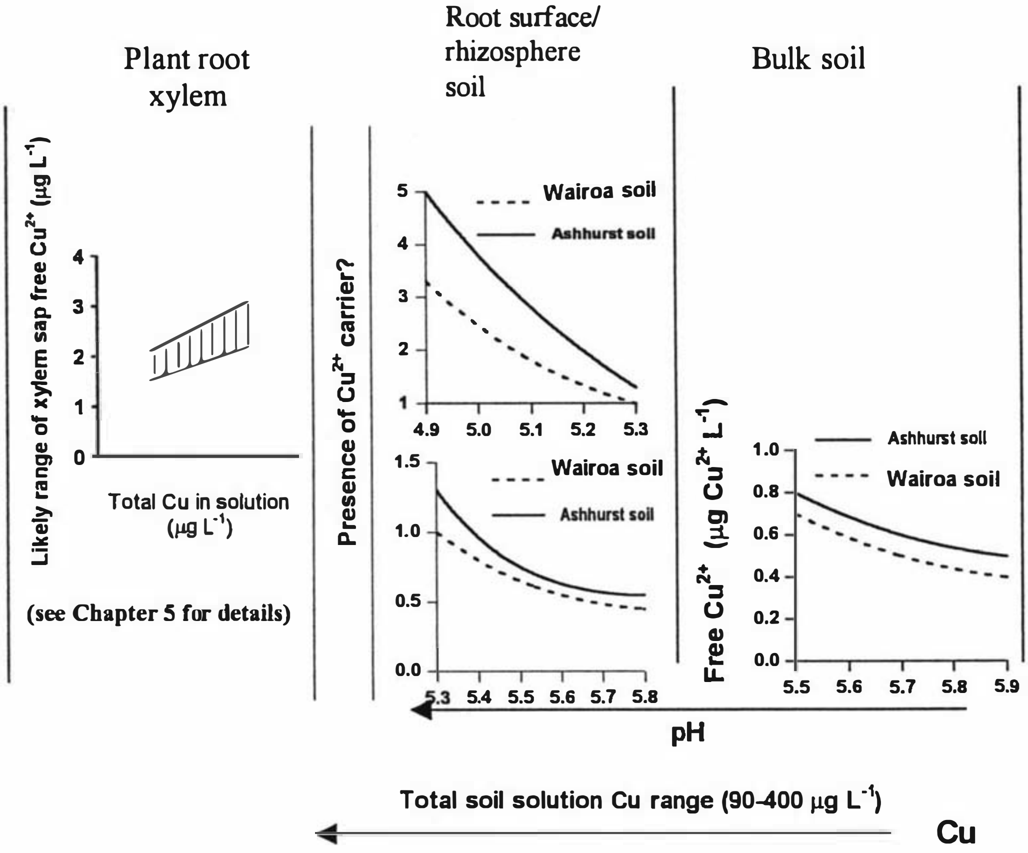

Fig. 9.9 The conceptual model of plant Cu uptake from soils.

9.4 CONCLUSIONS AND IMPLICATIONS

It is hypothesised that the rate of Cu uptake by plants depends both on the concentration of organically complexed Cu in the soil solution and the stability of this complex to pH change. Further investigations of stability of Cu-SOM complexes in a wider range soils could lead to a fuller understanding of the factors limiting the effectiveness of $CuSO_4$ topdressing as a strategy to ameliorate Cu deficiency in grazing animals.

A concept of a pH-sensitive two-phase mechanism of Cu transport to root, low rhizosphere pH-driven release of free Cu^{2+}, and uptake by plant roots was proposed. If pH change across the rhizosphere/root boundary does not occur, a root surface carrier with a high association constant for Cu must be present.

CHAPTER 10
SUMMARY

10.1 BACKGROUND

Copper deficiency in grazing animals is widespread in New Zealand, whether caused by low Cu in forage (primary deficiency) or by dietary interaction with other elements (eg. Mo, S and/or Fe) (secondary deficiency). Low uptake of Cu by pasture plants is a possible cause of primary Cu deficiency in New Zealand's grazing animals. The philosophy of this thesis was to examine the mechanisms of plant Cu uptake and transport from root to shoot in a range of plants capable of greater Cu uptake than pasture plants. The factors constraining Cu uptake by plants from Cu fertilised soils were also examined with the aim of developing strategies to improve Cu uptake by pasture crops.

10.2 SUMMARY

Chapters 2 and 3 — Copper uptake by heavy-metal hyperaccumulator plants

Three experiments were conducted to investigate Cu uptake by the Cu/Co “hyperaccumulator” *Haumaniastrum katangense* (Chapter 2). In experiment one, *H. katangense* plants were grown in an artificial substrate with 0 or 500 mg kg^{-1} Cu additions. In experiment two, plants were grown hydroponically in a Nutrient Film Technique system (NFT) in a modified half-strength Hoagland solution with 0.05 mg L^{-1} background Cu, with additional 0, 5, 10, 20 mg Cu L^{-1} supplied as $CuSO_4$. In experiment three, plants were grown in pots containing a sample of soil collected from the field in Zaïre. Cu hyperaccumulation in shoots of *H. katangense* was not found despite high Cu concentrations in various rooting media.

The Ni hyperaccumulators *Alyssum bertolonii* and *Berkheya coddii* were grown in NFT to test if they could co-accumulate Cu (Chapter 3). *A. bertolonii* did not exhibit Cu tolerance, whereas *B. coddii* exhibited a much greater degree of tolerance to this metal. A second experiment investigated the effect of high substrate concentrations of Cu and/or Ni on uptake by *B. coddii*. *B. coddii* did not accumulate Cu but remained Cu tolerant and continued to hyperaccumulate Ni. This suggests that the tolerance of *B. coddii* to Cu is not at the expense of Ni uptake. We conclude that *B. coddii* should be considered as a possible plant for phytoremediation of soils contaminated with both Cu and Ni.

Chapters 4 and 5 — Copper uptake and translocation in chicory and tomato plants

After failing to find the Cu hyperaccumulation in *H. katangense* and *B. coddii*, experiments were design to study how chicory, which is recognised as having slightly higher shoot Cu concentration than other forage plants, absorbs and distributes Cu between shoot and root. In these experiments, Cu distribution in chicory was compared to that with tomato. Again, all plants were grown using the Nutrient Film Technique system (NFT) with addition of 0.05, 5, 10 and 20 mg Cu L^{-1} (Chapter 4). Biomass production of shoots and roots of both chicory and tomato was strongly depressed by Cu concentrations higher than 5 mg Cu L^{-1} in the rooting media. At all Cu concentrations, Cu was predominantly retained in roots with less than 21-43% of plant Cu translocated to shoots except when plants were grown in solution Cu concentrations of 0.05 mg Cu L^{-1}. Relationships between Cu concentrations in the xylem sap and rooting media indicated that Cu adsorption by the root buffers against increased Cu concentrations in the rooting media. Large differences between measured and predicted Cu accumulation by shoots of tomato and chicory suggest that some xylem-transported Cu is recirculated to roots via the phloem.

Amino acid concentrations and chemical species of Cu in the xylem sap of chicory and tomato plants were measured using 6-week-old plants hydroponically grown in a NFT system. More than 99.7% of the total Cu in xylem sap was found to be in a complexed form. When exposed to high Cu concentrations in the rooting media, amino acid concentrations in the sap increased. Relative to other amino acids, the concentrations of glutamine (Gln), histidine (His), asparagine (Asn), valine (Val), nicotianamine (NA) and proline (Pro) in tomato xylem saps, and His, γ-aminobutyric acid (Gaba), glutamic acid (Glu), leucine (Leu), NA and phenylalanine (Phe) in chicory xylem saps, showed the greatest increases. The data indicate induced synthesis of some free amino acids as a specific and proportional response to Cu treatment. Solution Cu^{2+} concentration vs pH titration curves showed that NA and His in chicory and tomato xylem saps can account for all the bound Cu found in the sap.

Chapter 6 — Copper recirculation within plants

To explain the lack of correlation between predicted and actual plant Cu uptake, a solution culture experiment was conducted to investigate the redistribution of Cu in tomato plants. Tomatoes were grown in a split-root system exposed to low/low or low/high Cu solution concentration treatments. Different light intensities, relative humidities and major nutrient solution concentrations were also imposed in attempts to alter relative phloem and xylem flow rates. Significant amounts of Cu were translocated from the roots bathed in the high Cu concentration to shoots and finally to the half root system exposed to low Cu. A positive correlation was found between shoot Cu concentration and plant water use. High Cu treatments (10 mg L^{-1} solution Cu concentration) on half root systems strongly depressed plant biomass. Because of this Cu toxicity the effect of other treatments could not be evaluated.

