A COMPARATIVE STUDY OF THE INFLUENCE OF DIFFERENT COPPER CONCENTRATIONS ON THE GROWTH, CHEMICAL COMPOSITION AND ACTIVITIES OF CERTAIN COPPER CONTAINING ENZYMES IN *Atriplex nummularia* Lindl. AND *A. vestita* (Thunb.) Aell.

BY

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DECLARATION

"I declare **A comparative study of the influence of different copper concentrations on the growth, chemical composition and activities of certain copper containing enzymes in Atriplex nummularia Lindl. and Atriplex vestita (Thunb.) Aell.**, is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references."

.....

D. H. GREEN

DEDICATED TO MY MOTHER

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INTRODUCTION

Copper is essential for the normal growth and development of plants and animals. Animals depend on plants for their copper supply, which in turn mine the soil for available copper. Abnormalities in the diet of animals can thus be related to the availability of copper in the soil and its uptake by palatable plants. The relationship between soil copper and plant available copper have been well studied in overseas countries. In South Africa, however, little work has been done in this field and this project is an attempt to address the problem.

The key objectives of this project were:

(i) To investigate the effect of different copper concentrations on the growth and chemical composition of Atriplex nummularia Lindl. and Atriplex vestita (Thunb.) Aell.

(ii) To determine which one of the two species grows better under conditions of copper deficiency.

(iii) To investigate the relationship between copper supply and the activities of the copper enzymes, cytochrome oxidase, superoxide dismutase, ascorbate oxidase and laccase.

To facilitate the immediate dissemination of information to current journals, this thesis has been written in paper form:

In **Chapter 1** an overview is given of copper in the biotic and abiotic environment.

In **Chapter 2** the effect of different copper concentrations on the growth and chemical composition of A. nummularia Lindl. and A. vestita (Thunb.) Aell. is investigated and discussed. A comparison is drawn between copper treatments and species.

Chapter 3 deals with the relationship between copper supply and the activities of the copper enzymes, cytochrome oxidase, superoxide dismutase, ascorbate oxidase and laccase. The effects of copper treatment and species on enzyme activity are compared.

In conclusion a brief **summary** is given of the major findings of the investigation.

CHAPTER 1

OVERVIEW OF COPPER IN THE BIOTIC AND ABIOTIC ENVIRONMENT

1.1 COPPER IN THE SOIL:

The total amount of native copper in soil depends on the amount of copper in the parent material (Tisdale et al. 1968). It is usually in greater concentration in the soil than in parent rock because of weathering of the parent rock and the concentration of the element in the upper soil horizons by growing plants. Copper in parent rock exhibits typical chalcophile behaviour in that its abundant and stable minerals are sulfides rather than silicates or oxides (Mordtveldt et al. 1972). By far the most abundant of the copper minerals is chalcopyrite ($CuFeS_2$), which is common in igneous and sedementary rocks.

The average copper content in the soil ranges between 2 to 100g.m^{-3} (Tisdale et al. 1968), while normal soil solutions contain about 0.01g.m^{-3} copper (Devlin 1983). Concentrations higher than this are often regarded as toxic to plants. Two valences of copper are found in naturally occuring compounds i.e. Cu^{+} and Cu^{2+}. The Cu^{2+} ion is more commonly found in soils, adsorbed to clay minerals or tied up with organic matter. In soil solutions up to 98% of the copper is complexed to low molecular weight organic compounds (Marschner 1986). Smaller amounts of neutral insoluble salts, water-soluble compounds and copper minerals may be present. The cuprous ion, Cu^{+}, is unstable at ordinary temperatures in concentrations greater than 10^{-7}M; at higher concentrations it can exist in solution only in the form of anionic complexes like $CuCl_2^{-}$ (Mordtveldt et al.

1972). Cu^{2+} is readily reduced to the unstable Cu^{+}. In this respect copper is similar to iron. Most of the functions of copper as a plant nutrient are based on the participation of enzymatically bound copper in redox reactions. In the reactions of terminal oxidases, copper enzymes react directly with molecular oxygen. Therefore the terminal oxidation in living cells is catalyzed by copper and not by iron (Mordtveldt et al. 1972).

1.2 FACTORS INFLUENCING COPPER AVAILABILITY IN THE SOIL:

The availability of copper to plants is determined by several factors: level of soil organic matter, soil pH, and the presence of other metallic ions such as iron, zinc or aluminium (Tisdale et al. 1968).

Copper retention increases with an increase in soil organic matter (Tisdale et al. 1968). Copper-humus complexes vary in their degree of stability. In some cases the copper is bound so tightly that it is not available to plants, in others, plants can absorb copper from the organic compounds. Hence, soils high in organic matter are more subject to copper deficiencies than those with smaller amounts.

Wallace (1984) has shown that the amount of exchangeable copper decreased as the pH increased. Other workers, however, have found no relationship between copper availability and soil pH (Tisdale et al. 1968). They found

that increasing aluminium concentrations in solution cultures decreased the uptake of copper. It was thus concluded that both pH and aluminium activity affect the uptake of copper by plants as an increase in both causes a decrease in activity of ionic Cu^{2+}.

The absolute level of a trace element in the rooting medium is not the most important factor in relation to plant growth (Tisdale et al. 1968). More important is the amount of the elements in relation to one another. Scientists in North Carolina have found that maximum growth of plants is not related to the absolute amounts of copper or iron, but rather to the ratio of copper to iron in the rooting medium. They, for example, found maximum yield in lettuce at a copper to iron ratio of 2.5:3.0 (Tisdale et al. 1968). Similar relationships were discovered between copper and molybdenum and between copper and aluminium. The use of absolute quantities of elements in plant and soil diagnostic work may thus be misleading; these values, especially for the microelements, should be considered in relation to one another. It is further evident that a great deal of research is still needed, for if maximum plant growth is indeed the product of some unique ratio among the various nutrient elements it is of obvious interest to determine what those ratios are.

1.3 COPPER AVAILABILITY IN KAROO SOILS.

Ellis (1988) used the following norms, based on the copper

requirements of crop plants and not of the natural vegetation, to describe the copper status of Karoo soils:

Status	: Low	Medium	High	Very High
$mg.Kg^{-1}$ dry Soil	: 0 - 0.3	0.3 - 0.6	0.6 - 5.0	>5.0

Using these norms, Ellis (1988) found low copper availability in the A horizons of the West Coast (south) and Knersvlakte soils as well as in the B horizons of West Coast (north and south) soils. He also found high copper availabilty in the A horizons in Namaqualand closed mountains and open mountains, the Great Karoo (west and east), the Little Karoo (west and east), the Central, Eastern and Northern Karoo as well as Boesmanland (west and east). The rest of the Karoo soils are classified as having a medium copper availability.

The same low copper availabilty as found in the littoral and near-littoral sands of the West Coast, is also found in the sandy soils of the Southern Cape (Ellis, personal communication).

1.4 EFFECT OF COPPER AVAILABILITY ON PLANTS

Copper plays a catalytic role in plants (Bidwell 1974) and either directly or indirectly influences the activity of especially the photosynthetic enzymes in the plant. Copper

forms an important constituent of certain plant proteins eg. ascorbate oxidase (Devlin 1983), laccase (Berlanger et al. 1986), superoxide dismutase, cytochrome oxidase and plastocyanin (Lehninger 1982). Copper, like the other trace elements, is essential for normal growth, but toxic at relatively low concentrations.

Major deficiency symptoms include a decrease in vegetative growth, a decrease in leaf area, leaf chlorosis, necrosis, withering of leaves and die-back of shoots, also refered to as exanthema (Stiles 1961, Adedeji et al. 1984, Agarwala et al. 1985, Dell 1981 & Rey et al. 1987). Copper deficiency terminates physiological processes in the plant as it inhibits the synthesis of various enzymes eg. catalase (Agarwala et al. 1985 & 1986), cytochrome c oxidase (Agarwala et al. 1986, Bligny et al. 1986 & Walker et al. 1981), aldolase, ascorbate oxidase, superoxide dismutase and polyphenol oxidase (Agarwala et al. 1985 & Walker et al. 1981). On the contrary, a reduction in tissue copper causes an increase in enzymatic activity of peroxidase and ribonuclease enzymes and a corresponding decrease in DNA and RNA production (Agarwala et al. 1985). The decrease in DNA can be ascribed to the direct involvement of copper in DNA synthesis or to a fewer number of cells in the interphase. The decrease in RNA is due to the stimulation of ribonuclease

synthesis under copper stress (Agarwala et al. 1985). Copper deficiency causes male sterility in plants (Dell 1981). Dell found a reduction in the lignification of anther walls and the absence of the endothecia of anthers in plants subjected to copper deficiency. This resulted in reclamation disease, i.e. reduced seed set due to reduced pollen fertility and failure of the stomia to rupture in the absence of lignified anther wall thickenings. Dell also believes that copper deficiency interrupts microsporogenesis at or near the meiotic stage, an effect shared by deficiencies in boron and molybdenum.

Photosynthetic effects include a decrease in leaf chlorophyll (Adedeji et al. 1984); a decrease in plastocyanin (Agarwala et al. 1985); a depletion of carotenoids and plastoquinone (Droppa 1984); a reduction in reducing sugar concentration eg. sucrose (Agarwala et al. 1985) and a reduction in soluble proteins. Copper deficiency delays plant senescence as it retards ethylene production by delaying acetylene reduction (Dell 1981 & Snowball et al. 1980). This result in the development of short stems, blind shoots and the abortion of flowers (Rey et al. 1987, Walker et al. 1981 & Snowball 1980).

Copper toxcity shows similar effects to copper deficiency. For most crop species the critical toxicity level of copper in the leaves is considered to be above 0.02 to 0.03g.kg^{-1}

dry mass (Tisdale et al. 1968). Excessive amounts of copper depress the activity of iron and causes iron-deficiency symptoms to appear in plants. Major toxic symptoms include interveinal chlorosis (Rey et al. 1987), reduced dry matter production, degradation of grana stacks and stroma lamellae, an increase in the number and size of plastoglobuli and intrathylakoidal inclusions (Baszynski et al. 1988), a decrease in photosynthetic activity and early death of the plant (Adedeji et al. 1984). The low sucrose content of sugarbeet roots under copper stress might be due to low photosynthetic activity resulting from depressed concentrations of the copper containing protein, plastocyanin (Agarwala et al. 1985).

1.5 COPPER PROTEINS

Copper is a constituent of copper proteins and enzymes and is essential for their function in plant metabolism. Copper proteins act as terminal oxidases, mono - and dioxygenases in the elimination of superoxide radicals and in electron transport pathways (Sandman & Boger 1983).

Copper is present in three different forms of proteins:

(a) blue proteins without oxidase activity e.g. plastocyanin, which functions in electron transfer;

(b) non-blue proteins, which produce peroxidases and oxidize monophenols to diphenols; e.g superoxide dismutase;

(c) multicopper proteins containing at least four copper

atoms per molecule, which act as oxidases e.g. ascorbate oxidase and laccase.

Under conditions of copper deficiency the activity of these enzymes decreases fairly rapidly. However, in several cases it is difficult or impossible to correlate directly the decrease in activity of a certain copper enzyme with gross metabolic changes or with inhibition of plant growth. For example, in copper-deficient cells a drastic decrease in cytochrome oxidase activity was without effect on the respiration rate, indicating that this enzyme might be present in large excess in the mitochondria (Bligny & Douce 1977).

1.5.1. Plastocyanin

More than 50% of the copper localized in the chloroplasts is bound to plastocyanin. This compound has a molecular weight of 10,000 and contains one copper atom per molecule. Plastocyanin is a component of the electron transport chain of photosystem I. The copper atom of this molecule is the actual carrier of the electrons, since it is capable of undergoing Cu^{+} and Cu^{2+} cycles. The plastocyanin is reduced by cyt f during electron transfer of photosystem II and transfers the electron to P700 i.e. the photochemical reaction centre of photosystem I (Lehninger 1982).

With copper deficiency, there is a greater decrease in the plastocyanin content and the activity of photosystem I than

in the content of other chloroplast pigments and the activity of photosystem II (Marschner 1986), causing a decrease in photosynthetic rate. The decrease in photosynthesis can also directly be related to other roles of copper in the chloroplast e.g. copper is a component of certain chloroplast enzymes e.g. superoxide dismutase and copper is required for the synthesis of quinones. This is reflected in the decrease of plastoquinone under conditions of copper deficiency (Marschner 1986).

1.5.2. Cytochrome oxidase

Cytochrome oxidase is an important respiratory enzyme. It forms the terminal oxidase of the mitochondrial electron transport chain (Walker et al. 1981). The enzyme is a multipeptide aggregate containing two hemes and two copper atoms (Morrison et al. 1963) which are involved in the electron transport system and oxidative phosphorylation. The cytochrome c oxidase complex or cyt aa_3 is a structural element of the mitochondrial inner membrane. It spans the mitochondrial membrane assymetrically (Eytan et al. 1975) with heme a facing the outer membrane, while heme a_3 is situated on the matrix side and it has been demonstrated that there is a topological sequence a-Cu-Cu-a_3 (Malmstrom 1973). Cytochrome oxidase catalyzes the transfer of electrons from cytochrome c to oxygen, to form water. The two copper atoms undergo oxidation - reduction between the Cu^{+} and Cu^{2+} forms and are responsible for electron transport between the iron

components of cytochromes a and a_3. The copper and iron components of cyt aa_3 are responsible for transfer of four electrons to oxygen to form water (Lehninger 1982).

If oxygen is only partially reduced by accepting only two electrons, the product is hydrogen peroxide or superoxide radicals in the acceptance of one electron. Both products are extremely toxic to the cells because they attack unsaturated fatty acid components of membrane lipids, thus damaging membrane structure (Lehninger 1982). This is prevented in aerobic cells by the action of superoxide dismutase, a copper containing enzyme which converts the superoxide radical into hydrogen peroxide which is then split up into water and molecular oxygen by catalase activity.

1.5.3. Superoxide dismutase (SOD)

As mentioned in the previous paragraph, superoxide dismutase is a copper containing enzyme acting as a cellular defence against the toxic superoxide radical generated in a wide range of biochemical and photolytic reactions (Halliwell 1978). Hence, the activity pattern of SOD may indicate its ubiquitous requirement in the plant at a certain level to maintain the integrity of protoplasmic constituents. The activity of SOD in plants shows a close correlation to copper and nitrogen levels. By contrast the activities of the copper oxidases do not correspond with the parallel patterns of copper and nitrogen concentrations in the leaves (Walker *et al.* 1981). Ayala & Sandman (1988) distinguish between

three SOD isoenzymes in the leaves of pea plants grown with a normal copper supply viz. a plastidic Cu/Zn-SOD, a cytoplasmic Cu/Zn-SOD and a Mn-SOD. During copper deprivation the activities of both Cu/Zn-SOD decreased, with a concurrent increase of Mn-SOD. Nevertheless, total SOD activity decreases with a decrease in copper supply. The Cu/Zn-isoenzyme has a molecular mass of 32.000, and at the active site one copper and zinc atom are probably closely connected by a common histidine nitrogen (Sandman & Boger 1983). In green leaves most of this enzyme is localized in the chloroplasts, especially in the stroma (Marschner 1986).

1.5.4. Ascorbate oxidase

Ascorbate oxidase is a dimeric copper - containing enzyme, wide spread in plant tissues and involved in secondary metabolism (Esaka et al. 1988). It is linked to cytoplasmic dehydrogenases via a redox shuttle involving glutathione (Walker et al. 1981). It has a molecular mass of 140.000 with eight copper ions (Morpurgo 1988). Ascorbate oxidase catalyzes the oxidation of ascorbic acid to L-dehydro ascorbic acid. The reaction occurs in the cell walls and in the cytoplasm. Its physiological functions are still uncertain, but it may act as a terminal respiratory oxidase or in combinations with phenolases. Its activity is a very sensitive indicator of the copper nutritional status of the plant i.e. low ascorbate oxidase activity is associated with low copper status and vice versa. If copper is added, ascorbate oxidase activity increases. However, a large

excess of copper inhibits activity of the enzyme (Esaka et al. 1988).

1.5.5. Laccase

Laccase contains six copper atoms per molecule of which four are divalent. It occurs in the thylakoid membranes of chloroplasts, where it is required for the synthesis of plastoquinone, a constituent of photosynthetic electron transport chains (Marschner 1986).

1.5.6. Phenolase

Phenolase has two distinct enzyme functions:

(a) mono-oxygenation of monophenols,

(b) mono-oxygenation of o-diphenols leading to quinone production.

Phenolase is involved in the biosynthesis of lignin and alkaloids (Hanson & Havir 1979), the synthesis of electron - transfer intermediates (Bidwell 1974) and in the formation of brown melanotic substances, which are sometimes formed when tissues are wounded. o-Diphenol oxidase is produced in response to wounding, being inhibitors of invasive spore growth and acting as toxins against herbivores (Mayer & Harel 1979). Under conditions of copper deficiency, the decrease in phenolase is quite severe and is correlated with the accumulation of phenolics and a decrease in the formation of melanotic substances. The latter effect is reflected, for example in the close correlation between the colour of spores of **Aspergillus niger** and the copper nutritional status.