Chapters 7-9 —Strategies to improve copper uptake by pastures

In Chapters 4 and 5, the free amino acids histidine and nicotianamine were identified to be major Cu complexing ligands in chicory and tomato xylem sap. The value of using amino acids to enhance Cu uptake from fertilisers was examined in Chapter 7.

A greenhouse pot trial was conducted to investigate the effect of addition of amino acids and casein to different Cu fertilisers on Cu uptake by chicory plants (Chapter 7) grown in pots of Manawatu silt loam. The results indicated that the addition of amino acids or casein to Cu fertilisers could slightly increase chicory Cu uptake.

A subsequent glasshouse trial using perennial ryegrass as test plant was conducted with granular fertilisers to evaluate the effectiveness of Ca-caseinate-$CuSO_4$ and $Cu(OH)_2$-based fertilisers. Two soils, contrasting in soil organic matter content, were chosen for the study (Chapter 8). The $Cu(OH)_2$-based fertiliser was less effective than the $CuSO_4$-based fertilisers in supplying Cu to ryegrass. In general, Cu fertiliser effectiveness followed the sequence: Ca-caseinate-$CuSO_4$ > $CuSO_4.5H_2O$ granules, $CuSO_4.5H_2O$ powder > $Cu(OH)_2$-based product. The efficiency of Cu uptake from fertiliser was greater in the soil with lower organic matter content (Ashhurst stony silt loam).

Copper speciation was studied in the soil solutions extracted from the Cu-fertilised Ashhurst stony silt loam and Wairoa pumice soil (Chapter 9). Linear relationships between ryegrass Cu uptake and total soil solution Cu concentration were dependent on soil type, despite each soil having similar soil solution Cu concentrations. Between 98.5-99.5% of the soil solution Cu was complexed with dissolved soil organic matter. No relationship between Cu^{2+}concentration in soil solution (expressed as pCu^{2+}) and Cu concentration in plants was found. Free Cu^{2+} concentrations in soil solution were sensitive to pH change. The extent of the increase in free Cu^{2+} concentration per unit decrease in pH was dependent on soil type. It is suggested that the rate of Cu uptake by plants is likely to be dependent on both the concentration of organically complexed Cu in soil solution and the stability of this complex to pH change.

10.3 CONCLUDING REMARKS AND FUTURE STUDY DIRECTIONS

Further studies should investigate whether Cu transport from roots to shoots is greater in plants with naturally high NA or His concentrations in xylem sap. Such plants may be exploited by providing adequate Cu in forage grazed by ruminants. The role of mugineic acid in metal transport in a wider range of plants also needs further investigation.

High xylem flow rates relative to phloem flow rates (low water use efficiency), may explain why slow-growing plants in low fertility and hot environments may show Cu hyperaccumulation.

Experiments conducted in Chapter 6 to examine whether changes in the relative flow rates of xylem and phloem influenced Cu shoot concentrations were not as successful as they could have been if a non-toxic Cu solution concentration had been chosen. It is suggested that these experiments be repeated with a rooting media Cu concentration of 5 mg Cu L^{-1} or less.

The combination of low corrosiveness and marginally higher agronomic effectiveness of granulated Ca-caseinate-$CuSO_4$ fertilisers indicated that the commercial of this material should be further investigated with field trials.

The hypothesis that the rate of Cu uptake by plants depends both on the concentration of organically complexed Cu in the soil solution and the stability of this complex to pH change, needs future testing on a wider range of soils. This could lead to a fuller understanding of the factors limiting the effectiveness of $CuSO_4$ topdressing as a strategy to ameliorate Cu deficiency in grazing animals. The two-phase mechanism of complexed Cu transport to the root and then pH-sensitive Cu release to plant roots may explain why current extractive soil tests for Cu fail to explain Cu availability to pasture plants. If this mechanism proves relevant to all soils, new soil tests can be designed to accommodate this two-phase mechanism.

REFERENCES

Acock, B.; Charles-Edwards, D. A.; Hand, D. W. 1976: An analysis of some effects of humidity on photosynthesis by a tomato canopy under winter light conditions and a range of carbon dioxide concentrations. *Journal of experimental botany 27*: 933-941.

Adams, A. F. R.; Elphick, B. L. 1956: The copper content of some soils and pasture species in Canterbury. *New Zealand journal of science and technology 38A*: 345-358.

Adams, F. 1971: Ionic concentrations and activities in soil solutions. *Soil science society of America proceedings 35*: 421-426.

Allen, H. E.; Hall, R. H.; Brisbin, T. D. 1980: Metal speciation, effects on aquatic toxicity. *Environmental science and technology 14*: 441-443.

Allen, W. R.; Sheppard, P. M. 1971: Copper tolerance in some Californian populations of the monkey flower *Mimulus guttatus*. *Proceedings of royal society London B 177*: 177-196.

Alloway, B. J.; Jackson, A. R. 1991: The behaviour of heavy metals in sewage sludge-amended soils. *Science of the total environment 100*: 151-176.

Anderegg, G.; Ripperger, H. 1989: Correlation between metal complex formation and biological activity of nicotianamine analogues. *Journal of chemistry society chemical communication 1989:* 647-650.

Andrew, C. S.; Thorne, P. M. 1962: Comparative responses to copper by some tropical and temperate pasture legumes. *Australian journal of agricultural research 13*: 821-836.

Anonymous. 1975: New Zealand fertiliser manufacture's research association, annual report 1974-1975.

Archer, F. C. 1971: Factors affecting the trace element content of pastures. Pp. 150-158 *in*: Trace elements in soils and crops. *Ministry of agriculture, fisheries and food technical bulletin 21*. London, H.M.S.O.

Arduini, I.; Godbold, D. L.; Onnis, A. 1996: Cadmium and Cu uptake and distribution in Mediterranean tree seedlings. *Physiologia plantarum 97*: 111-117.

Asher, C. J. 1991: Beneficial elements, functional nutrients, and possible new essential elements. Pp. 703-723 *in*: Micronutrients in agriculture (2nd ed.). Mortvedt, J. J.; Cox, F. R.; Shuman, L. M.; Welch, R. M. Eds., Soil Science Society of America, Madison, WI.

Baker, A. J. M. 1981: Accumulators and excluders — strategies in response of plants to heavy metals. *Journal of plant nutrition 3*: 643-670.

Baker, A. J. M.; Brooks, R. R. 1989: Terrestrial higher plants which hyperaccumulate chemical elements – a review of their distribution, ecology and photochemistry. *Biorecovery 1*: 81-126.

Baker, A. J. M.; Reeves, R. D.; Hajar, A. S. M. 1994: Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. presl (*Brassicaceae*). *New phytologist 127*: 61-68.

Baker, A. J. M.; Walker, P. L. 1989: Ecophysiology of metal uptake by tolerant plants. Pp.155-173 *in*: Heavy metal tolerance in plants: Evolutionary aspects, Shaw A. J. Ed. CRC Press, Inc. Boca Raton, Florida.

Baker, A. J. M.; Walker, P. L. 1995: Physiological responses of plants to heavy metals and the quantification of tolerance and toxicity. *Chemical speciation and bioavailability 7*: 7-17.

Bakker, J. C. 1990: Effects of day and night humidity on yield and fruit quality of glasshouse tomatoes (*Lyopersicon esculentum* Mill.*). Journal of horticultural science 65*: 323-331.

Bakker, J. C.; Welles, G. W. H.; Van Uffelen, J. A. M. 1987: The effect of day and night humidity on yield and quality of glasshouse cucumbers. *Journal of horticultural science 62*: 363-370.

Beck, A. B. 1962: The levels of copper, molybdenum and inorganic sulphate in some Western Australian pastures. *Australian journal of experimental agriculture, animal and husbandry 2*: 40-45.

Beeson, K. C.; Gray, L.; Adams, M. B. 1947: The absorption of mineral elements by forage plants: I. The phosphorus, cobalt, manganese and copper content of some common grasses. *Journal of the American society of agronomy 39*: 356-362.

Bell, P. F.; Chaney, R. L.; Angle, J. S. 1991: Free metal activity and total metal concentrations of micronutrient availability to barley (*Hordeum vulgare* L. cv. Klages). *Plant and soil 130*: 51-62.

Beneš, I.; Schreiber, K.; Ripperger, H.; Kircheiss, A. 1983: Metal complex formation by nicotianamine, a possible phytosiderophore. *Experientia 39*: 261-262.

Bennett, F. A. 1998: Studies on Plants that Hyperaccumulate Copper, Cobalt and Nickel: Their Potential for Use in Phytomining and Phytoremediation. MSc. Thesis, Massey University, New Zealand.

Bernal, M. P.: McGrath, S. P.; Miller, A. J.; Baker, A. J. M. 1994: Comparative of the chemical changes in the rhizosphere of the nickel hyperaccumulator *Alyssum murale* with the non-accumulator *Raphanus sativus*. *Plant and soil 164*: 251-259.