With an ample copper supply the spores are black; with mild deficiency they are light brown; and with severe deficiency they are white. The decline in phenolase activity is also indirectly responsible for the delay in flowering and maturation often observed in copper-deficient plants.

1.5.7. Diamine oxidase

Diamine oxidase metabolizes polyamines such as spermine and spermidine, which are widely found in plant tissues and are precursors of alkaloids (Waller & Nowacki 1978). Delhaize et al. (1986) reported that diamine oxidase is synthesized only in young developing leaves and that apo-diamine oxidase is absent in copper deficient clover leaves .

1.6 COPPER FERTILIZER:

Should farmers experience a shortage of copper in their soils, copper can be applied by foliar application of soluble or slightly soluble salts or by applying fertilizer materials to the soil (Tisdale et al. 1968). Copper sulphate and copper ammonium phosphate are commonly used for this purpose. Lately chelates of copper and other micronutrients are used. These are especially suitable for soil application. Chelates are metalo-organic compounds, such as EDTA, which though soluble themselves, do not ionize to any degree (Tisdale et al. 1968). They retain copper and similar metals in a soluble form, permitting their absorption by plants, yet preventing their conversion to insoluble forms in the soil. Chelated copper compounds are commercially

available for soil or foliar application.

1.7 COPPER TOLERANCE

Genotypical differences in tolerance to copper and other heavy metals are well known in certain species and ecotypes of natural vegetation (Woolhouse 1983). Special plants tolerant to heavy metals, called metallophytes, have been known for centuries to grow in mining areas. Woolhouse (1983) grouped the mechanisms of copper tolerance in higher plants as follows:

(i) exclusion or restriction of copper uptake;

(ii) immobilization of copper in cell walls;

(iii) compartmentation of copper in soluble complexes;

(iv) compartmentation of copper in insoluble complexes;

(v) enzyme adaptation.

The exclusion or restriction of copper uptake is of minor importance in higher plants. The immobilization of copper in the cell walls is considered to be an important mechanism of copper detoxification, but by far the most important mechanism is the compartmentation of copper in soluble and insoluble complexes (Marschner 1986). In this case copper binds to specific proteins, called metallothioneins, the synthesis of which is induced by high copper concentrations. These proteins are rich in cysteine, to which copper is bound much more firmly than zinc or cadmium. The adaptation of enzymes to high copper concentrations is of limited importance and is presumably restricted to extracellular

enzymes such as wall-bound phosphatases in the free space of roots (Marschner 1986).

1.8 COPPER IN ANIMAL NUTRITION

The importance of copper in animal nutrition was recognized in 1928 by scientists at the University of Wisconsin who showed that both copper and iron are necessary for hemoglobin formation in rats suffering from anemia (Mordtveldt et al. 1972). Soon after demonstrating the essential role of copper in hematopoiesis, several enzymes with oxidase functions were shown to contain copper. Among these are tyrosinase (polyphenoloxidase), laccase and ascorbate oxidase. Mahler (1953) has shown butyryl CoA-dehydrogenase to be a cupro-flavoprotein containing copper as a part of the prosthetic group.

Certain diseases of cattle and sheep were shown to be related to copper deficiency, symptomized by diarrhea, loss of appetite and anemia. These were found in sheep and cattle in Florida in 1931 (Mordtveldt et al. 1972). Bennets and Chapman (1937) in Australia showed that a disease of lambs called "enzootic ataxia" was due to a copper deficiency and could be prevented by feeding copper to the ewes during pregnancy.

A normal human adult contains approximately 100 to 150mg of copper, or 1.5 to 2.0g.m^{-3}. Similar concentrations of copper occur in the bodies of most adult animal species

(Mordtveldt et al. 1972). The largest concentrations of copper occur in the liver of animals and the liver copper-level reflects the dietary intake of copper. Hence it was found that the blood and liver copper-levels of deficient animals were well below normal and they fail to thrive unless extra copper is supplied, either directly or indirectly.

A wide range of different clinical syndromes are caused by copper deficiency in animal species. They include anaemia (general symptom), depressed growth, bone disorders, depigmentation of hair, fur or wool, abnormal wool growth, demyelination of the spinal cord, fibrosis of the myocardium, gastrointestinal disturbances (diarrhea or scours). All have been shown to be caused by copper deficiency and all have been shown to be alleviated by or prevented by the administration of adequate amounts of copper (Mordveldt et al. 1972).

1.8.1 Bone disorders

Bone defects are characterized by spontaneous fractures and a condition similar to rickets in young calves and osteoporosis in older animals. The bone changes occur under conditions of normal phosphorous nutrition and were shown to be a specific effect of copper deficiency and not anaemia, since severe anaemia caused by iron deficiency, failed to produce similar effects.

1.8.2 Demyelination of the Central Nervous System

Copper deficiency causes enzootic ataxia commonly called "swayback" or "swingback" (Mordveldt et al. 1972). Two types of neonatal ataxia are recognised: (i) A common acute form which occurs in newborn lambs; and (ii) a delayed type in which clinical symptoms are not observed for weeks or months after birth. In both cases the symptoms are characterized by spastic paralysis, poor coordination of the hind legs, a stiff and staggering gait and an exaggerated swing of the hind quarters. Some lambs are completely paralyzed or ataxic at birth and die immediately.

1.8.3 Effect on Pigmentation of Hair and Wool

In sheep, copper deficiency produces a lack of pigment of blackwooled sheep, and characteristic loss of "crimp" from the fibres of all wool. The copper containing enzyme, polyphenol oxidase, is known to catalyze production of melanin from L-tyrosine. Hence low copper concentrations will inhibit this reaction influencing the pigmentation of the wool and hair. The lack of "crimp" is believed by Marston (1952) to be due to the need for copper for the normal oxidation of cysteine to the -S-S- bond required for maintaining the proper helix structure in the protein molecules of the wool.

1.8.4 Fibrosis of the Myocardium

"Falling disease" is believed to be caused by copper deficiency in association with a selenium deficiency. It is

characterized by sudden death, usually without any preliminary signs. The anaemia associated with "falling disease" is of the macrocytic hypochromic type. The major lesion is an atrophy of the myocardium with a replacement fibrosis (Mordveldt et al. 1972).

1.8.5 Diarrhea in cattle

Severe diarrhea, also known as "scouring disease", has been observed in many parts of the world and is known to be accentuated in areas with excess molybdenum and low amounts of copper.

1.8.6 Aortic Rupture

Copper deficiency in chicks produced dissecting aneurysm of the aorta and various bone deformities (O'Dell et al. 1961). This was caused by the absence of amine oxidase enzyme in chicks dosed with water from copper deficient dams. Amine oxidase increases the incorporation of lysine into the elastins of the aorta and it is dependant on copper to perform this function. The enzyme oxidizes the lysine molecules by deamination and in the absence of copper the number of oxidized lysine residues are reduced to condense for the formation of desmosine.

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CHAPTER 2

THE INFLUENCE OF COPPER ON THE GROWTH AND CHEMICAL COMPOSITION OF *Atriplex nummularia* Lindl. AND *A. vestita* (Thunb.) Aell.

ABSTRACT

Atriplex nummularia Lindl. and Atriplex vestita (Thunb.) Aell. plants were subjected to different copper concentrations viz. 0.0005, 0.005, 0.05, 0.5 and 5.0 $g.m^{-3}$ copper. Plants were grown in a Conviron growth chamber under twelve hour illumination, with day and night temperatures of 26°/20°C and relative humidity of 60%/80% respectively. Treatment and species significantly influenced the growth and chemical composition of the plants. Maximum elongation, fresh and dry mass production, occured at 0.05 $g.m^{-3}$ copper. No typical deficiency symptoms were seen, while the toxic copper concentration severely inhibited plant growth in both species. Leaves were small and chlorotic, with fewer and shorter lateral branches. The largest part of the root systems was dead, with new growth showing as short protrusions. Tissue copper increased with the increasing copper concentration, while leaf nitrogen and phosphorous responded inversely to copper treatment. The potassium, sodium, calcium, magnesium, iron, zinc and manganese concentrations were not significantly affected by the lower copper treatments, while 5.0$g.m^{-3}$ copper significantly reduced nutrient uptake in both species. Atriplex vestita grew significantly better than A.nummularia at the lower copper treatments with higher yields for all of the parameters tested. However, A. vestita is more susceptible to copper toxicity than A.nummularia.

UITTREKSEL

Atriplex nummularia Lindl. en Atriplex vestita (Thunb.) Aell. plante is aan verskillende koper konsentrasies onderwerp, nl. 0.0005, 0.005, 0.05, 0.5 en 5.0g.m^{-3} koper. Die plante is in potte met sand in 'n Conviron groeikabinet gekweek, onder twaalf uur beligting, met dag en nag temperature van 26°C/20°C en relatiewe voggehalte van 60%/80% onderskeidelik. Behandeling en spesies het die groei en chemiese samestelling van die plante betekenisvol beinvloed. Lengtegroei, vars- en droëmassa produksie was maksimaal by 0.05g.m^{-3} koper. Tipiese tekort simptome het nie voorgekom nie, terwyl kopertoksisiteit groei by albei spesies betekenisvol onderdruk het. Blare was klein en chloroties, met minder en korter sytakke. Die grootste deel van die wortelstelsel was dood, met nuwe groei in die vorm van uitsteeksels. Die koperkonsentrasie het in albei plantsoorte toegeneem, terwyl die stikstof- en fosforinhoud van die blare afgeneem het met toename in koperkonsentrasie. Die laer koperkonsentrasies het geen betekenisvolle invloed op die kalium-, natrium-, kalsium-, magnesium-, yster-, sink- en mangaankonsentrasies van die plante gehad nie, terwyl die behandeling met 5.0g.m^{-3} koper minerale voeding betekenisvol onderdruk het. Atriplex vestita het betekenisvol beter as A. nummularia gegroei by die lae koperkonsentrasies, terwyl dit meer vatbaar was vir kopertoksisiteit as A. nummularia.

Keywords: Atriplex, copper deficiency, copper toxicity, growth.

2.1 INTRODUCTION

Copper is an essential trace element for normal plant development. Agricultural plants often show toxicity or deficiency of this element (Hill, 1973). In South Africa, areas of copper deficiency have been identified in the southern and western Cape Province, while copper toxicity is more common towards the interior of the country (Ellis, 1987; and Vosloo, 1990; personal communication). Sheep in the southern and western Cape Province often show severe deficiency symptoms like enzootic ataxia ("sway back"), poor wool quality, osteoporosis or early death. Farmers artificially dose their sheep with copper-containing salt licks or "solkupro" in their drinking water.

Atriplex nummularia Lindl. and Atriplex vestita (Thunb.) AELL., two drought resistant, halophytic species, are planted in some of these areas for grazing purposes. Little is known about the reaction of these plants to soil copper availability, hence the effect of copper on the growth and chemical composition of these species was investigated. Since past research on copper nutrition primarily delt with copper deficiencies in plants, with fewer articles published on copper toxicity, copper concentrations ranging from deficient to toxic were used during this investigation.

2.2 MATERIALS AND METHODS

2.2.1 Cultivation, treatment and harvesting of plants

Atriplex nummularia and A. vestita seeds were supplied by the Agricultural Research Institute for the Karoo Region, Middelburg, Cape. Sodium chloride tends to inhibit seed germination and must be removed prior to sowing, so seeds were leached with running tap water to remove excess salt. Seeds were sown in seperate containers, treated with half strength Hoagland nutrient solution and germinated in a Fisons growth cabinet (model no. 600G3) under twelve hour illumination, with day and night temperatures and relative humidity of 26°C/20°C and 60%/80% respectively. Sand (20/40 grade) was washed in a 1:1 mixture of 15% HCl and 1% oxalic acid solution (Hewitt 1966)

After two months, each seedling was transplanted into a seperate polythene container with sand, prior to which the roots were rinsed in distilled water to remove all adhering sand particles. Three weeks were allowed for the seedlings to recover from the transplant before treatments were started. All nutrients, except copper, were uniformly applied. Five copper treatments were used (0.0005; 0.005; 0.05; 0.5 and 5.0 $g.m^{-3}$), replicated seven times in a random block design. Sodium chloride was added to a final concentration of 0.05M, because of the halophytic nature of the plants. Analytical grade reagents were used throughout

the experiment. The degree of contamination of the nutrient salts and distilled water was determined via polarography and final copper concentrations were obtained by the standard addition method. The pH was controlled between 6.5 and 7.0 to prevent the precipitation of mineral nutrients and the suppression of copper uptake by the plants (Wallace 1984). Fresh nutrient solution was applied weekly after the sand was rinsed with distilled deionised water and allowed to drain. Photographs were taken to record visual symptoms that occured during the experiment.

Increase in plant height was measured on a weekly basis for ten weeks after which the plants were harvested. The roots were excised, rinsed in distilled deionised water to remove soil particles and mineral nutrients, and oven dried at 60°C. The number and length of the lateral branches were recorded. The shoots were weighed, first the stems and leaves combined and then seperately. The seven replicates were divided into two groups: Three were used for chlorophyll determinations and thus stored in a freezer at -40°C until the time of analysis. The remainder of the replicates were oven dried at 60°C and the dry mass of the stems, leaves and roots were determined seperately.

2.2.2 Plant analysis

Plant samples were ground in a Wiley Intermediate Mill (60 mesh) and digested in a sulphuric acid-peroxide mixture (Allen *et al.* 1986). In the case of plants with high yields, samples

of 0.4 grams of material were used for the digest, while 0.2 grams were used in plant samples with low yields. The digested mixtures were diluted with $20cm^3$ distilled deionised water and filtered through Whatman no. 1 filter paper. The filtrates were made up to $100cm^3$ by further addition of distilled deionised water and transfered to brown plastic bottles for storage.

The following analyses were performed:

2.2.2.1 Total nitrogen was determined by a Micro-Kjeldahl method (Allen et al. 1986).

2.2.2.2 Macronutrients were determined with a Pye-Unicam SP9 Atomic Absorption Spectrophotometer. Potassium and sodium were diluted in distilled deionised water (1:15), with a Hamilton Microlab diluter, while calcium and magnesium were diluted (1:9) with Lanthanum(111)oxide solution to suppress interferences in the atomic absorption flame (Allen et al. 1986).

2.2.2.3 Micronutrients: iron, zinc, manganese and copper were determined directly with a Pye-Unicam SP9 Atomic Absorption Spectrophotometer.

2.2.2.4 Phosphorous was determined using the phospho-molybdo-blue method of Allen et al. (1986).

2.2.2.5 Chloride was determined with a Radiometer TTT 80 automatic titrator fitted with a silver electrode (Horwitz 1980).

2.2.2.6 Chlorophyll was determined by the method of Knudson et al. (1977).

2.2.3 Statistical Analysis

Results were subjected to statistical analysis of variance using ANOVA12 and a Significant Statistics (SIGSTAT) package. Least significant differences between species and treatments were calculated at the p=0.01 and p=0.05 levels.

2.3 RESULTS AND DISCUSSION

2.3.1 Visual symptoms

Optimal growth in both Atriplex species occured at 0.05g.m^{-3} copper (Fig.s 1, 2 & 3), while both grew slower at 0.0005g.m^{-3} copper (Fig.s 1 & 2). Atriplex vestita was less affected by the low copper concentration (Fig. 4). Both species showed toxic symptoms at 5.0g.m^{-3} copper (Fig. 1, 2 & 5). Atriplex vestita, with smaller, more chlorotic leaves, appeared more susceptible to copper toxicity than A. nummularia. Toxic symptoms appeared first in the new growth as a reddish colour, followed by severe interveinal chlorosis of the leaves. Plant growth was reduced, and by the end of the experiment the young shoots died. Two months after treatments were started plants were desiccated and stunted. Similar effects were reported in 'Samantha' roses (Rey et al. 1987) and in sugarbeet (Agarwala et al. 1985).

Root growth of both species was strongly inhibited by the 5.0g.m^{-3} copper concentration (Fig. 6). A. vestita has the better developed root system and therefor the root/shoot ratio could be more favourable. The largest part of the root systems died and the reduced new growth showed severe discoloration with few lateral branches. This effect was also reported for the roots of 'Samantha' roses (Rey et al. 1987), A. nummularia (Green 1986) and Chenopodium album (Green 1988). The inhibition of root elongation is caused by a decrease in IAA oxidase activity in roots exposed to high copper concentrations (Marschner 1986). This results in increased indole-3-acetic acid (IAA) concentrations in the roots and the subsequent inhibition of root elongation (Salisbury & Ross 1985). Part of this inhibition is caused by ethylene, because the increased auxin concentration stimulates the synthesis of ethylene, which in turn retards root and stem elongation. The roots of A. vestita were affected more by the toxic copper levels than those of A. nummularia, while root development in A. nummularia was suppressed more by the low copper concentrations. Optimum root development occured at 0.05g.m^{-3} copper in both plant species.