Berry, W. L. 1986: Plant factors influencing the use of plant analysis as a tool for biogeochemical prospecting. Pp. 18-27 *in*: Mineral exploration: biological systems and organic matter, Vol. 5, Carlisle, D.; Berry, W. L.; Kaplan, I. R.; Watterson, J. R., *eds*., Prentice-Hall, Englewood Cliffs, NJ.

Bineev, R. G.; Grigor'yan, B. R.; Yul'met'ev., R. M.; Kazakov, Kh. Sh. 1985: Effects of amino acids on the absorption of copper from the soil by plants. *Biologicheskie nauki 8*: 81-85.

Bingham, F. T.; Sposito, G.; Strong, J. E. 1984: The effect of chloride on the availability of cadmium. *Journal of environment quality 13*: 71-74.

Bingham, F. T.; Strong, J. E.; Sposito, G. 1983: Influence of chloride salinity on cadmium uptake by Swiss chard. *Soil science 135*: 160-165.

Bradshaw, A. D.; Mcneilly, T.; Gregory, R. P. G. 1965: Industrialization, evolution and development of heavy metal tolerance in plants. Pp. 327-344 *in*: *The British ecological society symposium No.5*. Goodman, G. T.; Edwards, R. W. *ed*. Ecology and the Industrial Society. Lambert Blackwell Scientific Publishers, Oxford.

Brams, E. A.; Fiskell, J. G. A. 1971: Copper accumulation in citrus roots and desorption with acid. *Soil science society of America proceedings 35*: 772-775.

Brooks, R. R 1977: Copper and cobalt uptake by *Haumaniastrum* species. *Plant and soil 48*: 541-544

Brooks, R. R. 1987: Serpentine and its vegetation—a multidisciplinary approach. Dioscorides Press, Portland, OR.

Brooks, R. R.: Baker, A. J. M.; Malaisse, F. 1992: Copper flower. *National geographic research and exploration 8*: 338-351.

Brooks, R. R.: Lee, J.; Reeves, R. D.; Jaffré, T. 1977: Detection of nickeliferous rocks by analysis of herbarium speciments of indicator plants. *Journal of geochemical exploration 7*: 49-57.

Brooks, R. R.; Malaisse, F. 1985: The Heavy Metal-tolerant Flora of Southcentral Africa: A Multidisciplinary Approach. Balkema A. A. *ed*. Rotterdam, Netherlands.

Brooks , R. R.; Naidu, S. D.; Malaisse, F.; Lee, J. 1987: The elemental content of metallophytes from the copper/cobalt deposits of Central Africa. *Bulletin, société royale de botanique de belgique 119*: 179-191.

Brooks, R. R.; Reeves, R. D.; Morrison, R. S.; Malaisse, F. 1980: Hyperaccumulation of copper and cobalt – a review. *Bulletin, société royale de botanique de belgique 113*: 166-172.

Brooks, R. R.; Robinson, B. H. 1998a: The potential use of hyperaccumulators and other plants for phytomining. Pp. 327-356 *in*: Plants that hyperaccumulate heavy metals: their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. Brooks, R. R. *ed.* CAB International, Wallingford, UK.

Brooks, R. R.; Robinson, B. H. 1998b: Aquatic phytoremediation by accumulator plants. Pp. 203-226 *in*: Plants that hyperaccumulate heavy metals: their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. Brooks, R. R. *ed.* CAB International, Wallingford, UK.

Brown, A. L.; Yamaguchi, Sh.; Leal-Diaz, J. 1965: Evidence for translocation of iron in plants. *Plant physiology 40*: 35-38.

Budêšínský, M.; Budzikiewiez, H.; Procházka, Z.; Ripperger, H.; Römer, A.; Scholz, G.; Schreiber, K. 1980: Nicotianamine, a possible phytosiderophore of general occurrence. *Phytochemistry 19*: 2295-2297.

Bukovac, M. J.; Wittwer, S. H. 1957: Absorption and ability of foliar applied nutrients. *Plant physiology 32*: 428-435.

Bunce, J. A. 1984: Effects of humidity on photosynthesis. *Journal of experimental botany 35*: 1245-1251.

Chaney, R. L. 1983: Plant uptake of inorganic waste constituents. Pp 50-76 *in*: Land treatment of hazardous wastes. Parr, J. F.; Marsh, P. B.; Kla, J. M. *eds.* Noyes Data Corp., Park Ridge, N.J.

Chaney, R. L.; Brown, J. C.; Tiffin, L. O. 1972: Obligatory reduction of ferric chelates in iron uptake of soybeans. *Plant physiology 50*: 208-213.

Checkai, R. T.; Corey, R. B.; Helmke, P. A. 1987: Effects of ionic and complexed metal concentrations on plant uptake of cadmium and micronutrient metals from solution. *Plant and soil 99*: 335-345.

Chui, V. W. D.; Chang, G. Y. S.; Cheung, Y. H.; Wong, M. H. 1988: Contamination of soil and plant by recycling of scrap plastics. *Environment international 14*: 525-529.

Clarkson, D. T.; Hanson, J. B. 1986: Proton fluxes and the activity of a stelar proton pump in onion roots. *Journal of experimental botany 37*: 1136-1150.

Clarkson, D. T.; Williams, L.; Hanson, J. B. 1984: Perfusion of onion root xylem vessels: a method and some evidence of control of the pH of the xylem sap. *Planta 16*: 361-369.

Cunningham, I. J. 1946: Copper deficiency in cattle and sheep on peat lands. *New Zealand journal of science and technology 27A*: 381-396.

Cunningham, I. J. 1950: Copper and molybdenum in relation to diseases of cattle and sheep in New Zealand. Pp. 246-250 *in*: A Symposium on copper metabolism. McElroy, W. D.; Glass, B. *eds*., The Johns Hopkins Press. Baltimore.

Cunningham, I. J.; Hogan, K. G.; Lawson, B. M. 1959: The effect of sulphate and molybdenum on copper metabolism in cattle. *New Zealand journal of agricultural research 2*: 145-152.

Cunningham, I. J.; Perrin, D. D. 1946: Copper compounds as fertilisers for pastures deficient in copper. *New Zealand journal of science and technology 28A*: 252-265.

De Boer, A. H.; Wegner, L. H. 1997: Regulatory mechanisms of ion channels in xylem parenchyma cells. *Journal of experimental botany 48*: 441-449.

Dekock, P. C.; Mitchell, R. L. 1957: Uptake of chelated metals by plants. *Soil science 84*: 55-62.

Dragun, J.; Baker, D. E.; Risius, M. L. 1976: Growth and element accumulation by two single-cross crown hybrids as affected by copper in solution. *Agronomy journal 68*: 466-470.

Duvigneaud, P.; Denaeyer-De Smet, S. 1963: Cuivre et végétation au Katanga, *Bulletin de la Société royale de botanique de belgique 96*: 93-31.

El-Falaky, A. A.; Aboulroos, S. A.; Lindsay, W. L. 1991: Measurement of Cd activities in slightly acidic to alkaline soils. *Soil science society of America journal 55*: 974-979.

Elkhatib, E. A.; Hern, J. L.; Staley, T. E. 1987: A rapid centrifugation method for obtaining soil solution. *Soil science society of America journal 51*: 578-583.

Fekkes, D. 1996: State-of-the-art of high-performance liquid chromatographic analysis of amino acids in physiological samples. *Journal of chromatography B 682*: 3-22.

Fiedler, P. L. 1985: Heavy metal accumulation and the nature of edaphic endemism in the genus *Calochortus* (Liliaceae). *American journal of botany 72*: 1712-1718.

Fierabracci, V.; Masiello, P.; Novelli, M.; Bergamin, E. 1991: Application of amino acid analysis by high-performance liquid chromatography with phenyl isothiocyanate derivatization to the rapid determination of free amino acids in biological samples. *Journal of chromatography 570*: 285-291.

Ford, M. A.; Thorne, G. N. 1974: Effects of atmospheric humidity on plant growth. *Annual botany 38*: 441-452.

Gabbrielli, R.; Mattionic, C.; Vergnano, O. 1991: Accumulation mechanisms and heavy metal tolerance of a Ni hyperaccumulator. *Journal of plant nutrition 14*: 1067-1080.

Gahoonia, T. S.; Nielsen, N. E. 1992: Control of pH at the soil-root interface. *Plant and soil 140*: 49-54.

Gartrell, J. W. 1981: Distribution and correction of copper deficiency in crops and pastures. Pp. 313-343 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*., Academic Press, New York.

Geering, H. R.; Hodgson, J. F. 1969: Micronutrient cation complexes in soil solution: III. Characterization of soil solution ligands and their complexes with Zn^{2+}, Cu^{2+}. *Soil science society of America proceedings 33*: 54-59.