Except for slower growth, no typical deficiency symptoms such as necrosis of the leaves, bending of the leaf tips and grey leaf blades (Reuther & Labanaskus 1966) were detected. This was also the case in 'Samantha' roses (Rey et al. 1987) and subterranean clover (Reuter et al. 1981). Rey et al. (1987) attribute this to the Cu:Fe ratio in the plants. Although the copper level is below the critical value, the Cu:Fe ratio was sufficient to

counteract the deficiency symptoms. However, the optimal range for the Cu:Fe ratio is still to be determined through a manipulation of iron and copper from deficient to toxic levels. Leaf senescence and internode elongation of the lateral branches were delayed by the lower copper concentrations, supporting Reuters' (1981) theory that copper deficiency delays, rather than decreases, the development of lateral branches and leaves. This stands in contrast with copper toxicity which completely inhibits cell elongation and plant development in copper sensitive species. The more erect habit observed in the copper deficient plants is primarily related to a reduction in internode elongation and the reduction in dry matter production of lateral branches (Reuter et al. 1981). Thus stronger apical dominance is present under conditions of lower copper supply, perhaps supporting the "indirect theory" of apical dominance (Hillman, 1984).

2.3.2 Growth of plants

Treatment and species had a highly significant ($p \leq 0.01$) influence on the growth of Atriplex (table 1). Optimum elongation, fresh mass and dry mass production occured at $0.05 g.m^{-3}$ copper (Fig.s 7, 8 & 9). Atriplex vestita grew better than A. nummularia at the deficient copper concentrations with highly significant ($p \leq 0.01$) higher yields in fresh and dry mass production (Fig.s 8 & 9). Although not significant, A. vestita appeared more susceptible to toxic copper than A. nummularia with slower growth and lower yields in fresh and dry mass. Using elongation growth as an indicator of growth became

a problem when lateral branches formed. The number of lateral branches and the longest lateral branch showed a similar effect to the other growth indicators (Fig.s 10 & 11). Optimal lateral branching occured at $0.05g.m^{-3}$copper in both species. Atriplex vestita invested highly significantly ($p \leq 0.01$) more energy in stem production than A. nummularia, resulting in a more woody species, except at $5.0g.m^{-3}$ (Fig. 12), where copper toxicity inhibited stem growth.

2.3.3 Nitrogen concentration of the plants

The interaction between copper treatment and species had no significant influence on the nitrogen concentration of the leaves and stems of Atriplex (table 1). However, treatment alone, caused a highly significant ($p \leq 0.01$) decrease in the nitrogen concentration of the stems and leaves of both A. nummularia and A. vestita as the copper concentration increased (table 2). The decrease in leaf and stem nitrogen is consistent with the suggestion of Snowball et al. (1980) that copper toxicity interferes with nitrogen fixation in the plants. This interference is attributed to the depression in activity of copper containing enzymes. The nitrogen concentration in the leaves did not differ significantly between the two species, while the stems of A. nummularia contained significantly ($p \leq 0.05$) more nitrogen per gram dry mass than A. vestita at the lower copper levels. The nitrogen concentration in the roots showed no significant change between the different copper treatments and between the two Atriplex species (Tab. 1 & 2).

2.3.4 Phosphorous concentration of the plants

The phosphorous concentration in the leaves of both Atriplex species decreased highly significantly ($p \leq 0.01$), with the increase in copper concentration, while the phosphorous concentration in all plant organs was significantly ($p \leq 0.05$) higher in A. vestita than in A. nummularia (table 3). Spencer (1966), in Mordtveldt et al. (1972), reported that high soil copper reduces phosphorous and iron uptake.

2.3.5 Chlorophyll

Both treatment and species had a highly significant ($p \leq 0.01$) influence on the chlorophyll content of the Atriplex plants (table 1). Leaves of A. vestita contained highly significantly ($p \leq 0.01$) more chlorophyll than A. nummularia (table 4), except at 5.0g.m^{-3} copper. This can be attributed to the more xeromorphic character of A. vestita, with its greater number of smaller leaves suggesting a higher volume to area ratio, smaller intercellular spaces and more palisade tissue (Esau 1977). Relatively small changes in chlorophyll a and chlorophyll b content were detected at the low copper concentrations in both species, suggesting that copper marginally affects the ratio of the two pigments in the plant. Baszynski et al. (1978) also found slight changes in chlorophyll in copper deficient spinach chloroplasts. Small changes in chlorophyll content were also recorded by Ayala & Sandman (1988) in copper deficient pea plants, making it difficult to assume a specific effect of copper deficiency on chlorophyll metabolism. Horvath et al. (1983), however, found

a decrease of leaf chlorophyll, carotenoids and plastoquinone when the foliar copper was decreased by fifty percent and concluded that copper regulates the synthesis of these pigments. From the results of this experiment and that of Ayala & Sandman (1988) it can be concluded that this regulatory effect of copper could be species dependent.

Maximum chlorophyll was recorded at the lower (0.0005 & $0.005g.m^{-3}$) copper concentrations in both species. It was found that leaves of copper deficient plants often contain higher chlorophyll and protein contents (Marschner 1986). This possibly compensates for lowered photosynthetic efficiency and reduced carbohydrate synthesis. Unfortunately this was not determined.

The lowest chlorophyll content was measured at $5.0g.m^{-3}$ copper, which caused severe chlorosis of the leaves. This is in agreement with Lolkema & Vooijs (1986) who found a decrease in chlorophyll content in copper sensitive Silene cucubalus when exposed to toxic copper concentrations. Chlorosis is a direct result of the action of high copper concentrations on lipid peroxidation and thus the destruction of thylakoid membranes (Sandman & Boger 1983). The most visible symptom of copper toxicity in spinach is the striking loss of chloroplast membrane constituents such as chlorophyll, carotenoids and lipoquinones (Baszynski et al. 1988). Copper toxicity results in the degradation of grana stacks and stroma lamellae and an increase in the number and size of the plastoglobuli and

intrathyllakoidal inclusions. This results in leaf chlorosis, a decrease in photosynthetic activity and early death of the plant. This feature was also recorded by Adedeji et al. (1984) in two tropical crops and by Hsu et al. (1988) in spinach chloroplasts.

2.3.6 Macronutrients

The concentration of macronutrient elements in the stems and the leaves of both A. nummularia and A. vestita did not differ significantly among the four lower copper treatments. However, a significant drop in macronutrient uptake occured at 5.0g.m^{-3} copper. Atriplex plants are known halophytes (Osmond et al. 1980). They survive saline conditions by storing excess sodium chloride in small vesicles on the leaves. This explains the extremely high sodium concentrations recorded in the leaves of both plants (Tab. 5). A highly significant ($p=0.01$) drop in sodium was recorded in the stems and leaves of both species at 5.0g.m^{-3} copper . The concentration of potassium, calcium and magnesium ions, were expectedly lower than the sodium concentration in both species. In most cases the potassium, calcium and magnesium ion concentrations decreased significantly ($p \leq 0.05$) with the increase in copper concentration (Tab. 6, 7 & 8). This decrease is attributed to the leakage of cations from the roots to the surrounding medium due to structural damage of cellular membranes in the roots (Sela et al. 1988, Mordtveldt et al. 1972).

2.3.7 Micronutrients

Copper treatment and species had a significant effect on the micronutrient content of Atriplex (table 1). The iron concentration was lowest at 5.0g.m^{-3} copper in both A. nummularia and A. vestita, although not significantly (table 9). This decrease in iron corresponds with the reduction in leaf and root iron concentration recorded in 'Samantha' roses (Rey et al. 1987) and in bush bean plants (Wallace 1984) in the presence of excess copper. According to Wallace (1984), effects associated with copper toxicity, are often the result of iron deficiency caused by excess copper in the plant. Copper interferes with iron uptake by competing for adsorption sites in the roots (Lingle et al. 1963) and reduces the movement of iron within the plant (Daniels et al. 1973). The secondary effect of iron deficiency is enhanced by phosphorous deficiency in the presence of excess copper, but it can be reversed by increasing the pH which decreases copper uptake (Wallace 1984). Copper also causes iron deficiency by oxidizing iron to an insoluble ferric state, eliminating soluble ferrous iron from the nutrient solution (Erkama 1950).

The zinc and manganese concentrations also decreased significantly ($p \leq 0.05$) with the increase in copper in both species (Tab. 10 & 11). Like iron, copper interferes with zinc uptake by competing for absorption sites in the roots. This was illutrated in barley roots (Mordtveldt et al. 1972). Manganese is absorbed by a different mechanism and its decrease can be regarded as an indirect effect of copper toxicity.

Manganese loss, for example, can be the result of leakage caused by structural damage to root membranes. The zinc concentration did not differ significantly between the two species, while the manganese concentration in the leaves and roots were significantly higher in A. nummularia at 0.0005 $g.m^{-3}$ copper.

Tissue copper increased highly significantly ($p \leq 0.01$) in the leaves, stems and roots of both species (table 12) at the highest copper concentration in the nutrient solution. Similar increases in tissue copper were recorded in 'Samantha' roses (Rey et al. 1987).

2.3.8 Chloride concentration of the plants

The chloride concentration in the leaves and the roots did not differ significantly between the different copper treatments and Atriplex species (table 1). However, the chloride concentration in the stems decreased significantly with the increase in copper treatment (table 13). The extremely high chloride concentrations of the leaves corresponds well with the high sodium concentrations recorded in table 5 and the halophytic nature of the plants (Osmond et al. 1980).

2.4 CONCLUSION

Copper deficiency significantly ($p \leq 0.05$) depressed yield in A. nummularia compared to A. vestita. Typical deficiency symptoms such as leaf chlorosis, distortion and necrosis in young

leaflets (Snowball et al. 1980, Adedeji et al. 1984, Agarwala et al. 1985 & Rey et al. 1987) were absent. The lack of specific symptoms make it difficult to recognize copper deficiency in the field and highlights the need for developing reliable diagnostic procedures to define the copper status of the plant (Reuter et al. 1981). The more erect habit of the copper deficient plants is primarily related to the reduction in dry matter of lateral branches associated with delayed plant development. Atriplex vestita grew significantly better than A. nummularia at the lowest copper concentration, with higher fresh and dry matter production, better nutrient assimilation and more chlorophyll. This is attributed to the better developed root systems of A. vestita under conditions of low copper (Fig. 6).

Optimum growth in both A. nummularia and A. vestita occurred at $0.05g.m^{-3}$ copper. Optimal plant elongation, lateral branching, fresh and dry matter production were recorded at $0.05g.m^{-3}$ copper. Atriplex nummularia and A. vestita are highly susceptible to copper toxicity with A. vestita the more sensitive species. Copper toxicity in both species resulted in decreased growth and plant development, severe leaf chlorosis, increased leaf abscission and death of young shoots. The high degree of leaf chlorosis is attributed to structural damage of the chloroplast lamellae caused by copper excess (Baszynski et al. 1988). This results in a decrease in leaf chlorophyll and decreased photosynthetic activity. However, many of the symptoms associated with copper stress, whether deficient or

toxic, are often not the result of copper stress itself, but can be attributed to the depression in uptake of other nutrients. Chlorophyll deficiency for example can also be caused by a decrease in iron uptake under conditions of copper excess.

The nutrient status of the two Atriplex species was not significantly affected by the low copper concentrations, while copper toxicity strongly inhibited nutrient uptake. The absorption of both nitrogen and phosphorous were inhibited by increasing copper concentrations. The mineral nutrient concentrations did not differ significantly between the two species. However, when related to total dry mass, it is evident that A. vestita is more nutritious to grazers than **A. nummularia** (see appendix). It contains more mineral nutrients than A. nummularia at all the different copper concentrations, except at $5.0 g.m^{-3}$. It is thus concluded that A. vestita is better adapted and will grow better in the low copper soils of the southern and the western Cape. It would be more advantageous to farmers to plant A. vestita rather than A. nummularia, but further field trials on copper poor soils are required to confirm the result of this growth chamber study.

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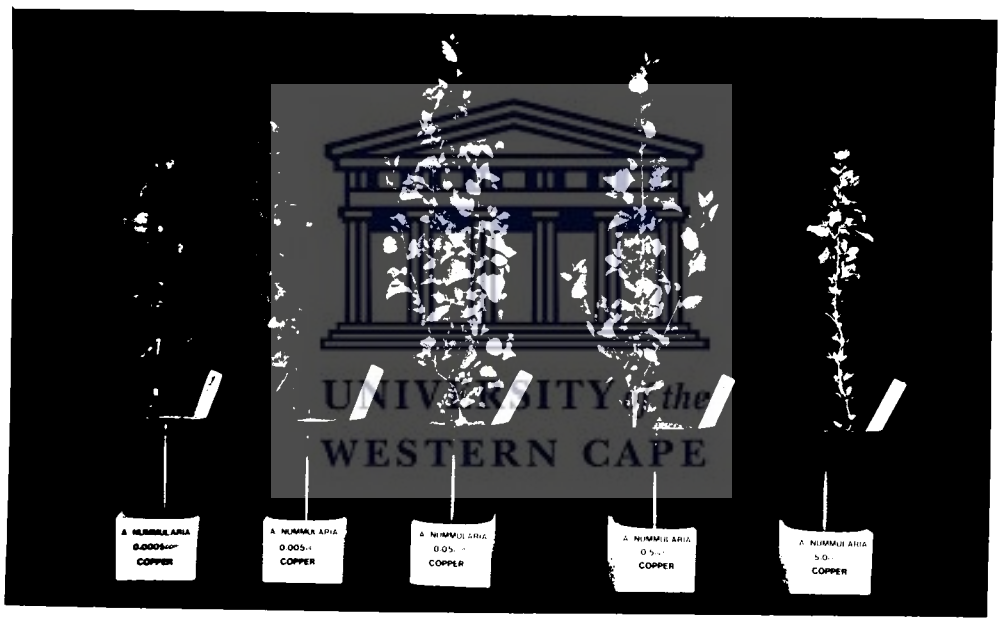

Figure 1: The effect of copper supply on the growth of A. nummularia.

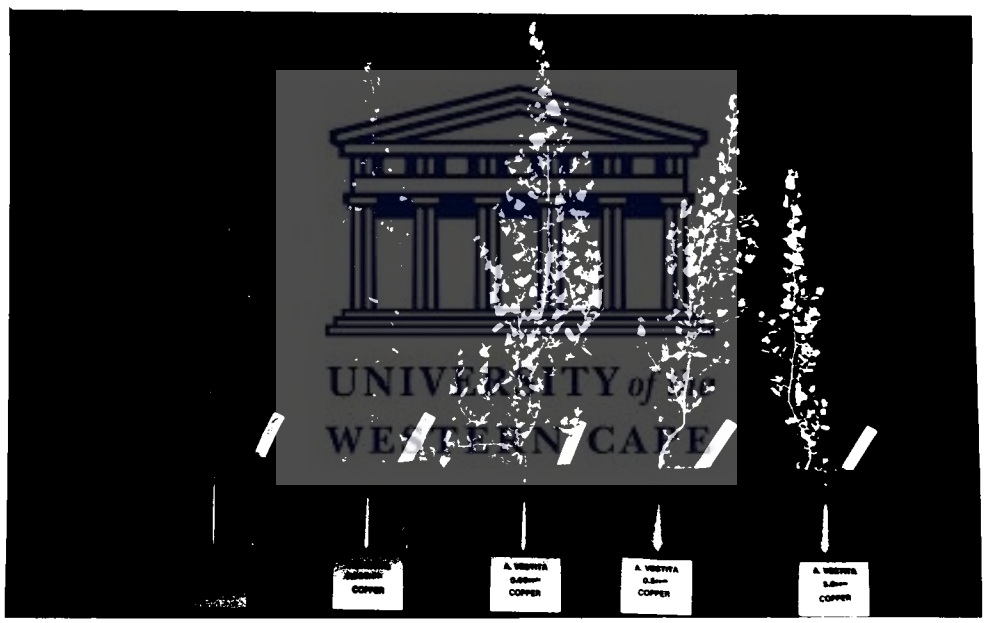

Figure 2: The effect of copper supply on the growth of A. vestita.

Figure 3: A. nummularia and A.vestita both grew best with a copper supply of $0.05g.m^{-3}$.

Figure 4: A. vestita was less affected by the low copper concentration than A. nummularia.

Figure 5: A. vestita was more susceptible to copper toxicity than A. nummularia.

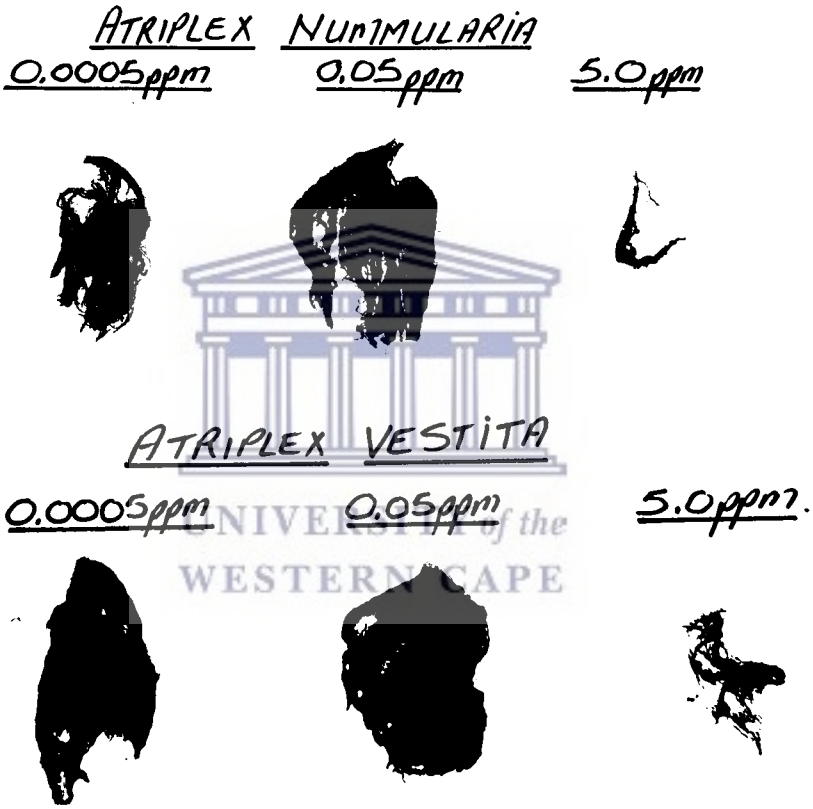

Figure 6: The effect of different copper concentrations on root development in A. nummularia and A. vestita.

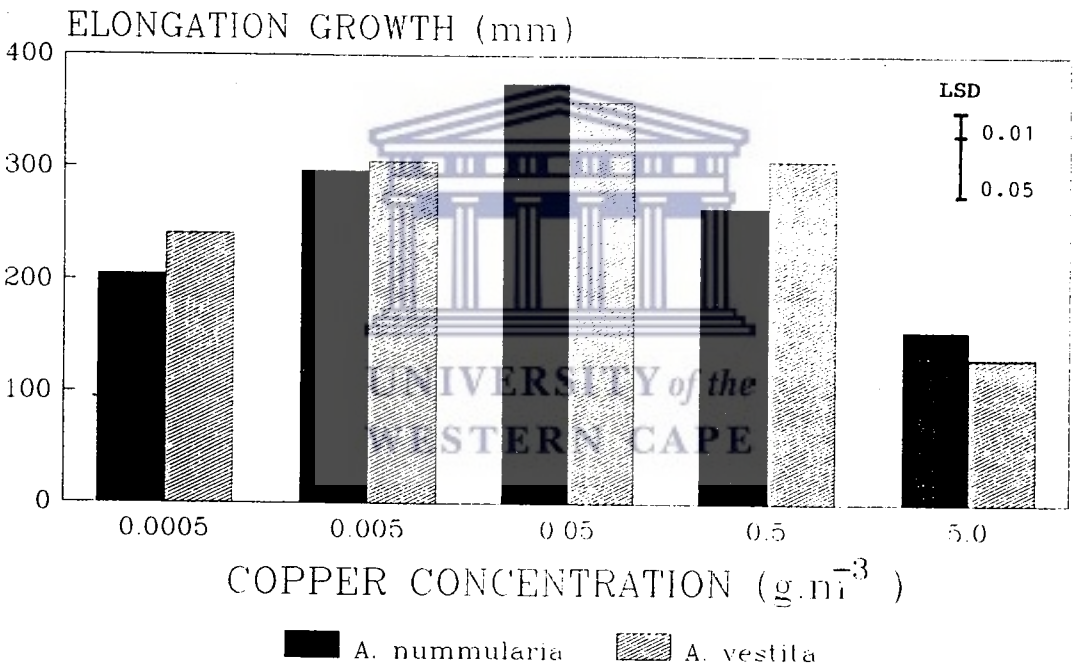

Figure 7: The effect of copper on the elongation growth of A. nummularia and A. vestita (n = 7).

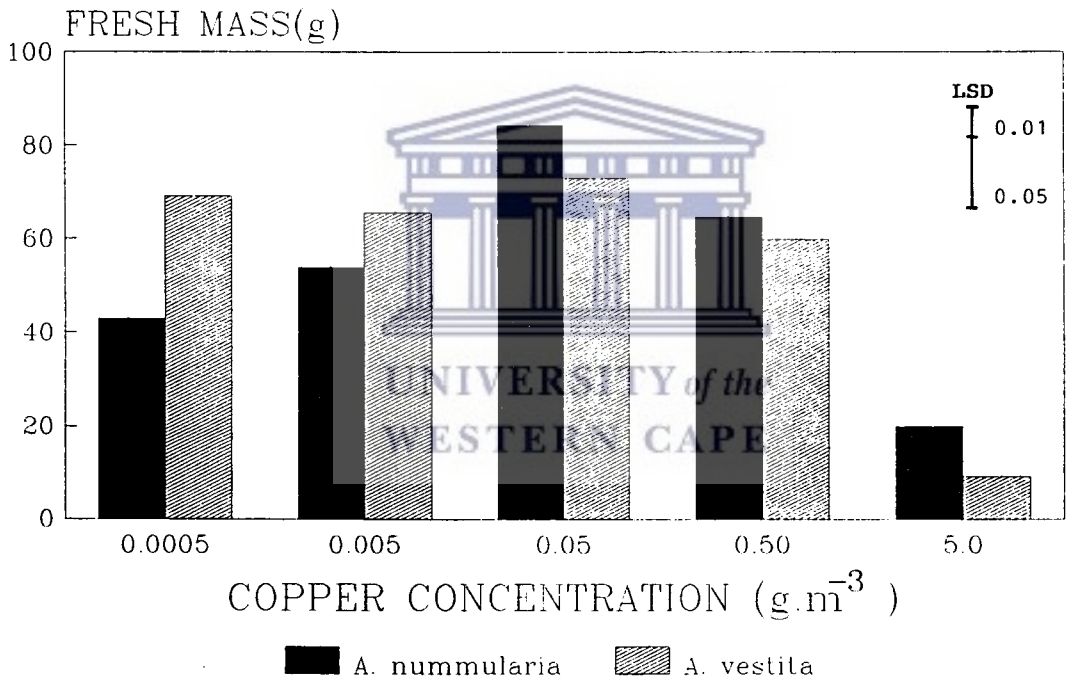

Figure 8: The effect of copper on the fresh mass of A. nummularia and A. vestita (n = 7).

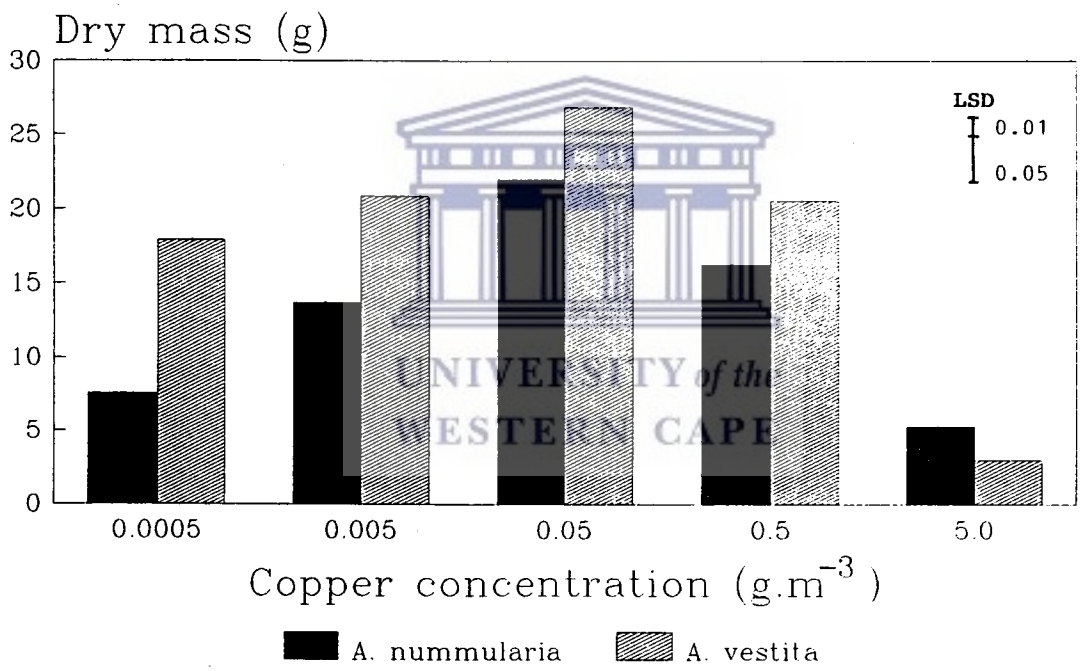

Figure 9: The effect of copper on the dry mass of A. nummularia and A. vestita (n = 4).

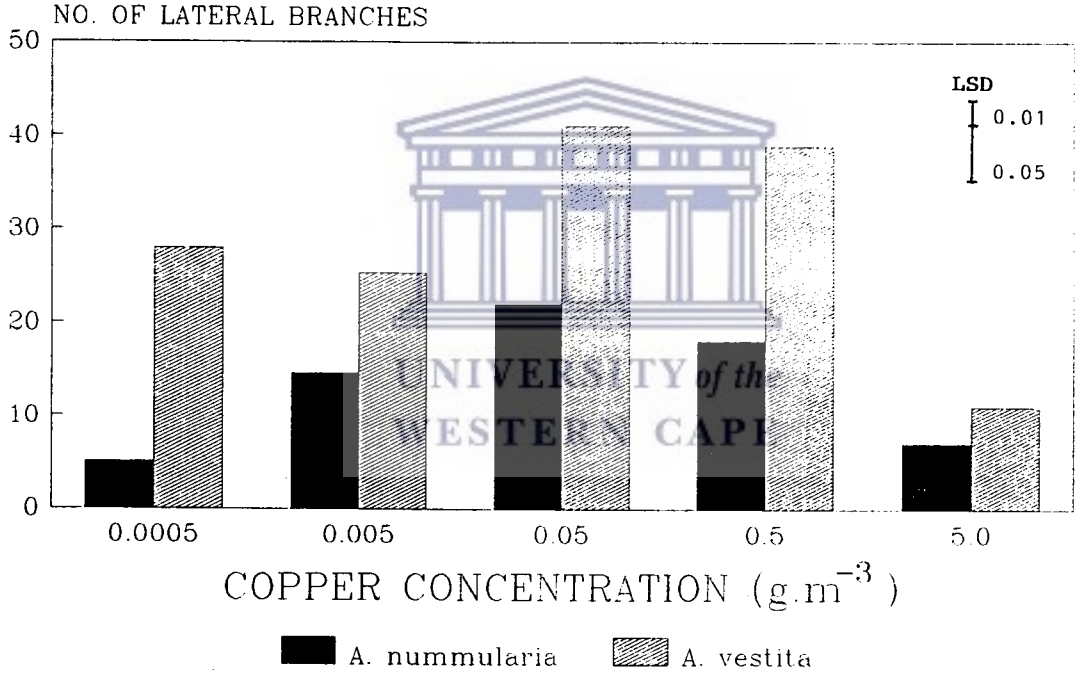

Figure 10: The effect of copper on lateral branching in A. nummularia and A. vestita (n = 7).

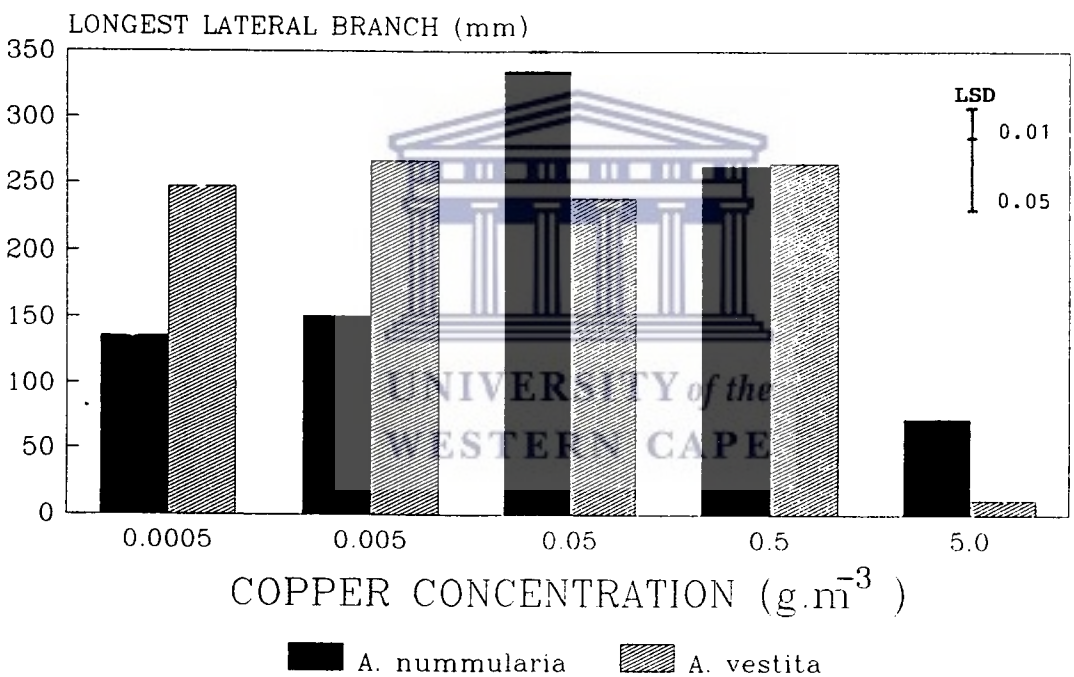

Figure 11: The relationship between copper supply and the longest lateral branches in A. nummularia and A. vestita (n = 7).

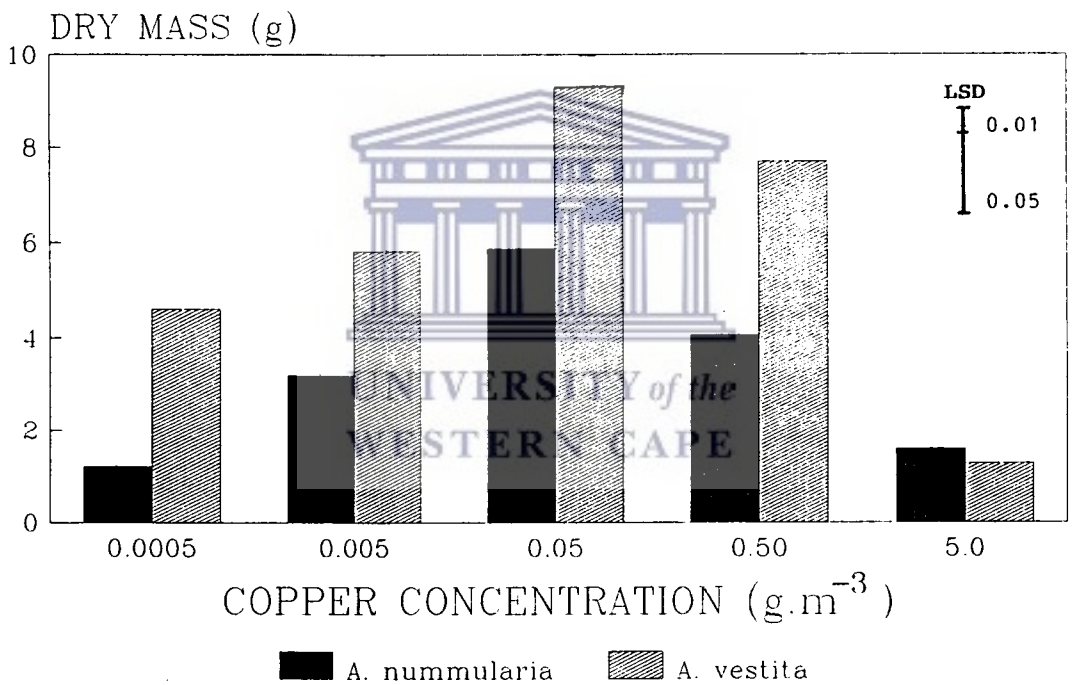

Figure 12: The dry mass of the stems versus copper supply in A. nummularia and A. vestita (n = 4).

Table 1: Effect of treatment, species, and treatment & species on the growth and chemical composition of *Atriplex*.