Gilkes, R. J. 1981: Behaviour of Cu additives — fertilisers. Pp. 97-117 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Gladstones, J. S.; Loneragan J. F.; Simmons, W. J. 1975: Mineral elements in temperate crop and pasture plants. III Copper. *Austrialian journal of agricultural research 26*: 113-126.

Gomide, J. A.; Noller, C. H.; Mott, G. O.; Conrad, J. H.; Hill, D. L. 1969: Mineral composition of six tropical grasses as influenced by plant age and nitrogen fertilization. *Agronomy journal 61*: 120-123.

Grace, N. D. 1983: Copper (Cu). Pp. 57-66 *in*: The mineral requirements of grazing ruminants. Grace, N. D. *ed*. *New Zealand society of animal* *production occasional publication, No. 9*. Keeling and Mundy Limited, Palmerston North, New Zealand.

Grace, N. D. 1994: Managing trace element deficiencies. AgResearch, Palmerston North, New Zealand.

Grace, N. D.; Knowles, S. O.; Rounce, J. R.; West, D. M.; Lee, J. 1998: Effect of increasing pasture copper concentrations on the copper status of grazing Romney sheep. *New Zealand journal of agricultural research 41*: 377-386.

Graham, R. D. 1979: Transport of copper and manganese to the xylem exudate of sunflower. *Plant, cell and environment 2*: 139-143.

Graham, R. D. 1981. Absorption of copper by plant roots. Pp. 141-163 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Grange, R. I.; Hand, D. W. 1987: A review of the effects of atmospheric humidity on the growth of horticultural crops. *Journal of horticultural science 62*: 125-134.

Gregory, R. P. G.; Bradshaw, A. D. 1965: Heavy metal tolerance in populations of *Agrostics tenuis* Sibth. and other grasses. *New phytologist 64*: 131-143.

Griffin, R. A.; Jurinak, J. J. 1973: Estimation of activity coefficients from the electrical conductivity of natural aquatic systems and soil extracts. *Soil science 116*: 26-30.

Hajar, A. S. M. 1987: Comparative Ecology of *Minuartia verna* (L.) Hiern. and *Thlaspi alpestre* (L.) in the Southern Pennines, with Special Reference to Heavy-metal Tolerance. Ph.D. Thesis, University of Sheffield, UK.

Harsh, J. B.; Doner, H. E. 1984: Specific adsorption of copper on a hydroxy-aluminum montmorillonite complex. *Soil science society of America journal 48*: 1034-1039.

Herbik, A.; Giritch, A.; Horstmann, C.; Becker, R.; Balzer, H.; Bäumlein, H.; Stephan, U. W. 1996: iron and copper nutrition-dependent changes in protein expression in a tomato wild type and the nicotianamine-free mutant chloronerva. *Plant physiology 111*: 533-540.

Herren, T.; Feller, U. 1994: Transfer of zinc from xylem to phloem in the peduncle of wheat. *Journal of plant nutrition 17*: 1587-1598.

Hewitt, A. E. 1992: New Zealand soil classification. *DSIR land resources scientific report No. 19,* Lower Hutt, New Zealand.

Hill, J. M. 1973: The changes with age in the distribution of copper and some copper-containing oxidases in red clover (*Trifolium pratense* L. cv. Dorset Marlgrass). *Journal of experimental botany 24*: 525-536.

Hoagland, D. R.; Arnon, D. I. 1941: The water culture method for growing plants without soil. Miscellaneous Publications No. 354. *Circular of California agricultural experimental station 347*: 461.

Hocking, P .J. 1980: The composition of phloem exudate and xylem sap from tree tobacco (*Nicotiana glauca* Grah.). *Annals of botany 45*: 633-643.

Hocking, P. J.; Pate, J. S. 1978: Accumulation and distribution of mineral elements in annual lupins *Lupinus albus* and *Lupinus angustifolius*. *Australian journal of agricultural research 29*: 267-280.

Hocking, P J.; Pate J. S.; Atkins, C. A.; Sharkey, P. J. 1978: Diurnal patterns of transport and accumulation of minerals in fruiting plants of *Lupinus angustifolius* L. *Annals of botany 42*, 1277-1290.

Hodgson, J. F.; Geering, H. R.; Norvell, W. A. 1965: Micronutrient cation complexes in soil solution: partition between complexed and uncomplexed forms by solvent extraction. *Soil science society of America proceedings 29*: 665-669.

Hodgson, J. F.; Lindsay, W. L.; Trierweiler, J. F. 1966: Micronutrient cation complexing in solution. Hg(II), Cd (II) and Cu (II) complexes. *Canadian journal of soil science 54*: 413-417.

Hogan, K. A.; Cunningham, I. J.; McNally, G. T. 1964: The effects of topdressing pastures grown on Te Kopuru sand with copper and molybdenum compounds. *New Zealand journal of agricultural research 7*: 174-179.

Homer, F. A.; Morrison, R. S.; Brooks, R. R.; Clements, J.; Reeves, R. D. 1991: Comparative studies of nickel, cobalt, and copper uptake by some nickel hyperaccumulators of the genus *Alyssum*. *Plant and soil 138*: 195-205.

Hsu, H. H. 1986a: The absorption and distribution of Metalosates from foliar fertilization. Pp. 236-254 *in*: Foliar feeding of plants with amino acid chelates. Ashmead, H. D.; Ashmead, H. H.; Miller, G. W.; Hsu, H. H. *eds*. Noyes Publications, Park Ridge, New Jersey, USA.

Hsu, H. H. 1986b: Chelates in plant nutrition. Pp. 209-218 *in*: Foliar feeding of plants with amino acid chelates. Ashmead, H. D.; Ashmead, H. H.; Miller, G. W.; Hsu, H. H. *eds*. Noyes Publications, Park Ridge, New Jersey, USA.

Hsu, H. H.; Ashmead, H. D.; Graff, D. J. 1982: Absorption and distribution of foliar-applied iron by plants. *Journal of plant nutrition 5*: 969-974.

Hsu, H. H.; Ashmead, H. D.; Graff, D. J. 1986: Absorption and distribution of foliar-applied iron by plants. Pp. 264-270 *in*: Foliar feeding of plants with amino acid chelates. Ashmead, H. D.; Ashmead, H. H.; Miller, G. W.; Hsu, H. H. *eds*. Noyes Publications, Park Ridge, New Jersey, USA.

Jarvis, S. C. 1978: Copper uptake and accumulation by perennial ryegrass grown in soil and solution culture. *Journal of the science of food and agriculture 29*: 12-18.

Jarvis, S. C. 1980: The uptake and distribution of copper in perennial ryegrass and white clover grown in flowing solution culture with a controlled supply of copper. *Journal of the science of food and agriculture 31*: 870-876.

Jarvis, S. C.; Robson, A. D. 1982: Absorption and distribution of copper in plants with sufficient or deficient supplies. *Annal botany*, 50: 151-160.

Jarvis, S. C.; Whitehead, D. C. 1981: The influence of some soil and plant factors on the concentration of copper in perennial ryegrass. *Plant and soil 60*: 275-286.

Jarvis, S. C.; Whitehead, D. C. 1983: The absorption, distribution and concentration of copper in white clover grown on a range of soils. *Plant and soil 75*: 427-434.

Jeffery, J. J.; Uren, N. C. 1983: Copper and zinc species in the soil solution and effects of soil pH. *Australian journal of soil research 21*: 479-488.

Jeppsen, R. B. 1991: Mineral supplementation in plants via amino acid chelation. Pp. 321-331 *in*: Biological trace element research. Subramanian, K. S.; Iyengar, G. V.; Okamoto, K. *eds*. American Chemical Society, Washington, DC.

Jeschke, W. D.; Pate, J. S. 1991a: Ionic interactions of petiole and lamina during the life of a leaf of Castor bean (*Ricinus communis* L.) under moderately saline conditions. *Journal of experimental botany 42*: 1051-1064.

Jeschke, W. D.; Pate, J. S. 1991b: Cation and chloride partitioning through xylem and phloem within the whole plant of *Ricinus communis* L. under conditions of salt stress. *Journal of experimental botany 42*: 1105-1116.

Jeschke, W. D.; Wolf, O.; Pate, J. S. 1991: Solute exchange from xylem to phloem in the leaf and from xylem to phloem in root. Pp. 96-105 *in*: Recent advances in phloem transport and assimilate compartmentation.

Bonnemain, J. L.; Delrot, S.; Lucas, W. J.; Dainty, J. *eds*. Ouest Edition, Nantes.

Khan, M. A. R.; Bolan, N. S.; Mackay, A. D.; Tillman, R. W. 1998: Reaction and plant availability of Cu fertilisers in pasture soils. Pp. 39-40 *in*: *Proceedings of New Zealand soil science society Wairoa conference*, 16-19 November, Wairoa, New Zealand.