Plant variable	Source of variation		
	Treatment	Species	Treatment & Species
ELONGATION	***	NS	NS
FRESH MASS:			
Foliage	***	NS	***
Leaves	***	NS	***
Stems	***	***	***
DRY MASS:			
Leaves	***	NS	NS
Stems	***	***	***
Roots	***	***	***
Total	***	***	*
LEAVES:			
Chlorophyll	***	***	***
Nitrogen	***	NS	NS
Phosphorous	***	***	NS
Chloride	NS	NS	NS
Sodium	***	NS	NS
Potassium	***	NS	***
Calcium	***	NS	NS
Magnesium	***	***	***
Iron	NS	*	NS
Zinc	*	NS	NS
Manganese	***	***	***
Copper	***	*	*
STEMS:			
Nitrogen	***	***	NS
Phosphorous	***	***	***
Chloride	***	***	***
Sodium	***	***	***
Potassium	***	***	***
Calcium	***	NS	*
Magnesium	***	***	***
Iron	NS	NS	NS
Zinc	***	NS	NS
Manganese	***	***	***
Copper	***	***	***
ROOTS:			
Nitrogen	NS	NS	*
Phosphorous	NS	***	NS
Chloride	NS	NS	NS
Sodium	NS	NS	NS
Potassium	***	***	***
Calcium	***	***	***
Magnesium	***	NS	***
Iron	*	NS	*
Zinc	***	NS	NS
Manganese	***	***	***
Copper	***	***	***

*** = highly significant at $p \leq 0.01$ NS = not significant.
* = significant at $p \leq 0.05$

Table 2: The effect of species and treatment on the nitrogen content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A . nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	25.200	24.748	14.605	11.430	19.087	17.878
0.005	20.515	19.330	13.005	10.135	16.215	16.988
0.05	16.533	15.000	10.268	9.008	18.788	13.260
0.5	13.803	16.828	10.398	10.058	17.525	13.978
5.0	7.990	9.568	8.523	8.035	17.943	14.973
LSD	($p \leq 0.01$) = 7.365 ($p \leq 0.05$) = 5.464		($p \leq 0.01$) = 3.359 ($p \leq 0.05$) = 2.494		($p \leq 0.01$) = 5.514 ($p \leq 0.05$) = 4.087	

LSD = least significant difference

Table 3: The effect of species and treatment on the phosphorous content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	3.156	4.375	2.313	3.531	3.438	3.625
0.005	3.063	3.500	2.469	3.063	2.813	3.188
0.05	1.969	2.625	1.250	2.875	2.500	2.656
0.5	1.844	3.063	1.313	3.406	2.500	3.375
5.0	1.313	1.969	1.250	4.000	1.833	3.767
LSD	($p \leq 0.01$) = 0.896 ($p \leq 0.05$) = 0.666		($p \leq 0.01$) = 0.910 ($p \leq 0.05$) = 0.675		($p \leq 0.01$) = 0.911 ($p \leq 0.05$) = 0.676	

Table 4: The effect of species and treatment on the chlorophyll concentration ($g.kg^{-1}$ dry mass) in the leaves of A. nummularia and A. vestita (n = 3).

Treatment	chlorophyll a		chlorophyll b		Total chlorophyll	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	3.283	10.739	1.350	6.555	4.633	17.295
0.005	3.689	12.903	1.691	8.522	5.380	21.431
0.05	2.057	8.916	1.527	6.404	3.584	15.321
0.5	3.229	9.627	2.204	6.691	5.433	16.318
5.0	0.218	0.801	0.144	0.599	0.362	1.399
LSD	($p \leq 0.01$) = 2.68 ($p \leq 0.05$) = 1.96		($p \leq 0.01$) = 2.01 ($p \leq 0.05$) = 1.47		($p \leq 0.01$) = 4.56 ($p \leq 0.05$) = 3.33	

Table 5: The effect of species and treatment on the sodium content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	181.62	172.89	32.31	39.61	27.08	23.58
0.005	156.80	183.12	30.61	50.04	27.08	26.86
0.05	164.26	186.97	30.12	38.08	31.03	25.82
0.5	151.00	173.88	27.04	36.61	29.29	27.70
5.0	79.35	83.66	22.49	29.60	29.16	27.42
LSD	($p \leq 0.01$) = 37.79 ($p \leq 0.05$) = 28.07		($p \leq 0.01$) = 7.46 ($p \leq 0.05$) = 5.54		($p \leq 0.01$) = 10.48 ($p \leq 0.05$) = 7.78	

Table 6: The effect of species and treatment on the potassium content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}$Cu	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	12.557	14.865	3.958	6.560	6.725	10.690
0.005	10.560	10.145	4.360	7.323	7.083	8.538
0.05	10.705	8.783	4.303	6.715	9.583	6.513
0.5	8.103	9.623	4.103	10.123	9.878	10.360
5.0	6.555	5.560	2.140	5.665	5.838	6.865
LSD	($p \leq 0.01$) = 2.786 ($p \leq 0.05$) = 2.069		($p \leq 0.01$) = 2.225 ($p \leq 0.05$) = 1.652		($p \leq 0.01$) = 3.824 ($p \leq 0.05$) = 2.840	

Table 7: The effect of species and treatment on the calcium content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}$Cu	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	7.365	6.828	9.320	6.965	11.212	6.002
0.005	6.565	6.848	6.603	6.760	8.250	6.158
0.05	6.695	6.815	6.495	6.833	6.970	6.648
0.5	6.140	6.553	6.315	6.190	6.090	6.473
5.0	5.948	6.228	7.740	6.035	10.288	10.635
LSD	($p \leq 0.01$) = 0.930 ($p \leq 0.05$) = 0.690		($p \leq 0.01$) = 2.514 ($p \leq 0.05$) = 1.867		($p \leq 0.01$) = 2.709 ($p \leq 0.05$) = 2.012	

Table 8: The effect of species and treatment on the magnesium content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	2.945	3.858	1.503	2.040	3.150	2.803
0.005	3.051	3.865	1.503	1.733	3.113	2.848
0.05	2.665	3.313	1.170	1.390	2.865	2.710
0.5	2.708	3.203	1.095	1.553	2.608	2.893
5.0	2.523	2.645	1.053	1.120	3.175	3.920
LSD	($p \leq 0.01$) = 0.682 ($p \leq 0.05$) = 0.506		($p \leq 0.01$) = 0.477 ($p \leq 0.05$) = 0.354		($p \leq 0.01$) = 1.426 ($p \leq 0.05$) = 1.059	

Table 9: The effect of species and treatment on the iron content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	0.215	0.310	0.229	0.162	0.855	0.453
0.005	0.205	0.274	0.146	0.204	0.700	0.525
0.05	0.207	0.210	0.155	0.180	0.643	0.742
0.5	0.162	0.199	0.159	0.242	0.422	0.858
5.0	0.150	0.255	0.172	0.215	0.480	0.415
LSD	($p \leq 0.01$) = 0.166 ($p \leq 0.05$) = 0.123		($p \leq 0.01$) = 0.107 ($p \leq 0.05$) = 0.079		($p \leq 0.01$) = 0.473 ($p \leq 0.05$) = 0.349	

Table 10: The effect of species and treatment on the zinc content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	0.114	0.072	0.058	0.067	0.069	0.076
0.005	0.084	0.056	0.040	0.047	0.061	0.065
0.05	0.049	0.072	0.034	0.052	0.072	0.062
0.5	0.031	0.059	0.025	0.040	0.038	0.047
5.0	0.044	0.060	0.036	0.033	0.037	0.057
LSD	($p \leq 0.01$) = 0.056 ($p \leq 0.05$) = 0.042		($p \leq 0.01$) = 0.028 ($p \leq 0.05$) = 0.021		($p \leq 0.01$) = 0.036 ($p \leq 0.05$) = 0.027	

Table 11: The effect of species and treatment on the manganese content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n=4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	0.074	0.048	0.034	0.068	0.101	0.050
0.005	0.068	0.053	0.028	0.035	0.061	0.046
0.05	0.067	0.045	0.023	0.026	0.042	0.040
0.5	0.045	0.048	0.027	0.032	0.043	0.041
5.0	0.046	0.042	0.033	0.025	0.038	0.038
LSD	($p \leq 0.01$) = 0.019 ($p \leq 0.05$) = 0.014		($p \leq 0.01$) = 0.000 ($p \leq 0.05$) = 0.000		($p \leq 0.01$) = 0.048 ($p \leq 0.05$) = 0.035	

Table 12: The effect of species and treatment on the copper content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	0.019	0.014	0.022	0.013	0.031	0.010
0.005	0.012	0.016	0.011	0.014	0.023	0.015
0.05	0.014	0.021	0.010	0.017	0.022	0.017
0.5	0.011	0.029	0.014	0.030	0.035	0.037
5.0	0.054	0.057	0.053	0.056	0.290	0.398
LSD	($p \leq 0.01$) = 0.039 ($p \leq 0.05$) = 0.029		($p \leq 0.01$) = 0.019 ($p \leq 0.05$) = 0.014		($p \leq 0.01$) = 0.148 ($p \leq 0.05$) = 0.110	

Table 13: The effect of species and treatment on the chloride content ($g.kg^{-1}$ dry mass) in the leaves, stems and roots of A. nummularia and A. vestita (n = 4).

Treatment	Leaves		Stems		Roots	
$g.m^{-3}Cu$	A. numm.	A. vest.	A. numm.	A. vest.	A. numm.	A. vest.
0.0005	197.56	197.21	34.21	40.77	27.77	21.72
0.005	191.00	211.39	40.06	58.68	26.50	25.09
0.05	198.98	225.03	37.49	43.25	31.64	22.96
0.5	194.46	207.31	24.77	45.91	30.22	29.61
5.0	204.39	208.46	23.85	28.27	29.25	21.98
LSD	($p \leq 0.01$) = 48.79 ($p \leq 0.05$) = 36.23		($p \leq 0.01$) = 13.83 ($p \leq 0.05$) = 10.27		($p \leq 0.01$) = 13.62 ($p \leq 0.05$) = 10.08	

CHAPTER 3

THE INFLUENCE OF COPPER ON THE ACTIVITY OF CERTAIN COPPER CONTAINING ENZYMES IN THE LEAVES OF *Atriplex nummularia* Lindl. and *A. vestita* (Thunb.) Aell.

ABSTRACT

The activity of copper containing enzymes, cytochrome oxidase, superoxide dismutase, ascorbate oxidase and laccase in the leaves of Atriplex nummularia Lindl. and A. vestita (Thunb.) Aell. was examined in relation to copper supply. Plants were grown in Hoagland nutrient solution in a Fisons growth cabinet at three different copper concentrations viz. 0.0005, 0.05 and 5.0g.m^{-3} copper. Treatment and species had a highly significant ($p \leq 0.01$) influence on the activities of cytochrome oxidase, superoxide dismutase and ascorbate oxidase. No significant differences were observed in laccase activity between the different copper treatments and species. Cytochrome oxidase and superoxide dismutase activity were maximal at 0.05g.m^{-3} copper. Enzyme activity was significantly inhibited at 5.0g.m^{-3} copper. Ascorbate oxidase activity increased consistently with the increase in copper treatment suggesting that the enzyme requires a copper concentration above 5.0g.m^{-3} for optimal action.

UITTREKSEL

Die effek van verskillende koperkonsentrasies op die aktiwiteit van die koperbevattende ensieme, sitochroom-oksidase, superoksieddismutase, askorbiensuuroksidase en lakkase in die blare van Atriplex nummularia Lindl. en A. vestita (Thunb.) Aell. is ondersoek. Plante is in Hoagland

voedingsoplossing in 'n Fisons groeikabinet by drie verskillende koper konsentrasies nl. 0.0005, 0.05 en 5.0g.m^{-3} koper gekweek . Behandeling en spesies het die aktiwiteit van sitochroomoksidase, superoksieddismutase en askorbiensuuroksidase hoogs betekenisvol ($p \leq 0.01$) beïnvloed. Geen betekenisvolle verskille in lakkase aktiwiteit is tussen die verskillende koperbehandelings en spesies waargeneem nie. Die aktiwiteit van sitochroom-oksidase en superoksieddismutase was optimaal by 0.05g.m^{-3} koper. Betekenisvolle stremming van die ensiem het by 5.0g.m^{-3} koper voorgekom. Die aktiwiteit van askorbiensuuroksidase het konstant toegeneem met toename in koperkonsentrasie, wat daarop dui dat die optimum waarde vir koper 5.0g.m^{-3} oorskry.

Keywords: Atriplex nummularia, Atriplex vestita, cytochrome oxidase, superoxide dismutase, ascorbate oxidase, laccase, copper.

3.1 INTRODUCTION

Many specific enzyme sites contain copper through which copper supply may exert major effects on the growth and development of plants (Walker et al. 1981). Copper influences enzyme activities either by being involved in the synthesis of the enzyme itself or some other protein might be implicated in the incorporation of copper into the enzyme. In cytochrome c oxidase for example mere incorporation of copper in the enzyme is involved (Walker et al. 1981).

Plants react differently to copper availability in the soil. Woolhouse (1983) distinguishes between copper-tolerant and copper-sensitive plants. Copper-tolerant plants grow equally well in soils with adequate or toxic copper levels, while copper-sensitive plants are killed by excess copper supply. Both groups, however, are adversely affected by a deficient copper supply.

Past research primarily dealt with copper deficiencies in plants with fewer articles published on copper toxicity. Copper deficiency has a direct impact on energy metabolism because it affects the synthesis of the copper-containing electron carriers, plastocyanin and cytochrome oxidase, resulting in decreased photosynthesis and respiration rates (Walker & Webb 1981). The effect of copper deficiency on a

number of copper-containing enzymes has been reported. Diamine oxidase, ascorbate oxidase, o-diphenol oxidase, superoxide dismutase and cytochrome c oxidase activities decreased in plants grown in copper depleted media (Hill 1973, Walker et al. 1981, Delhaize et al. 1985).

The primary effect of copper deficiency is to retard rather than inhibit plant development (Reuter et al. 1981). Upon addition of copper, these plants will often develop normally. Copper toxicity on the other hand causes structural damage of cellular membranes and chloroplast lamellae, resulting in decreased photosynthetic activity and ultimate death of the plant (Baszynski et al. 1988).

This study was undertaken to asses the relation between copper supply and the activities of a number of copper containing enzymes viz. laccase, ascorbate oxidase, cytochrome oxidase and superoxide dismutase in the leaves of Atriplex nummularia Lindl. and A. vestita (Thunb.) Aell.

3.2 MATERIALS AND METHODS

3.2.1 Cultivation of test plants

A. nummularia and A. vestita seeds were supplied by the Agricultural Research Institute for the Karoo Region, Middelburg, Cape. A quantity of seeds of both species were rinsed with running tap water for 48 hours, to remove excess

sodium chloride from the seed coats. The salt tends to inhibit seed germination and must be removed prior to sowing. The seeds were germinated and grown in purified sand in a Fisons growth cabinet (model no. 600G3) under twelve hour illumination with day temperature of 28°C and night temperature of 16°C. The relative humidity was 60% for day and 80% for night temperatures. The sand (20/40 grade) was washed in a 1:1 mixture of 15% HCl and 1% oxalic acid solution (Hewitt 1966). The seedlings were treated, for two months, with half-strength Hoagland solution minus copper, before they were transfered to water culture.

Seedlings, of approximately the same size, were transplanted into 500cm^3 milk bottles. The bottles were first painted black and then white to prevent illumination and overheating of the developing roots. The bottles were thoroughly washed and repeatedly rinsed with distilled-deionised water. Initially the seedlings were treated with a half strength Hoagland solution minus copper. Two weeks were allowed for the seedlings to recover from the transplant before treatments were started.

Three treatments, 0.0005, 0.05 and 5.0g.m^{-3} copper were used. Analytical grade reagents were used throughout the experiment. The degree of copper contamination of the salts and water supply was determined polarographically and ranged between 0.0001 and 0.0005g.m^{-3}copper. The final copper

concentrations of the test solutions were obtained by the standard addition method. Fresh nutrient solution was supplied on a weekly basis and daily supplemented with distilled deionised water to avoid the concentration of mineral elements due to water loss. The experiment was laid out according to a random block design. Fifteen plants were used per treatment. These were divided into five groups of three each. Each group represented one replicate of a specific treatment. This was done to ensure sufficient test material for enzyme analysis.

Due to the early establishment of toxic effects, plants were harvested a month after treatments started. Photographs were taken to record the visual effects (Figs 1, 2 & 3) and only the leaves were collected for enzyme analysis. The leaves were weighed and stored in plastic bags at -40°C in a freezer.

3.2.2 Extraction of protein

The leaf material was freeze dried and ground with a Wiley intermediate mill (40 mesh). This lyophyllized powder was collected in glass vials and stored at 0°C until the time of analysis. Enzyme extraction was done on 0.2gram of the ground material. The powder was homogenized in $10cm^3$ ice cold extraction buffer with a mortar and pestle. The buffer consisted of 0.1M potassium phosphate buffer, pH 7.8 and 0.1mM potassium EDTA (Giannopolitis & Ries 1977). A few

grains of purified sand were added to enhance the extraction of protein from the cellular tissue. The homogenate was filtered through two layers of cheese cloth and centrifuged twice at 13000xg in a Beckman J21 centrifuge. The supernatant was collected in a volumetric flask, made up to $10cm^3$ with extraction buffer and stored at -30°C in a freezer.

3.2.3 Determination of protein content

The method of Bradford (1976) was modified to determine protein content. This method involves the binding of Coomassie Brilliant Blue G-250 to protein. A standard curve was prepared using bovine serum albumin (BSA) as protein stock solution (25mgBSA + 500ml water). Standard concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 4.5 & 5.0ug protein were used (10µl = 0.5µg protein), three replicates each. The stock solution was made up to 100ul with distilled deionised water in a 1mm plastic cuvette. Three millilitres Bradford reagent was added and thoroughly mixed by inversion. Ten minutes were allowed for protein-dye binding and the optical density was measured at 595nm on a Milton Roy Spectronic 601 spectrophotometer. This data was represented in a standard curve from which the protein content of the test samples was determined.

The reaction mixture of the test samples consisted of 10µl diluted (1:1) protein extract, 90µl distilled deionised

water and $3cm^3$ Bradford reagent. Three determinations were performed per sample and the average protein content was determined from the standard curve and expressed in milligrams protein per gram freeze dried material (table 2).

3.2.4 Assay of enzyme activities

3.2.4.1 Cytochrome oxidase

Cytochrome oxidase activity was determined by the microspectrophotometric method of Cooperstein et al. (1950). A reduced cytochrome c solution was prepared by adding $0.166cm^3$ freshly prepared 1.2M sodium thiosulphate to $50cm^3$ 1.7M x 10^{-5} cytochrome c in 0.03M potassium phosphate buffer. The mixture was continuously stirred to remove excess sodium thiosulphate and prevent autoxidation of the reduced cytochrome c. Reaction mixtures were prepared in 10mm plastic cuvettes.

	Sample	Blank
Reduced cytochrome c	$0.3cm^3$	$0.3cm^3$
Enzyme extract	$0.05cm^3$	--
Distilled deionised water	--	$0.05cm^3$

The change in optical density at 550nm was measured every thirty seconds. After 3 minutes two drops of saturated potassium ferricyanide solution were added (to completely oxidize the cytochrome c) and the total extinction determined. Temperature was kept at 25°C.

Cytohrome c oxidase activity is expressed as the first order velocity constant for the oxidation; the reaction is first order with respect to the reduced cytochrome c (Smith 1955 & Cooperstein et al. 1950). The value for the optical density (OD) of the totally oxidized cytochrome c is subtracted from the values for optical density determined at definite intervals during the oxidation. The value of this difference plotted against time should give a straight line on semilog paper (OD values on log scale). From this plot:

$$k=\{\log(OD_{t1}-OD_{ox})-\log(OD_{t2}-OD_{ox})\}.\{t_2-t_1\}^{-1}$$

where OD_{t1}, OD_{t2} and OD_{ox} represent the extinctions at t_1, t_2 and after the addition of ferricyanide respectively.

3.2.4.2 Superoxide dismutase (SOD)

Because of the instability of its substrate all available assays of superoxide dismutase (SOD) are indirect and depend upon its ability to scavenge superoxide radicals from reaction mixtures and thus inhibit reactions caused by these radicals (Beauchamp & Fridovich 1971). Hence SOD activity was determined based on the photoreduction of nitroblue tetrazolium (NBT) by superoxide radicals. SOD inhibits the formation of blue formazan and was quantified on this basis. The photochemical procedure was chosen as being

independent of other enzymes and proteins and therefore more reliable in the case of crude extracts (Giannopolitis & Ries 1977). The original assay of Beauchamp & Fridovich (1971) was modified. The reaction mixture consisted of 13mM methionine; 63µM nitroblue tetrazolium, 1.3µM riboflavin, 0.05M sodium carbonate, 0.333cm^3 sample extract and 0.02mM potassium ferricyanide in a final volume of 1cm^3 in a 10mm plastic cuvette. The solution was thoroughly mixed by inversion and the change in optical density measured at 560nm against a distilled deionised water blank. The initial rate of the reaction was determined as the increase in absorbance at 560nm in the absence of enzyme extract. In the presence of plant extract or SOD the increase in absorbance is inhibited and the percentage inhibition was used to quantify the enzyme activity.

3.2.4.3 Ascorbate oxidase

Ascorbate oxidase activity was assayed at 25°C by following the decrease in absorbance at 265nm (Vines & Oberbacher 1963). A matched pair 10mm quartz cuvettes were used. The reaction mixtures consisted of:

	Sample	Blank
0.1M Phosphate/EDTA buffer pH5.6	2.9cm^3	2.9cm^3
0.005M L-ascorbic acid	0.1cm^3	0.1cm^3
Enzyme extract	0.1cm^3	--

Blank solutions were prepared fresh to prevent autoxidation of the L-ascorbic acid. The blank cell was placed in the sample compartment and the test cell in the reference compartment of the spectrophotometer and the increase in absorbance was monitored at 265nm for 3 minutes. Enzyme activity was expressed as the change in absorbance per minute.

3.2.4.4 Laccase

The method of Reinhammar (1969) was used to determine laccase activity. Reaction mixtures were prepared as follows:

	Sample	Blank
0.6mM N,N-dimethyl-		
p-phenyleneamine pH7.5	3.0cm^3	3.0cm^3
Enzyme extract	0.05cm^3	--
Distilled deionized water	--	0.05cm^3

The solutions were thoroughly mixed by inversion and the increase in absorbance at 323nm was measured for 3 minutes in a Hitachi U3201 spectrophotometer, equipped with a time drive. Temperature was kept constant at 25°C and enzyme activity was expressed as increase in absorbance per minute.

3.2.5 Statistical analysis

Results were subjected to statistical analysis of variance using ANOVA12, a Significant Statistics (SIGSTAT) package, and least significant differences between species and treatments were calculated at the p=0.01 and p=0.05 levels.

3.3 RESULTS AND DISCUSSION

3.3.1 Visual symptoms:

Atriplex nummularia and A. vestita exhibited normal growth at 0.05g.m^{-3} copper (Fig. 1, 2 & 3). When compared to the preceeding experiment (chapter 2) it was evident that copper stress effects established quicker in water than in sand culture. This is attributed to the even, easily accessible spread of mineral nutrients through a liquid medium and the absence of a possible adsorbant for the copper ions. Three weeks after treatments were started, plants at 5.0g.m^{-3} copper showed severe toxic symptoms. Leaves were chlorotic and leaf growth was strongly inhibited (Fig. 4). Toxic symptoms appeared first on the new growth as a reddish colour before severe interveinal chlorosis started. Plants appeared dessicated and stunted and an early harvest was necessary to ensure sufficient fresh material for enzyme analysis. Similar symptoms have been described for 'Samantha' roses (Rey et al. 1987), sugarbeet (Agarwala et el. 1985) and subterranean clover (Reuter et al. 1981). No typical deficiency symptoms like necrosis, bending of leaf tips and grey leaf blades (Reuther & Labanaskus 1966) were observed (Fig. 5). Rey et al. (1987) attributed the lack of deficiency symptoms to the Cu:Fe ratio in the plant. Although the copper level is below the critical level, the Cu:Fe ratio is still sufficient to counteract the deficiency symptoms.

3.3.2 Protein concentration of the plants

The interaction between copper treatment and species had no significant effect on the protein concentration of the leaves of the Atriplex species, although highly significant ($p \leq 0.01$) differences were observed between species and treatments seperately (table 1). The protein concentration in A. vestita was higher than that of A. nummularia, with the highest concentrations measured at $0.05g.m^{-3}$ copper in both species (table 2). In both species no significant differences were observed between the two lower copper levels, while the protein concentration at $5.0g.m^{-3}$ copper decreased highly significantly ($p \leq 0.01$). In subteranean clover this decrease in protein content was caused by a decrease in nitrogen fixation, which is caused by the depression in activity of copper containing enzymes in the presence of excess copper (Snowball et al. 1980).

3.3.3 Cytochrome oxidase:

Both copper treatment and species highly significantly ($p \leq 0.01$) influenced cytochrome oxidase activity in the leaves of Atriplex (table 1). Copper stress significantly ($p \leq 0.05$) inhibits cytochrome oxidase activity in A. vestita. In A. nummularia no significant differences were observed in cytochrome oxidase activity between the different copper treatments (Fig. 6). Maximal activity was recorded at $0.05g.m^{-3}$ copper for both species. Cytochrome oxidase activity was highly significantly ($p \leq 0.01$) higher in A. vestita than in A. nummularia at $0.05g.m^{-3}$ copper.

Cytochrome oxidase is the terminal oxidase in the mitochondrial electron transport chain (Walker et al. 1981). Due to the more xeromorphic nature, the greater number and smaller leaves, a greater volume to leafarea ratio, smaller intercellular spaces and more palisadic tissue (Esau 1977), it can be concluded that A. vestita contains more mitochondria than A. nummularia explaining the higher activity readings. The decrease in cytochrome oxidase activity, at deficient copper levels, corresponds with the decrease in cytochrome oxidase activity recorded in cultured sycamore cells (Bligny and Douce 1979), subterranean clover (Walker et al. 1981) and in red clover (Hill 1973).

3.3.4 Superoxide dismutase:

Superoxide dismutase activity is expressed as the percentage inhibition of the photoreduction of nitro-blue tetrazolium (Fig. 7). Copper treatment and species had a highly significant ($p \leq 0.01$) influence on the SOD activity in the leaves of Atriplex (table 1). The percentage inhibition recorded at 5.0g.m^{-3} copper was highly significantly ($p \leq 0.01$) lower compared to the other treatments implicating low SOD activity. SOD activity at the low and adequate copper levels showed no significant differences between the species and the treatments (Fig. 7). Maximal inhibition i.e. SOD activity occured at 5.0g.m^{-3} copper in both cases.

The lack of NBT inhibition at the low copper levels can be attributed to the presence of three SOD isoenzymes viz a plastidic Cu/Zn-SOD, a cytoplasmic Cu/Zn-SOD and a Mn-SOD (Ayala & Sandman 1988). During copper deprivation the activities of both Cu/Zn-SOD isoenzymes decreased, but a concurrent increase in Mn-SOD suppress the establishment of deficiency symptoms (Ayala & Sandman 1988). Nevertheless, total SOD activity normally decreases with a reduction in tissue copper.

3.3.5 Ascorbate oxidase:

Ascorbate oxidase activity in Atriplex was significantly ($p \leq 0.01$) influenced by the interaction between treatment and species (table 1). Ascorbate oxidase activity increased with the increase in copper concentration in both A. nummularia and A. vestita (Fig. 8), with activities in A. vestita highly significantly ($p \leq 0.01$) higher than that of A. nummularia. The inhibition of ascorbate oxidase activity at the low copper level corresponds with the low activities recorded by Hill (1973) in red clover. The high activity readings at 5.0g.m^{-3} copper can be attributed to additional oxidation of L-ascorbate by other enzymes such as o-diphenol oxidase (Hallaway et al. 1970). Cu^{2+} ions also catalyse the oxidation of ascorbate (Hallaway et al. 1970). However, EDTA was added during the assay procedure, chelating the Cu^{2+} ions and neutralizing their effect. The influence of o-diphenol oxidase was not investigated, but pH and temperature specificity should eliminate possible

effects of o-diphenol oxidation of the substrate. Another possibility is that the critical level (Rey et al. 1987) above which copper will decrease ascorbate oxidase activity may well exceed $5.0g.m^{-3}$ in A nummularia and A. vestita. According to Rey et al.(1987) the critical value for enzyme action is species dependent, explaining the unique behaviour of the two species in question. However, this needs to be further researched.

3.3.6 Laccase:

No significant differences in laccase activity were observed between the different copper treatments and between the species (table 1). Laccase activity in A. vestita was slightly higher than in A. nummularia (Fig. 9). Although little difference was observed between the different treatments, the trend exhibited optimal enzyme action at $0.05g.m^{-3}$ copper. Slight inhibition occured at $0.0005g.m^{-3}$ and $5.0g.m^{-3}$ copper.

3.4 CONCLUSION

Copper availability has a definite influence on the activity of certain copper containing enzymes in the leaves of A. nummularia and A. vestita, with activities in A. vestita normally higher than that of A. nummularia. Low copper reduces the activity of laccase, ascorbate oxidase, cytochrome oxidase and superoxide dismutase in both species. Optimal activity occurs at $0.05g.m^{-3}$ copper,

while excess copper strongly inhibits enzyme activity, with the exception of ascorbate oxidase. This can be attributed either to a high critical copper level or to additional catalization of L-ascorbic acid by other enzymes such as o-diphenol oxidase (Hallaway et al. 1970). However, this needs to be researched.

The low enzyme activities in copper deficient leaves may be a result of a copper limiting holoenzyme assembly, in which the apoproteins of these enzymes are still synthesized (Delhaize et al. 1986). This was the case for cytochrome c oxidase in copper deficient yeast (Keyhani et al. 1975) and for galactose oxidase in copper deficient Dactylium dendroides (Shatzman & Kosman 1978). An alternative mechanism would be for copper to regulate the synthesis of the apoprotein so that no apoprotein is synthesized in its absence. Delhaize et al. (1985) presented indirect evidence that diamine oxidase, ascorbate oxidase and o-diphenol oxidase are synthesized only during early leaf development and that in copper deficient leaves, apoproteins of these enzymes are either absent or cannot be reactivated, even if copper becomes available.

Atriplex nummularia and Atriplex vestita are both copper-sensitive species. The decrease in enzyme activity at toxic copper levels can be attributed to structural damage of cell membranes and chloroplast lamellae (Baszynski et al. 1988), which result in decreased photosynthetic

activity and ultimate death of the plants. Copper toxicity therefore not only inhibits, but eventually terminates the activity of laccase, ascorbate oxidase, cyotchrome oxidase and superoxide dismutase in A. nummularia and A. vestita, which leads to the death of the plants.

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Table 1: The effect of copper treatment and species on the protein concentration and activities of certain copper-containing enzymes in Atriplex.

Variable:	Source of variation		
	Treatment	Species	Treatment & Species
Protein concentration	***	***	NS
Enzyme avtivity:			
Laccase	NS	NS	NS
Ascorbate oxidase	***	***	***
Cytochrome oxidase	***	***	***
superoxide dismutase	***	***	***

*** = highly significant at $p \leq 0.01$
NS = not significant

Table 2: The effect of copper on the protein concentration in the leaves of A. nummularia and A. vestita (n = 5).

Copper Treatment $g.m^{-3}$	Protein concentration $g.kg^{-1}$ dry mass	
	A. nummularia	A. vestita
0.0005	22.30	22.90
0.05	20.40	28.00
5.0	7.00	13.97
*LSD	($p \leq 0.01$) = 8.38 ($p \leq 0.05$) = 6.12	

* = least significant difference

Figure 1: The effect of different copper concentrations on the growth of A. nummularia.

Figure 2: The effect of different copper concentrations on the growth of A. vestita.

Figure 3: Both A. nummularia and A. vestita grew best at 0.05g.m^{-3} copper.

Figure 4: A. nummularia and A. vestita showing toxic symptoms at 5.0g.m^{-3} copper.

Figure 5: Atriplex species showing no deficiency symptoms at 0.0005g.m^{-3} copper.

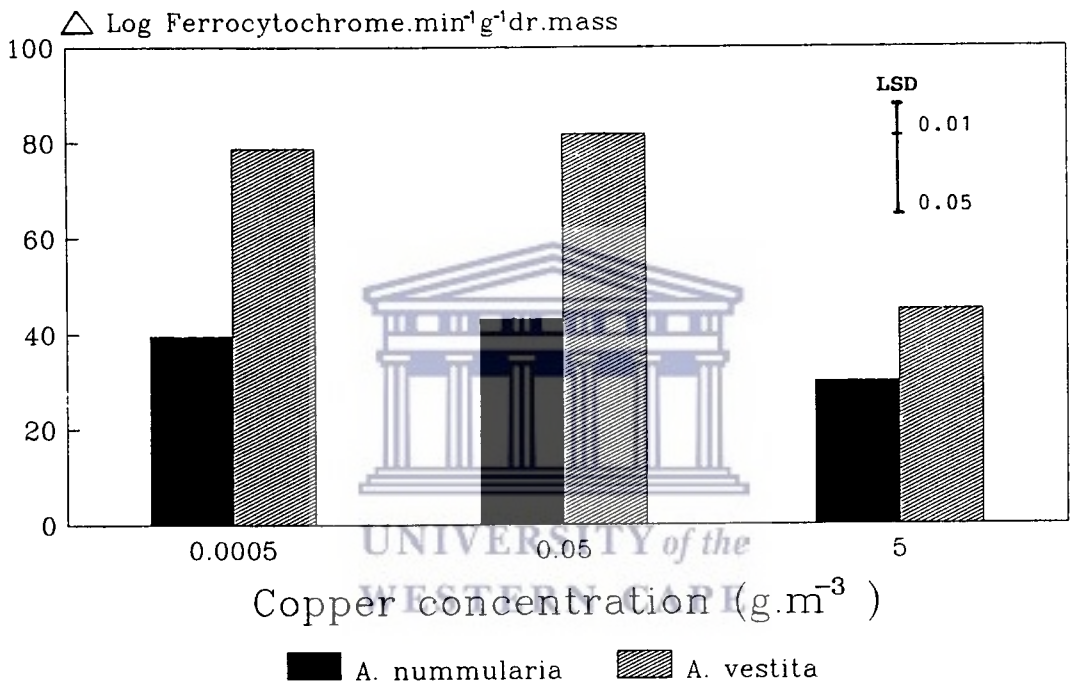

Figure 6: Effect of copper on the activity of cytochrome oxidase in the leaves of Atriplex (n = 5).