Khasawneh, F. E. 1971: Solution ion activity and plant growth. *Soil science society of American, proceedings 35*: 426-436.

Kirkham, M. B. 1980: Movement of cadmium and water in split-root wheat plants. *Soil science 129*: 339-344.

Kochian, L.V. 1991: Mechanisms of micronutrient uptake and translocation in plants. Pp. 230-296 *in*: Micronutrients in agriculture (2nd ed.). Soil Science Society of America, USA.

Köckenberger, W.; Pope, J. M.; Xia, Y.; Jeffrey, K. R.; Komor, E.; Callaghan, P. T. 1997: A non-invasive measurement of phloem and xylem water flow in castor bean seedlings by nuclear magnetic resonance microimaging. *Planta 201*: 53-63.

Korte, C. J.; Smith, D. R.; Deaker, J. M.; Scotland, T.; Willimott, M. 1996: Copper and sulphur deficiencies and interactions with other factors in the Wairoa Region. *Unpublished survey-report to New Zealand meat research and development council*, Wellington.

Krämer, U.; Cotter-Howells, J. D.; Charnock,J. M.; Baker, A. J. M.; Smith, J. A. C. 1996: Free histidine as a metal chelator in plants that accumulate nickel. *Nature 379*: 635-638.

Krijger, G. C.; Van Vliet, P. M.; Wolterbeen, H. T. 1999: Metal speciation in xylem exudate of *Lycopersion esculentum* Mill - technetium. *Plant and soil 212*: 165-173.

Kumar, P. B. A. N.; Dushenkov, V.; Motto, H.; Raskin I. 1995: Phytoextraction: The use of plants to remove heavy metals from soils. *Environmental science & technology 29*: 1232-1238.

Kuo, S.; Jellum, E. J. 1991: Affinity constants and behaviour of Cd sorption in some acid soils. *Water, air and soil pollution 57*-58: 369-376.

Kuppelwieser, H.; Feller, U. 1990: Influence of fusiecoccin and xylem wall adsorption on cation transport into maturing wheat (*Triticum aestivumi)* ears. *Plant nutrition and physiological application 41*: 117-120.

Lasat, M. M.; Baker, A. J. M.; Kochian, L. V. 1996: Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and non-accumulator species of *Thlaspi*. *Plant physiology 112*: 1715-1722.

Liao, M. T.; Hedley, M. J.; Woolley, D. J.; Brooks, R. R.; Nichols, M. A. 1999a: Effect of amino acids and casein on Cu uptake by chicory from soil. *Proceedings of New Zealand grasslands association 61*: 181-184.

Liao, M. T.; Hedley, M. J.; Woolley, D. J.; Brooks, R. R.; Nichols, M. A. 1999b: Copper-induced amino acid synthesis in the xylem saps of tomato and chicory plants. Pp. 1061-1062 *in*: *Proceedings 5th international conference on biogeochemchemistry of trace elements*, July 11-15th, Vienna, Austria.

Liao, M. T.; Hedley, M. J.; Woolley, D. J.; Brooks, R. R.; Nichols, M. A. 2000a: Copper uptake and translocation in chicory (*Cichorium intybus* L. cv Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv Rondy) plants grown in NFT system. I. Copper uptake and distribution in plants. *Plant and soil* (Accepted).

Liao, M. T.; Hedley, M. J.; Woolley, D. J.; Brooks, R. R.; Nichols, M. A. 2000b: Copper uptake and translocation in chicory (*Cichorium intybus* L. cv Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv Rondy) plants grown in NFT system. II. The role of nicotianamine and histidine in xylem sap copper transport. *Plant and soil* (Accepted).

Liao, M. T.; Hedley, M. J.; Woolley, D. J.; Longanthan, P.; Brooks, R. R.; Nichols, M. A. 2000c: Agronomic effectiveness of selected fertilisers in supplying copper to perennial ryegrass (*Lolium perenne* cv. Nui). *Nutrient cycling in agroecosystems* (in preparation)

Lindsay, W. L. 1979: Cu in soil solution. Pp. 222-236 *in*: Chemical equilibria in soils. A Wiley-inter Science Publication, John Wiley & Sons, New York.

Lindsay, W. L. 1991: Inorganic equilibria affecting micronutrients in soils. Pp. 89-112 *in*: Micronutrients in agriculture (2nd ed.). Mortvedt, J. J.; Cox, F. R.; Shuman, L. M.; Welch, R. M. *eds.*, Soil Science Society of America, Madison, WI, USA.

Linehan, D. J.; Shepherd, H. 1979: A comparative study of the effects of natural and synthetic ligands on iron uptake by plants. *Plant and soil 52*: 281-289.

Lipton, W. J. 1970: Growth of tomato plants and fruit production in high humidity and at high temperature. *Journal of American horticultural science society 95*: 674-680.

Liu, W. C.; Tzeng, J. S.; Li, S. W.; Wang, M. C.; Wang, Y. P. 1994: Metal pollutants in landfill soils and their effect on sugarcane. *Environmental geochemistry and health* 115-129 (*special issue*).

Loganathan, P.; Hedley, M. J. 1997: Limiting factors controlling copper uptake and concentration in grazed pasture. A report submit to Ravensdown Fertilizer Coop Ltd. Feb 1997. Lime and Fertiliser Research Center, Massey University, New Zealand.

Loganathan, P.; Bretherton, M. R.; Hedley, M. J. 1998: Interim research progress report on Ravensdown Fertilizer Coop Ltd. Funded projects - to November 30th 1998. *A progress report submitted to Ravensdown Fertilizer Coop Ltd. Dec 1998.* Lime and Fertiliser Research Center, Massey University, New Zealand.

Loganathan, P.; Liao, M. T.; Bretherton, M. R.; Hedley, M. J. 1999a: Final progress report on Cu fertiliser studies 1998. *A final report submitted to Ravensdown Fertilizer Coop Ltd. Feb. 1999.* Lime and Fertiliser Research Center, Massey University, New Zealand.

Loganathan, P.; Liao, M. T.; Bretherton, M. R.; Hedley, M. J. 1999b: Interim research progress report on Ravensdown Fertilizer Coop Ltd. Funded projects - February 1st to November 30th 1999. *A progress report submitted to Ravensdown Fertilizer Coop Ltd. Nov. 1999.* Lime and Fertiliser Research Center, Massey University, New Zealand.

Loneragan, J. F. 1981: Distribution and movement of copper in plants. Pp. 165-188 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Luo, M ; Rimmer, D. L. 1995: Zinc-Cu interaction affecting plant growth on a metal-contaminated soil. *Environmental pollution 88*: 79-83.

Ma, Q. Y.; Lindsay, W. L. 1995: Estimation of Cd^{2+} and Ni^{2+} activities in soils by chelation. *Geoderma 68*: 123-133.

Maas, F. M.; Van De Wetering, D. A. M.; Van Beusichem, M. L.; Bienfait, H. F. 1988: Characterization of phloem iron and its possible role in the regulation of Fe-deficiency reactions. *Plant physiology 87*: 167-171.

Maire, R. H.; Cattanni, R. A. 1966: Growth and copper content of anatomical plant parts as influenced by copper supply and time. *Plant and soil 24*: 339-342.

Malaisse, F.; Brooks, R. R. 1982: Colonisation of modified metalliferous environments in Zaïre by the copper flower *Haumaniastrum katangense. Plant and soil 64*: 289-293.

Malaisse, F.; Brooks, R. R.; Baker, A. J. M. 1994: Diversity of vegetation communities in relation to soil heavy metal content at the Shinkolobwe copper/cobalt/uranium mineralization, upper Shaba, Zaïre. *Belgium journal of botany 127*: 3-16.

Malaisse, F.; Grégoire, J.; Brooks, R. R.; Morrison, R. S.; Reeves, R. D. 1978: *Aeolanthus biformifolius* De Wild.: A hyperaccumulator of copper from Zaïre. *Science 199*: 887-888.

Malaisse, F.; Grégoire, J.; Morrison, R. S.; Brooks, R. R.; Reeves, R. D. 1979: Copper and cobalt in vegetation of fungurume, Shaba Province, Zaïre. *Oikos*, 33: 472-478.

Marinussen, M. 1997: Heavy metal accumulation in earthworms exposed to spatially variable soil contamination. PhD thesis, Wageningen Agricultural University, Netherlands.

Marschner, H. 1986: Mineral Nutrition of Higher Plants. Academic Press, London.

May, P. M.; Linder, P. W.; and Williams, D. R. 1977: Computer simulation of metal-ion equilibria in biofluids: Models for the low-molecular-weight complex distribution of calcium (II), magnesium (II), manganese (II), iron (III), copper (II), Zinc (II) and lead (II) ions in human blood plasma. *Journal of chemical society* 588-595.