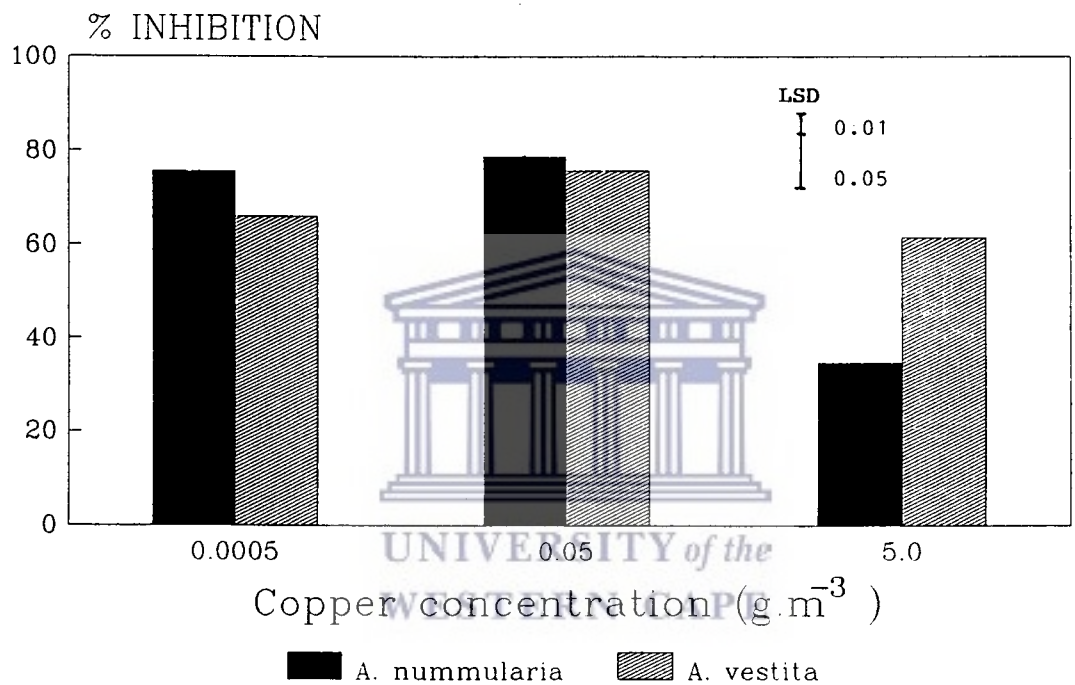

Figure 7: Effect of copper on the activity of superoxide dismutase in the leaves of Atriplex (n = 5).

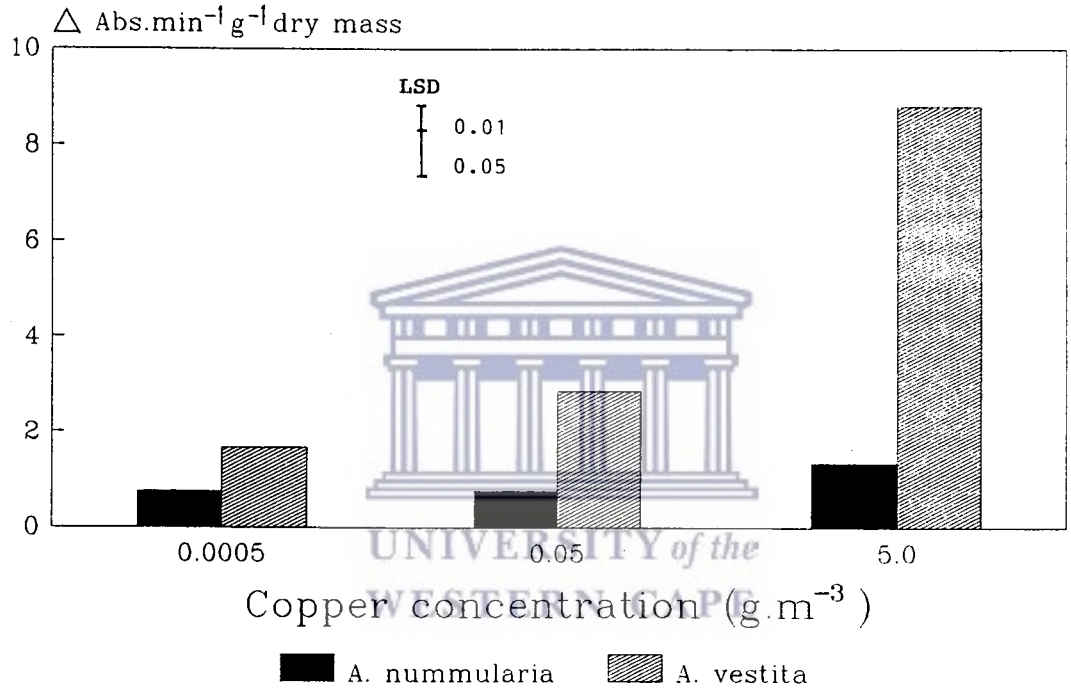

Figure 8: Effect of copper on the activity of ascorbate oxidase in the leaves of Atriplex (n = 5).

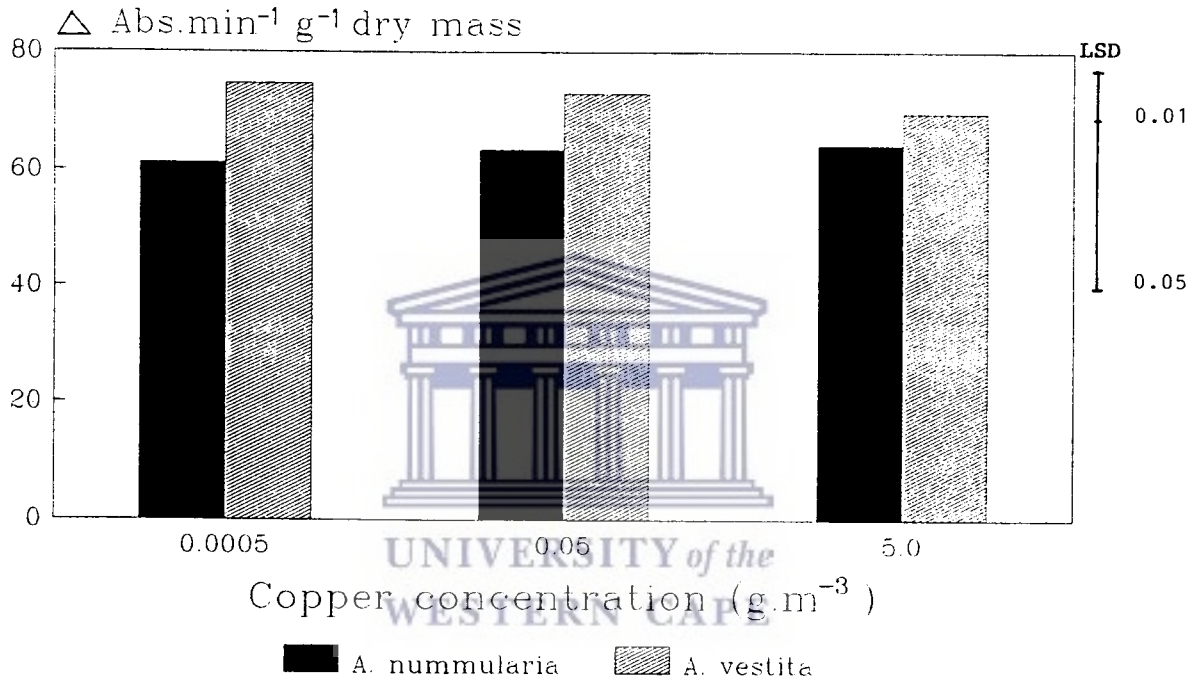

Figure 9: The effect of copper on the activity of laccase in the leaves of Atriplex (n = 5).

SUMMARY

The interaction between copper supply and species significantly influenced the growth, chemical composition and activities of copper-containing enzymes in Atriplex.

Maximal growth occured at 0.05g.m^{-3} copper in Atriplex nummularia Lindl. and Atriplex vestita (Thunb.) Aell. (=halimus). Except for slower growth, no typical deficiency symptoms were detected in both species. Copper toxicity significantly depressed plant growth as measured by internode elongation, lateral branching, and root development in both species (Chp. 2). The plants appeared desiccated and stunted, with small and chlorotic leaves. The largest part of the root systems were dead and the reduced new growth showed severe discoloration with few lateral branches.

Due to the halophytic nature of the plants, sodium and chloride contents were exceptionally high in the leaves of both plants. The lower copper concentrations had no significant influence on the mineral nutrient concentration of both species. However, when related to the total dry mass (appendix) it is evident that maximum growth and nutrient accumulation occured at 0.05g.m^{-3} copper. Copper toxicity significantly depressed the uptake and translocation of cations, with a concommitant decrease in nitrogen, phophorous and chloride in the leaves, stems and roots.

This is attributed to cation leakage, competition of copper with other cations for absorption sites in the roots, cellular damage, and the depression in activity of copper-containing enzymes. Leaf chlorosis, associated with copper toxicity, is the result of decreased iron uptake and structural damage to chloroplast lamellae.

Treatment and species had a highly significant ($p=0.01$) influence on the activities of cytochrome oxidase, superoxide dismutase and ascorbate oxidase. No significant differences were observed in laccase activity between the different copper treatments and species. Cytochrome oxidase and superoxide dismutase activity were maximal at $0.05 g.m^{-3}$ copper. Low copper hardly inhibited enzyme activity, while significant inhibition occured at the toxic copper concentration. Ascorbate oxidase activity increased with the increase in copper treatment suggesting that the enzyme has a critical value for copper adequacy above 5.0 $g.m^{-3}$ copper.

Atriplex vestita grows better than A. nummularia in low copper environments, with significantly higher yields in fresh mass, dry mass, and chlorophyll production. When related to total dry mass, it contains significantly more mineral nutrients and the activity of certain physiologically important copper-containing enzymes is higher in this species.

APPENDIX

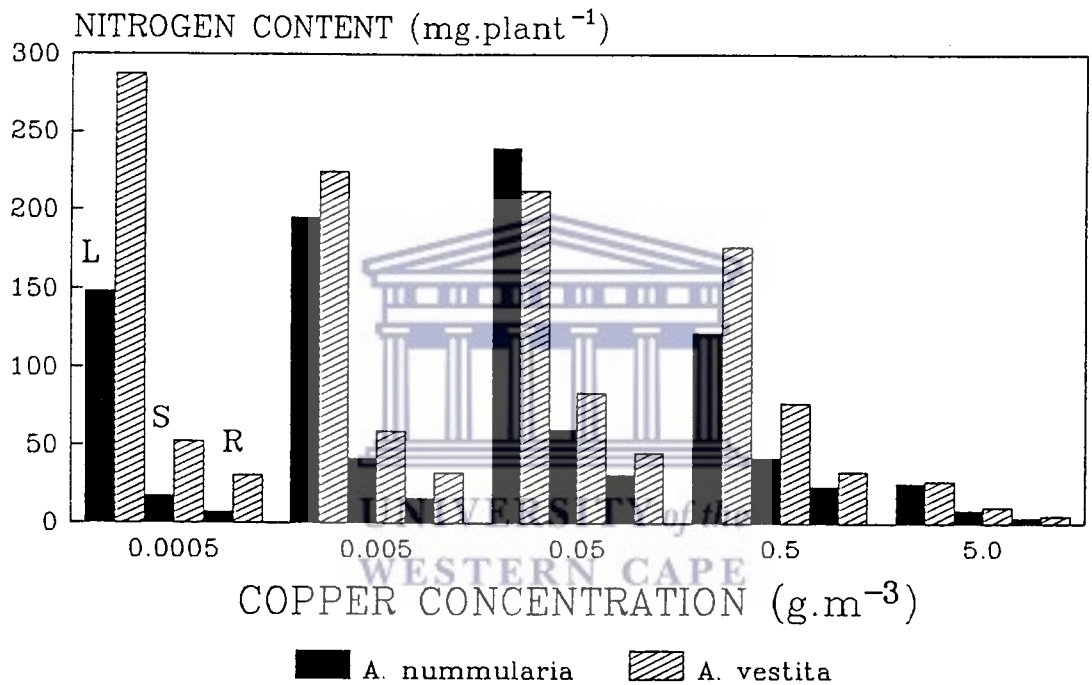

Figure 1: The effect of copper treatment on the total nitrogen content of A. nummularia and A. vestita.

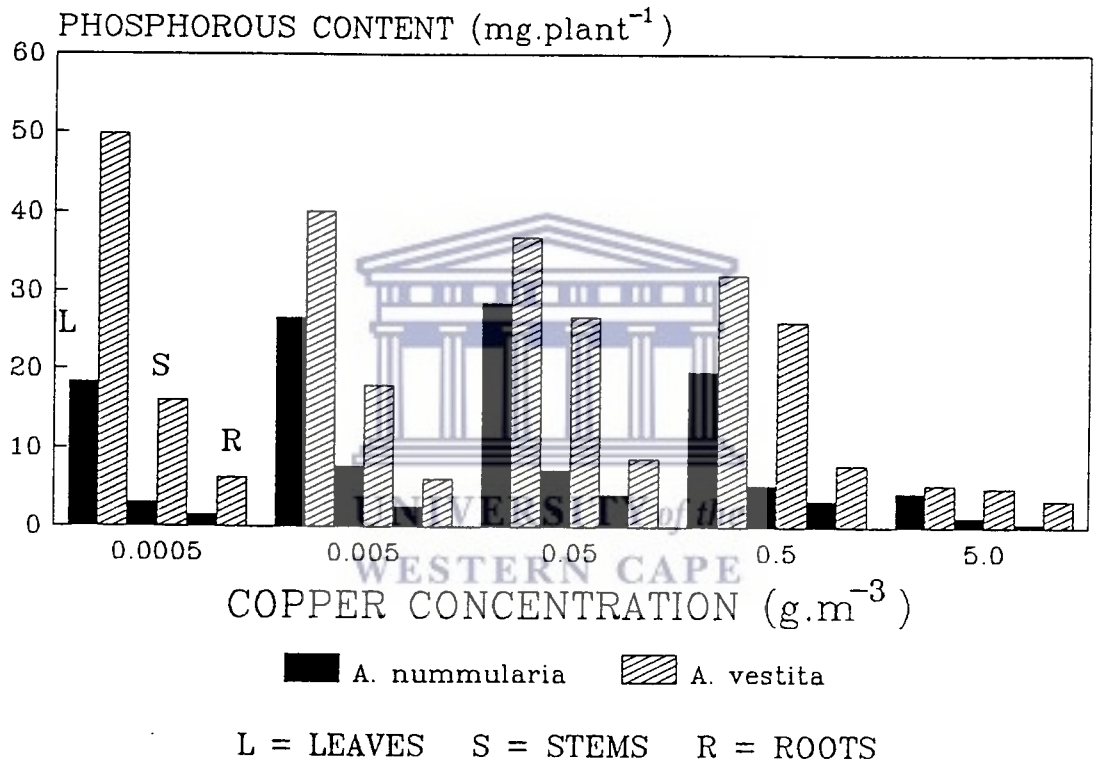

Figure 2: The effect of copper treatment on the phosphorous content of A. nummularia and A. vestita.

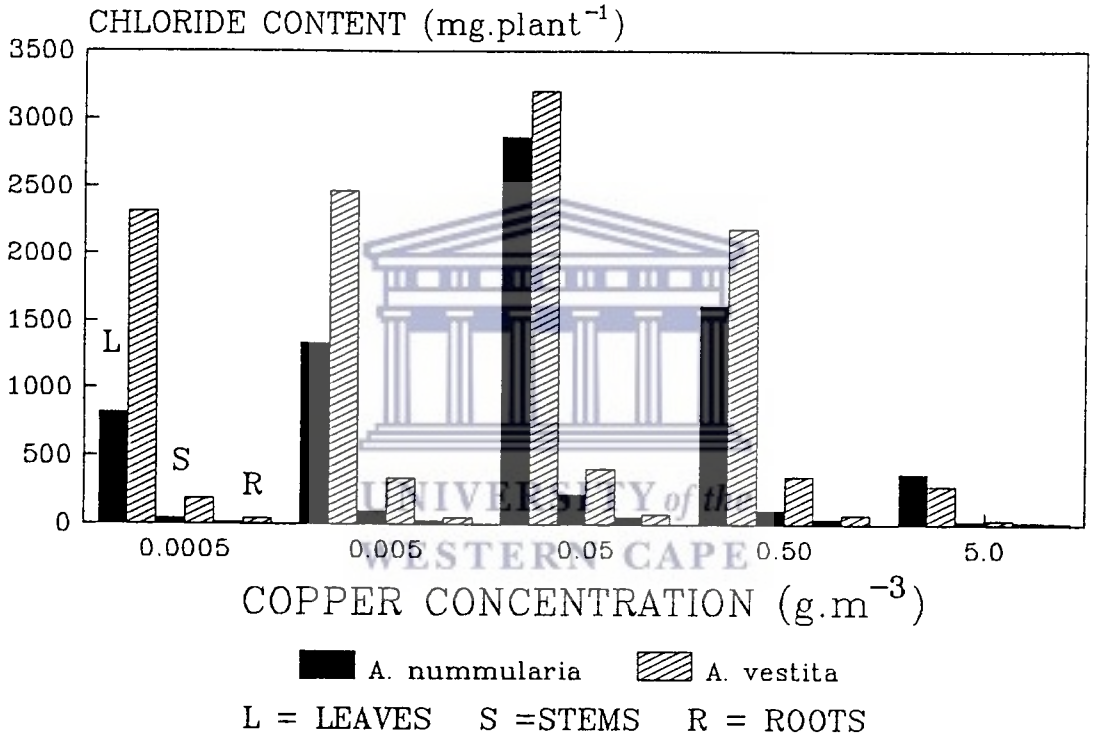

Figure 3: The effect of copper treatment on the chloride content of A. nummularia and A. vestita.