Mazhoudi, S.; Chaoui, A.; Ghorbal, M. H.; El-Ferjani, E. 1997: Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum,* M.). *Plant science 127*: 129-137.

McBride, M.; Martínez, C. E.; Sauvé, S. 1998: Copper (II) activity in aged suspensions of goethite and organic matter. *Soil science society of America journal 62*: 1542-1548.

McGrath, S. P. 1998: Phytoextraction for soil remediation. Pp 261-289 *in*: Plants that hyperaccumulate heavy metals---their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. Brooks, R. R. *ed.* CAB International. Wallingford, UK

McGrath, S. P.; Hamon, R. E.; McLaughlin, M. J.; Rogers, S. L. 1999: Phytoremediation - the green approach. Pp. 88-128 *in*: Bioremediation: Sustainable technology for the 21st century. Taylor francis, London.

McGrath, S. P.; Sanders, J. R.; Shalaby, M. H. 1988: The effects of soil organic matter levels on soil solution concentrations and extractabilities of manganese, zinc and copper. *Geoderma 42*: 177-188.

McGrath, S. P.; Shen, Z. G.; Zhao, F. J. 1997: Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant and soil 188*: 153-159.

McLaren, R. G.; Crawford, D. V. 1973: Studies of soil copper. I. The fractionation of copper in soils. *Journal of soil science 24*: 172-181.

McLaren, R. G.; Hogg, D. S.; Swift, R. S.1990: Some factors affecting the availability of native and applied soil copper in New Zealand soils. *Forest ecology and management 37*: 131-142.

McLaren, R. G.; Swift, R. S.; Quin, B. F. 1984: EDTA-extractable copper, zinc, and manganese in soils of the Canterbury Plains. *New Zealand journal of agricultural research 27*: 207-217.

McLaren, R. G.; Williams, J. G. 1981: Effects of adding chelated and non-chelated copper and cobalt to a deficient soil on the content of these nutrients in clover and ryegrass. *Journal of the science of food and agriculture 32*: 181-186.

McLaughlin, M. J.; Tiller, K. G.; Smart, M. K. 1997: Speciation of cdmium in soil solutions of saline/sodic soils and relationship with cadmium concentrations in potato tubers (*Solanum tuberosum* L.). *Australian journal of soil research 35*: 183-198.

Mench, M.; Morel, J. L.; Guckert, A. 1987: Metal binding properties of high molecular weight soluble exudates from maize (*Zea mays* L.) roots. *Biology and fertility of soils 3*: 165-169.

Mench, M.; Morel, J. L.; Guckert, A.; Guillet, B. 1988: Metal binding with root exudates of low molecular weight. *Journal of soil science 39*: 51-527.

Mesjasz-Przybylowicz, J.; Pineda, C. A. 1998: Elemental distribution in young leaves of the Ni hyperaccumulator - *Berkheya coddii. Proceedings of microscope society of Southern Africa 28*: 57-65.

Minnich, M. M.; McBride, M. B.; Chaney, R. L. 1987: Copper activity in soil solution: II. Relation to copper accumulation in young snapbeans. *Soil science society of America Journal*, 51: 573-578.

Mitchell, R. L.; Reith, J. W. S.; Johnston, I. M. 1957: Trace element uptake in relation to soil content. *Journal of the science of food and agriculture 8*: 51-59.

Morel, J. L.; Mench, M.; Guckert, A. 1986: Measurement of Pb^{2+}, Cu^{2+} and Cd^{2+} binding with mucilage exudates from maize (*Zea mays* L.) roots. *Biology and fertility of soils 2*: 29-34.

Mori, S.; Nishizawa, N.; Hayashi, H.; Chino, M.; Yoshimura, E.; Ishihara, J. 1991: Why are young rice plants highly susceptible to iron deficiency? Pp. 175-188 *in*: Iron nutrition and interactions in plants. Chen, Y.; Hadar, M. *eds*. Kluwer Academic Publishers, Netherlands.

Morrey, D. R.; Balkwill, K.; Balkwill, M. J. 1989: Studies on serpentine flora preliminary analyses of soils and vegetation associated with serpentinite rock formations in the south-eastern transvaal South Africa. *South African journal of botany 55*: 171-177.

Morris, R. L.; Swanson, B. T. 1980: Xylem pH and reduction potential levels of iron-stressed silver maple (*Acer saccharium* L.). *Plant physiology 65*: 387-388.

Morrison, R. S. 1980: Aspects of the Accumulation of Cobalt, Copper and Nickel by Plants. Ph.D. Thesis, Massey University, New Zealand.

Morrison, R.S.; Brooks, R. R.; Reeves, R. D.; Malaisse, E. 1979: Copper and cobalt uptake by metallophytes from Zaïre. *Plant and soil 53*: 535-539.

Nable, R. O.; Loneragan, J. F. 1984: Translocation of manganese in subterranean clover (*Trifolium subterraneum* L. cv. Seaton Park). I. Redistribution during vegetative growth. *Australian journal of plant physiology 11*: 101-111.

Noma, M.; Noguchi, M. 1976: Occurrence of nicotianamine in higher plants. *Phytochemistry 15*: 1701-1702.

Noma, M.; Noguchi, M.; Tamaki, E. 1971: A new amino acid, nicotianamine, from tobacco leaves. *Tetrahedron letters 22*: 2017-2020.

Nor, Y. M.; Cheng, H. H. 1986: Chemical speciation and bioavailability of copper: uptake and accumulation by *Eichornia*. *Environmental toxicology and chemistry 5*: 941-947.

Oertli, J. J.; Seresinhe, P. S. J. W.; Ruh, R. 1990: The influence of the nutritional status of tomato plants on the water use efficiency. *Acta horticulturae 278*: 227-235.

Ouariti, O.; Boussama, N.; Zarrouk, M.; Cherif, A.; Ghorbal, M. H. 1997: Cadmium and copper induced changes in tomato membrane lipids. *Phytochemistry 45*: 1343-1350.

Parker, D. R.; Chaney, R. L.; Norvell, W. A. 1995: Chemical equilibrium models: Applications to plant nutrition research. Pp. 163-200 *in*: Chemical equilibrium and reaction models. Loeppert, R. H. *ed. Soil science society of American special publication No. 42*, SSSA, Madison, WI, USA.

Pare, T.; Dinel, H.; Schnitzer, M. 1999: Extractability of trace elements during co-composting of biosolids and municipal solid wastes. *Biology and fertility of soils 29*: 31-37.

Parker, D. R.; Pedler, J. F. 1997: Re-evaluating the free-ion activity model of trace metal availability to higher plants. Pp. 107-112 *in*: Plant nutrition-for sustainable food production and environment, *Proceedings of the XIII international plant nutrition colloquium*, 13-19 September 1997, Tokyo, Ando, T. et al. *eds.*. Kluwer Academic Publishers, Japan.

Pate, J. S. 1975: Exchange of solutes between phloem and xylem and circulation in the whole plant. Pp. 451-473 *in* Transport in plants. I. Phloem transport. Zimmermann, M. H.; Milburn, J. A. *eds*. Springer Verlage, Berlin.

Pate, J. S. 1976: Nutrients and metabolites of fluids recovered from xylem and phloem: significance in relation to long-distance transport in plants. Pp. 253-281 *in*: Transport and transfer processes in plants. Wardlaw, I, F.; Passioura, J. B. *eds*. Academic Press, New York.

Pate, J. S. 1980: Transport and partitioning of nitrogenous solutes. *Annual review in plant physiology 31*: 313-340.

Pate, J. S.; Hocking, P. H. 1978: Phloem and xylem transport in the supply of minerals to a developing legume (*Lupinus albus* L.) fruit. *Annals botany 42*: 1517-1523.

Paton, A.; Brooks, R. R. 1996: A re-evaluation of *Haumaniastrum* species as geobotanical indicators of copper and cobalt. *Journal of geochemical exploration 56*: 37-45.

Pavan, M. A.; Bingham, F. T.; Pratt, P. F. 1982: Toxicity of aluminum to coffee in ultisols and oxisols amended with $CaCO_3$, $MgCO_3$ and $CaSO_42H_2O$. *Soil science society of America journal 46*: 1201-1207.

Pich, A.; Scholz, G. 1991: Nicotianamine and the distribution of iron into apoplast of tomato (*lycopersicon esculentum* Mill.). II. Uptake of iron by protoplasts from the variety Bonner Beste and its nicotianamine-less mutant chloronerva and the compartmentation of iron in leaves. *Journal of experimental botany 42*: 1517-1523.

Pich, A.; Scholz, G. 1996: Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill.): nicotianamine-stimulated copper transport in the xylem. *Journal of experimental botany 47*: 41-47.

Pich, A.; Scholz, G.; Stephan, U. W. 1994: Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. *Plant and soil 165*: 189-196.

Pierson, E. E.; Clark, R. B.; Coyne, D. P.; Maranville, J. W. 1986: Iron deficiency stress effects on total iron in various leaves and nutrient solution pH in sorghum and beans. *Journal of plant nutrition 9*: 893-907.

Pierzynski, G. M.; Schwab, A. P. 1993: Bioavailability of zinc, cadmium, and lead in a metal-contaminated alluvial soil. *Journal of environmental qualality 22*: 247-254.

Piper, C. S. 1942: Investigations on copper deficiency in plants. *Journal of agricultural science 32*: 143-178.

Piper, C. S.; Walkley, A. 1943: Copper, zinc, and manganese in some plants of agricultural interest. *Journal of council of science and industry research 16*: 217-234.

Procházka, Z.; Scholz, G. 1984: Nicotianamine, the 'normalizing factor' for the autotroph tomato mutant Chloronerva; a representative of a new class of plant effectors. *Experientia 40*: 794-801.

Punshon, T.; Dickinson, N. M. 1997: Heavy metal resistance in *Salix*: pre-treament and acclimation. Pp.35-36 *in: Proceedings of fourth international conference on the biogeochemistry of trace elements*,. Iskandar, K. I.; Hardy, S. E.; Chang, A. C.; Pierzynski, G. M. *eds*. June 23-26, 1997. Clark Kerr Campus, Berkeley, California, USA.

Punz, W. F.; Sieghardt, H. 1993: The response of roots of herbaceous plant species to heavy metals. *Environmental experimental botany 33*: 85-98.

Reeves, R. D.; Baker, A. J. M. 1984: Studies on metal uptake by plants from serpentine and non-serpentine populations of *Thlaspi goesingense* Hálácsy (Cruciferae). *New phytologist 98*: 191-204.

Reeves, R. D.; Baker, A. J. M.; Brooks, R.R. 1995: Abnormal accumulation of trace metals by plants. *Mining environmental management 3*: 4-8.

Reeves, R. D.; Baker, A. J. M.; Borhidi, A.; Berezain, R. 1996: Nickel-accumulating plants from the ancient serpentine soils of Cuba. *New phytologist 133*: 217-224.

Reeves, R. D.; Brooks, R. R. 1983: Hyperaccumulation of lead and zinc by two metallophytes from mining areas of central Europe. *Environmental pollution 31*: 277-85.

Reith, J. W. S. 1975: Copper deficiency in plants and effects of copper on crops and herbage. Pp. 25-37 *in*: *Copper in farming symposium*. London, Copper development Association.

Renner, R. 1997: Rethinking water quality standards for metal toxicity. *Environmental science and technology 31*: 466-468.

Reuter, D. J.; Hannam, R. J.; Judson, G. J.; Dodson, M. E. 1977: Copper fertiliser for pasture and sheep production on lateritic podzolic soils in South Australia. 2. Current requirements on developed soil. *Agricultural record 4*: 36-45.

Reuther, W.; Labanauskas, C. K. 1968: Copper. Pp 157-180 *in*: Diagnostic criteria for plants and soils. Chapman, H. D. *ed*. Riverside, University of California.

Robinson, B. H. 1997: The Phytoextraction of Heavy Metals from Metalliferous Soils. Ph.D. Thesis, Massey University, New Zealand.

Robinson, B. H.; Brooks, R. R.; Howes, A. W.; Kirkman, J. H.; Gregg, P. E. H. 1997a: The potential of the high-biomass Ni hyperaccumulator *Berkheya coddii* for phytoremediation and phytomining. *Journal of geochemical exploration 60*: 115-126.

Robinson, B. H.; Chiarucci, A.; Brooks, R. R.; Petit, D.; Kirkman, J. H.; Gregg, P. E. H.; Dominicis, V. De. 1997b: The Ni hyperaccumulator plant *Alyssum bertolonii* as a potential agent for phytoremediation and phytomining of Ni. *Journal of geochemical exploration 59*: 75-86.

Robinson, B. H.; Leblanc, M.; Petit, D.; Brooks, R. R.; Kirkman, J. H.; Gregg, P. E. H. 1998: The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. *Plant and soil 203*: 47-56.

Robson, A. D.; Reuter, D. J. 1981: Diagnosis of copper deficiency and toxicity. Pp. 287-307 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Römkens, P. F. A. M. 1998: Effect of land use changes on organic matter dynamics and trace metal solubility in soils. PhD thesis, University of Groningen, Netherlands.

Rudolph, A.; Becker, R.; Scholz, G.; Procházka, Z.; Toman, J.; Macek, T.; Herout, V. 1985: The occurrence of the amino acid nicotianamine in plants and microorganisms. A reinvestigation. *Biocheme und physiologie der pflanzen 180*: 557-563.

Ruiz, L.; Arvieu, J. C. 1990: Measurement of pH gradients in the rhizosphere. *Symbiosis 9*: 71-75.

Salam, A. K.; Helmke, P. A. 1998: The pH dependence of free ionic activities and total dissolved concentrations of copper and cadmium in soil solution. *Geoderma 83*: 281-291.

Salisbury, F. B.; Ross, C. W. 1992: Plant physiology (4th edn.). Wadsworth Belmont, CA, USA.

Sanders, J. R. 1982: The effect of pH upon the copper and cupric ion concentrations in soil solutions. *Journal of soil science 33*: 679-689.

Sarong, L. Q., Bouldin, D. R. and Reid, W. S. 1989. Total and labile zinc concentrations in water extracts of rhizosphere and bulk soils of oats and rice. *Communications in Soil Science and plant Analysis*, 20: 271-289.

SAS Institute, 1990: SAS/STAT user's guide, Version 6. SAS Institute, Inv. Cary, NC.

Sauvé, S.; Cook, N.; Hendershot, W. H.; McBride, M. B. 1996: Linking plant tissue concentrations and soil copper pools in urban contaminated soils. *Environmental pollution 94*: 153-157.

Schmidke, I.; Stephan, U. W. 1995: Transport of metal micronutrients in the phloem of castor bean (*Ricinus communis*) seedlings. *Physiologia plantarum 95*: 147-153.

Scholz, G. 1989: Effect of nicotianamine on iron re-mobilization in derooted tomato seedlings. *BiolMeatls 2*: 89-91.

Scholz, G.; Faust, J.; Ripperger, H.; Schreiber, K. 1988: Structure-function relationships of nicotianamine analogues. *Phytochemistry 27*: 2749-2754.

Schreiber, K. 1986: Identification and characterization of an endogenous cytometallophore of general distribution in plants. *Pure application chemistry 58*: 745-752.

Senden, M. H. M. N.; Meer, Van Der A. J. G. M.; Limborgh, J.; Wolterbeek, H. T. H. 1992: Analysis of major tomato xylem organic acids and PITC-derivatives of amino acids by RP-HPLC and UV detection. *Plant and soil 142*: 81-89.

Shen, Z. G.; Zhao, F. J.; McGrath, S. P. 1997: Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non-accumulator *Thlaspi ochroleucum*. *Plant, cell and environment 20*: 898-906.

Sherrell, C. G.; Rawnsley, J. S. 1982: Effect of copper application on copper concentration in white clover and perennial ryegrass on some Northland soils and a yellow-brown pumice soil. *New Zealand journal of agricultural research 25*: 363-368.

Shuman, L. M. 1991: Chemical forms of micronutrients in soils. Pp. 113-144 *in*: Micronutrients in agriculture (2nd ed.). Mortvedt, J. J.; Cox, F. R.; Shuman, L. M.; Welch, R. M. *eds*. Soil Science Society of America, Madison, WI, USA.

Smart, M. E.; Cymbaluk, N. F.; Christensen, D. A. 1992: A review of copper status of cattle in Canada and recommendiations for supplementation. *Canadian veterinary 33*: 163-170.

Smolders, E.; Mclaughlin, M. J. 1996a: Chloride increases Cd uptake in Swiss chard in a resin-buffered nutrient solution. *Soil science society of American journal 60*: 1443-1447.

Smolders, E.; Mclaughlin, M. J. 1996b: Effect of Cl and Cd uptake by Swiss chard in nutrient solutions. *Plant and soil 179*: 57-64.

Sommer, A. L. 1931: Copper as an essential element for plant growth. *Plant physiology 6*: 339-345.

Sparks, D. L. 1984: Ion activities: An historical and theoretical overview. *Soil science society of America journal 48*: 514-518.

Stephan, U. W.; Scholz, G. 1993: Nicotianamine: mediator of transport of iron and heavy metals in the phloem? *Physiologia plantarum 88*: 522-529.

Stephan, U. W.; Schmidke, I.; Pich, A. 1994: Phloem translocation of Fe, Cu, Mn, and Zn in *Ricinus* seedlings in relation to the concentrations of nicotianamine, an endogenous chelator of divalent metal ions, in different seedling parts. *Plant and soil 165*: 181-188.

Stephan, U. W.; Schmidke, I.; Stephan, V. W.; Scholz, G. 1996: The nicotianamine molecule is made-to-measure for complexation of metal micronutrients in plants. *BioMetals 9*: 84-90.

Stevenson, F. J. 1994: Humus Chemistry, 2nd Edition, Wiley, New York.

Stevenson, F. J.; Fitch, A. 1981: Reactions with organic matter. Pp. 69-97 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Stiff, M. J. 1971: Copper-bicarbonate equilibria in solutions of bicarbonate ion at concentrations similar to those found in natural water. *Water research 5*: 171-176.

Struckmeyer, B. E.; Peterson, L. A.; Tai, F. H. M. 1969: Effects of copper on the composition and anatomy of tobacco. *Agronomy journal 61*: 932-936.

Stumm, W.; Morgan, J. J. 1981: Aquatic Chemistry. Wiley and Sons Inc., New York.

Sugiura, Y.; Nomoto, K. 1984: Phytosiderophores: Structures and Properties of Mugineic Acids and Their Metal Complexes. *Structure and bonding 58*: 106-135.

Swalls, A. A.; O'Leary, J. W. 1975: The effect of relative humidity on growth, water consumption and Ca uptake in tomato plants. *Journal of the arizona academy of science 10*: 87-89.

Symeonidis, L.; McNeilly, T.; Bradshaw, A. D. 1985: Differential tolerance of three cultivars of *Agrostis capillaris* L. to cadmium, copper, lead, nickel and zinc. *New phytologist 101*: 309-315.

Tammes, P. M. L. 1958: Micro- and macronutrients in sieve tube sap of plants. *Acta botany netherlands 7*: 233-234.

Tammes, P. M. L.; Van, Die J. 1964: Studies on phloem exudation from *Yucca flaccida*. *Acta botany nertherlands 13*: 76-83.

Tang, S.; Wilke, B. M.; Huang C. 1999: The uptake of copper by plants dominantly growing on copper mining spoils along the Yangtze River, the People's Republic of China. *Plant and soil 209*: 225-232.

Temminghoff, E. J. M. 1998: Chemical speciation of heavy metals in sandy soils in relation to availability and mobility. PhD thesis, Wageningen Agricultural University, Netherlands.

Temminghoff, E. J. M.; Van Der Zee, S. E. A. T. M.; Haan, F. A. M. D. 1997: Mobility of Cu in Cu-contaminated soil: effects of complexation and competition. Pp.715-716 *in*: *Proceedings of fourth international conference on the biogeochemistry of trace elements*, Iskandar, K. I.; Hardy, S. E.; Chang, A. C.; Pierzynski, G. M. *eds*. June 23-26, 1997. Clark Kerr Campus, Berkeley, California, USA.

Thomas, B.; Thompson, A. 1948: The ash-content of some grasses and herbs on the Palace Leas hay plots at Cockle Park. *Empire journal of experimental agriculture 16*: 221-230.

Thomas, B.; Thompson, A.; Oyenuga, V. A.; Armstrong, R. H. 1952: The ash constituents of some herbage plants at different stages of maturity. *Empire Journal of experimental agriculture 20*: 10-22.

Thornton, B. 1991: Indirect compartmental analysis of copper in live ryegrass roots: comparison with model systems. *Journal of experimental botany 42*: 183-188.

Tiffin, L. O. 1972: Translocation of micronutrients in plants. Pp. 199-229 *in*: Micronutrients in agriculture. Mortvedt, J. J.; Giordano, P. M.; Lindsay, W. L. *eds*. American Society of Agronomy. Madison, WI, USA.

Tilstone, G. H.; Macnair, M. R. 1997: Ni tolerance and Cu-Ni co-tolerance in *Mimulus guttatus* from Cu mine and serpentine habitats. *Plant and soil 191*: 173-180.

Timperley, M. H.; Brooks, R. R.; Peterson, P. J. 1970: The significance of essential and non-essential trace elements in plants in relation to biogeochemical prospecting. *Journal of applied ecology 7*: 429-439.

Tolra, R. P.; Poschenrider, C.; Barcelo, J. 1996: Zinc hyperaccumulation in *Thlaspi caerulescens*. II. Influence on organic acids. *Journal of plant nutrition 19*: 1514-1550.

Turner, R. G. 1969: Heavy metal tolerance in plants. Pp 399-410 *in*: Ecological aspects of mineral nutrition of plants. *British ecological society symposium vol 9*. Rorison, I. H. *ed*. Blackwell Scientific Publishers, Oxford.

Urrestarazu, M.; Sanchez, A.; Lorente, F. A.; Guzmán, M. 1996: A daily rhythmic model for pH and volume from xylem sap of tomato plants. *Communications in soil science and plant analysis 27*: 1859-1874.

Van der Mark, F.; Van den Briel, M. L.; Van Oers, J. W. A. M.; Bienfait, H. F. 1982: Ferritin in bean leaves with constant and changing iron status. *Planta 156*: 341-344.

Vázquez, M. D.; Poschenrieder. Sh.; Barceló, J.; Baker, A. J. M.; Hatton, P.; Cope, G. H. 1994: Compartmentation of zinc in roots and leaves of the zinc hyperaccumulator *Thlaspi caerulescens*. J & C Presl. *Botanica acta 107*: 243-251.

Verkleij, J. A. C.; Schat, H. 1989: Mechanisms of metal tolerance in higher plants. Pp.179-189 *in*: Heavy metal tolerance in plants: Evolutionary aspects. Shaw A. J. *ed*. CRC Press, Inc. Boca Raton, Florida.

Von Frenckell-Insam, B. A. K.; Hutchinson, T. 1993a: Nickel and zinc tolerance and co-tolerance in *Deschampsia cespitosa* (L.) Beauv. Subject to artificial selection. *New phytologist 125*: 547-553.

Von Frenckell-Insam, B. A. K.; Hutchinson, T. 1993b: Occurrence of heavy metal tolerance and co-tolerance in *Deschampsia cespitosa* (L.) Beauv. From European and Canadian populations. *New phytologist 125*: 555-564.

Vose, P. B. 1963: The translocation and redistribution of manganese in *Avena. Journal of experimental botany 14*: 448-457.

Walker, C. D.; Webb, J. 1981: Copper in plants: forms and behaviour. Pp. 189-212 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Walker, C. D.; Welch, R. M. 1986: Nicotianamine and related phytosiderophores: their physiological significance and advantages for plant metabolism. *Journal of plant nutrition 9*: 523-534.

Wang, X. P.; Zabowki, D. 1998: Nutrient composition of Douglas-fir rhizosphere and bulk soil solutions. *Plant and soil 200*: 13-20.

Welch, R. M. 1986: Effects of nutrient deficiencies on seed production and quality. *Advance in plant nutrition 2*: 205-247.

Welch, R. M. 1995: Micronutrient nutrition of plants. *Critical reviews in plant sciences 14*: 49-82.

White, M. C.; Baker, F. D.; Chaney, R. L.; Decker, A. M. 1981a: Metal complexation in xylem fluid. II. Theoretical equilibrium model and computational computer program. *Plant physiology 67*: 301-310.

White, M. C.; Decker, A. M.; Chaney, R. L. 1981b. Metal complexation in xylem fluid. I. Chemical composition of tomato and soybean stem exudate. *Plant physiology 67*: 292-300.

Whitehead, D. C. 1987: Some soil-plant and root-shoot relationships of copper, zinc and manganese in white clover and perennial ryegrass. *Plant and soil 97*: 47-56.

Williams, R. F. 1955: Redistribution of mineral elements during development. *Annual review of plant physiology 6*: 1499-1512.

Wood, J. G.; Womersly, H. B. 1964: Development and metabolism of copper-deficient oat plant. *Australian journal of experimental biology and medical science 24*: 79-94.

Woolhouse, H. W.; Walker, S. 1981: The physiological basis of copper toxicity and copper tolerance in higher plants. Pp. 235-258 *in*: Copper in soils and plants. Loneragan, J. F.; Robson, A. D.; Graham, R. D. *eds*. Academic Press, New York.

Youssef, R. A.; Chino, M. 1989: Root-induced changes in the rhizosphere of plants. I. pH changes relation to the bulk soil. *Soil science and plant nutrition 35*: 461-468.

Youssef, R. A.; Chino, M. 1991: Movement of metals from soil to plant roots. *Water, air, and soil pollution 58*: 249-258.

Zoysa, A. k. N.; Longanathan, P.; Hedley, M. J. 1999: Phosphate rock dissolution and transformation in the rhizosphere of tea (*Camellia sinensis* L.) compared with other plant species. *European journal of soil science 49*: 477-486.