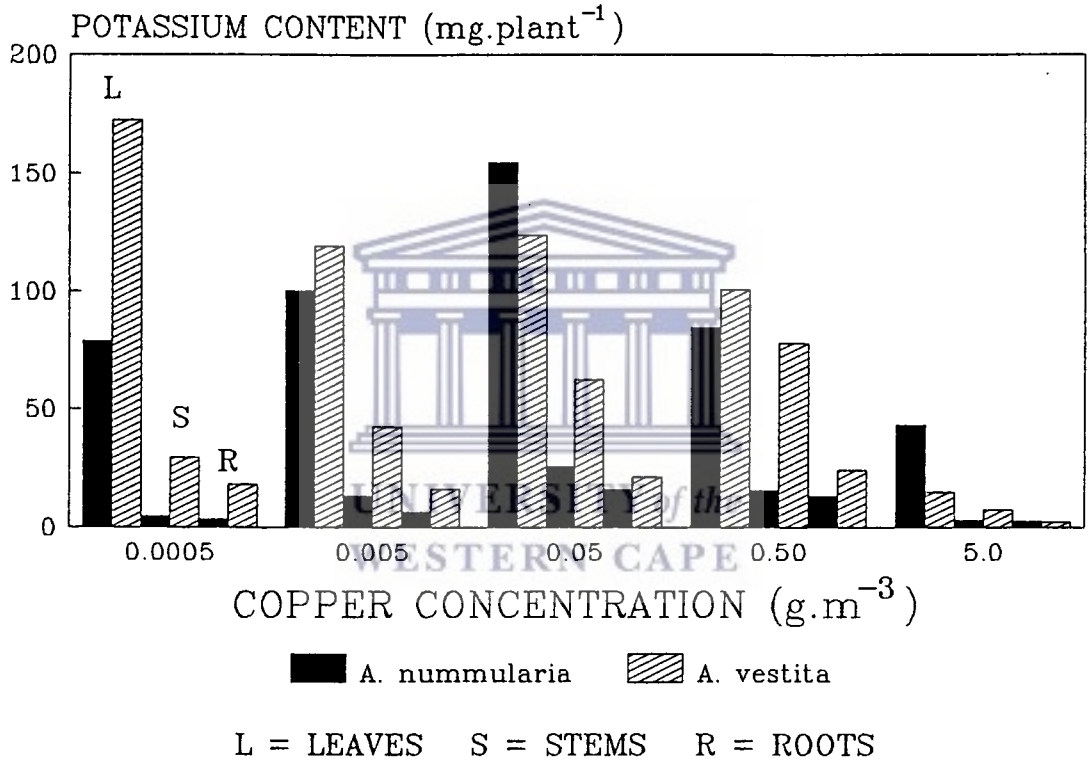

Figure 4: The effect of copper treatment on the potassium content of A. nummularia and A. vestita.

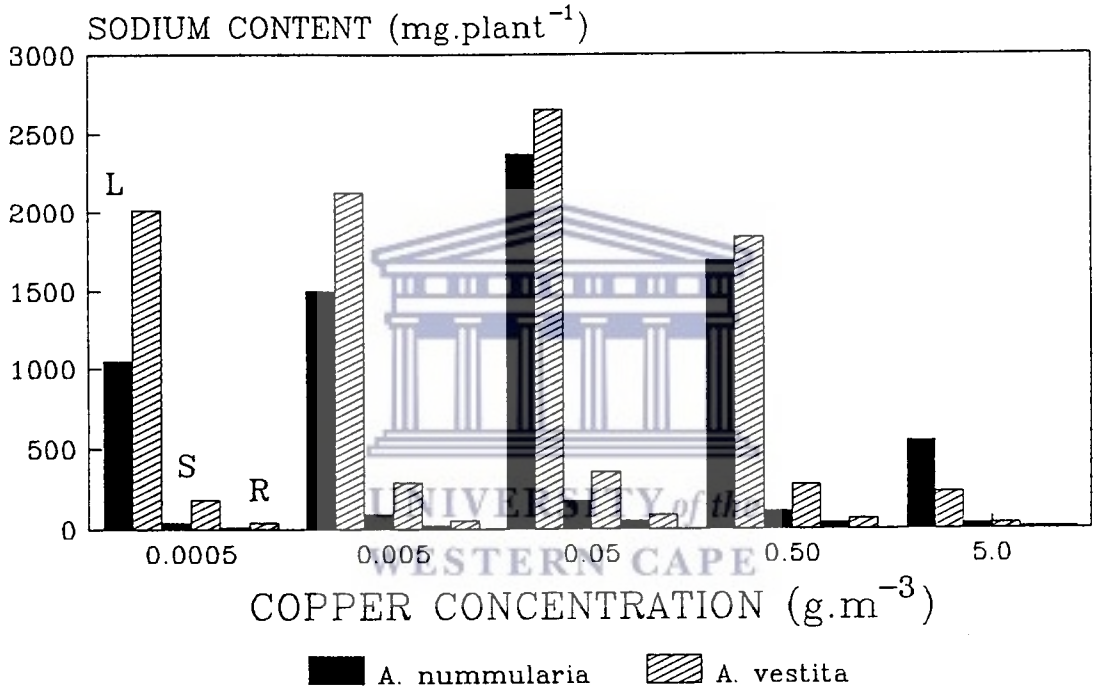

Figure 5: The effect of copper treatment on the sodium content of A. nummularia and A. vestita.

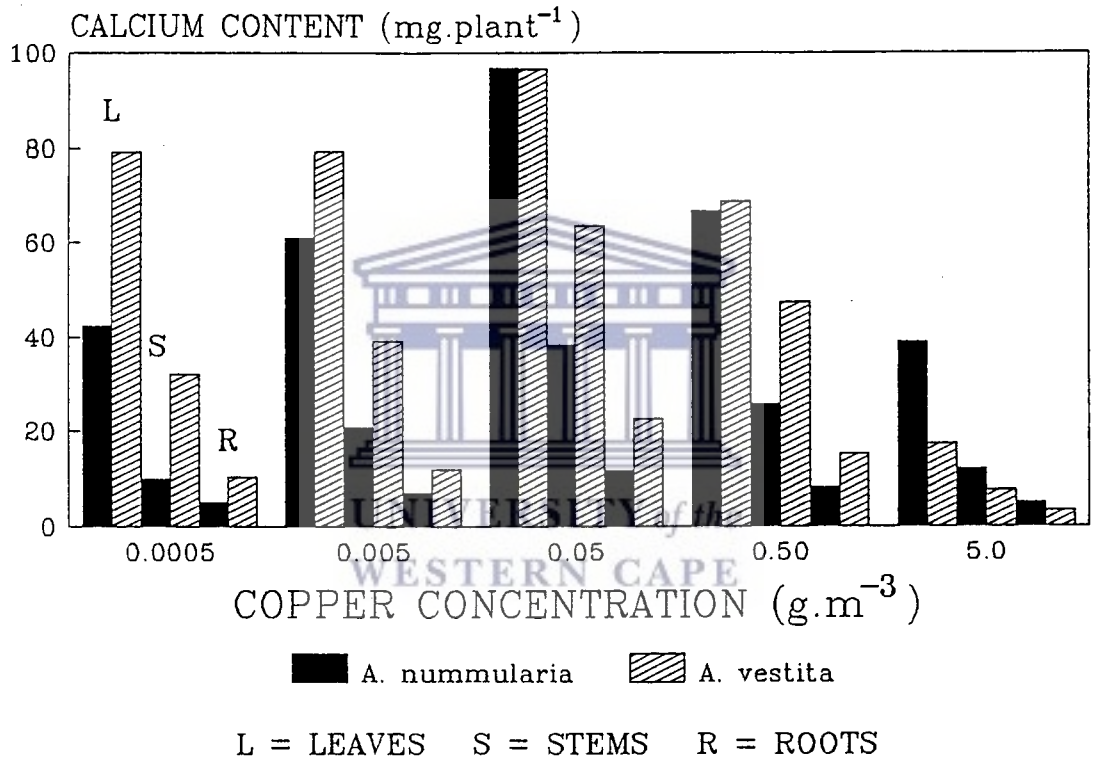

Figure 6: The effect of copper treatment on the calcium content of A. nummularia and A. vestita.

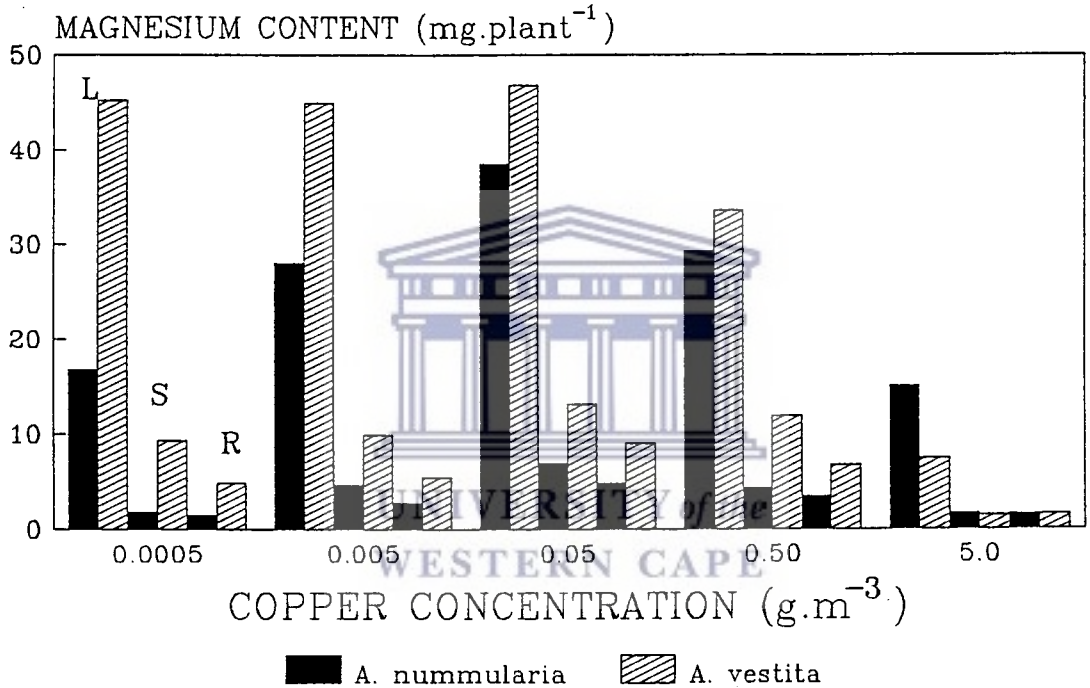

Figure 7: The effect of copper treatment on the magnesium content of A. nummularia and A. vestita.

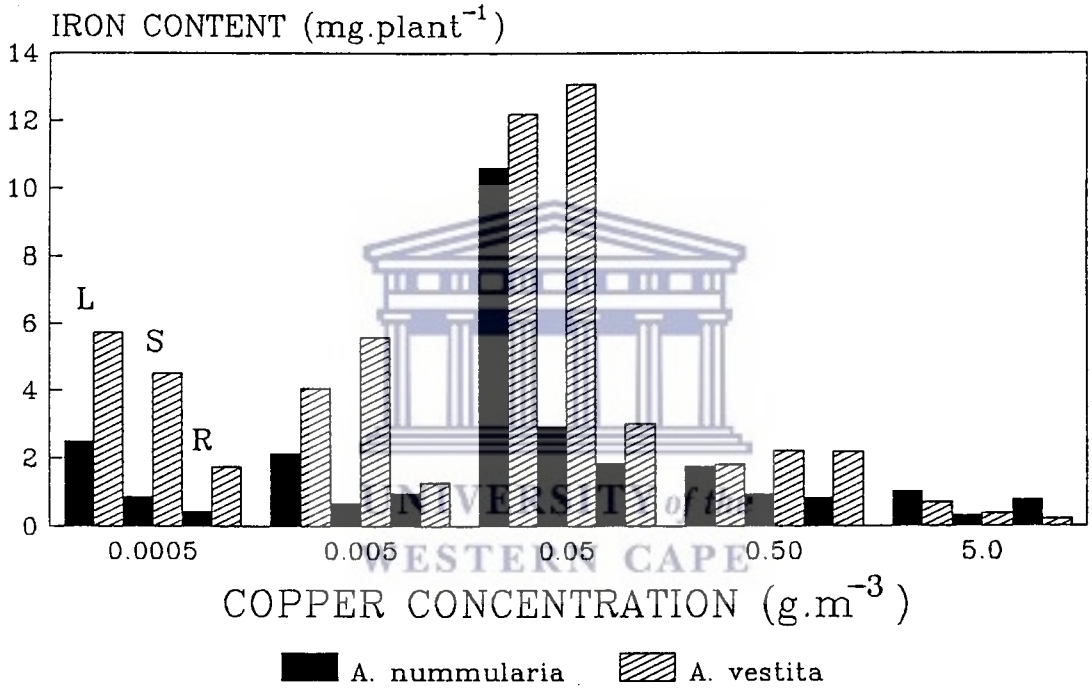

Figure 8: The effect of copper treatment on the iron content of A. nummularia and A. vestita.

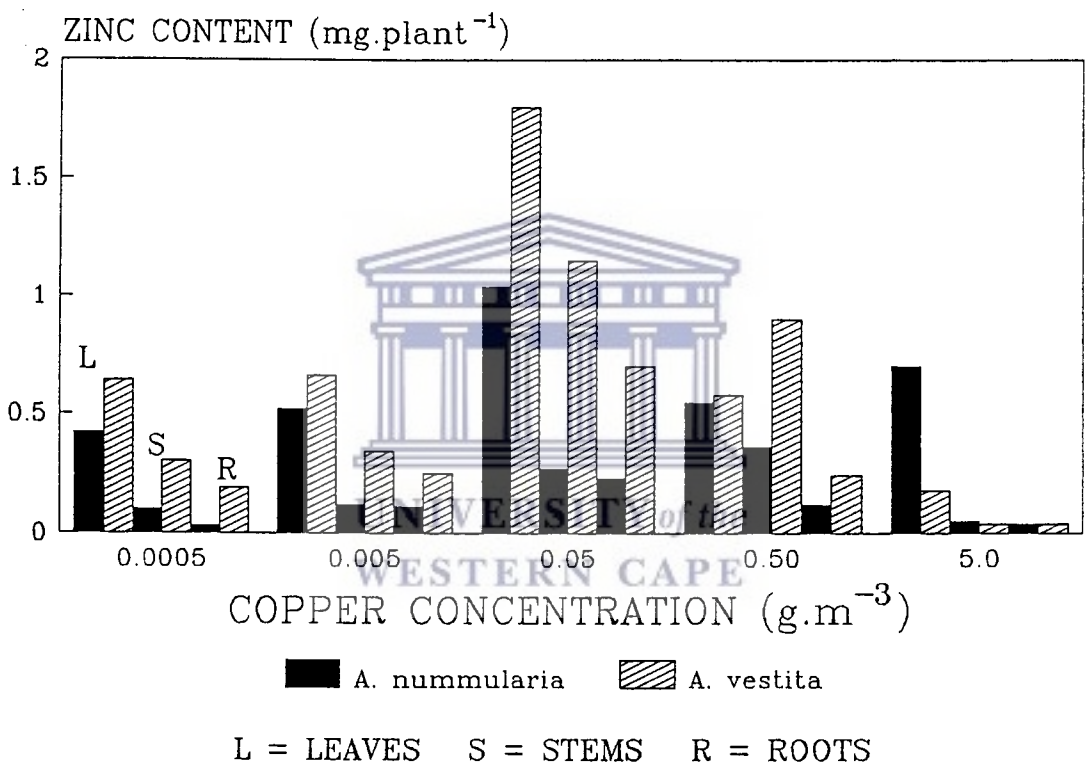

Figure 9: The effect of copper treatment on the zinc content of A. nummularia and A. vestita.

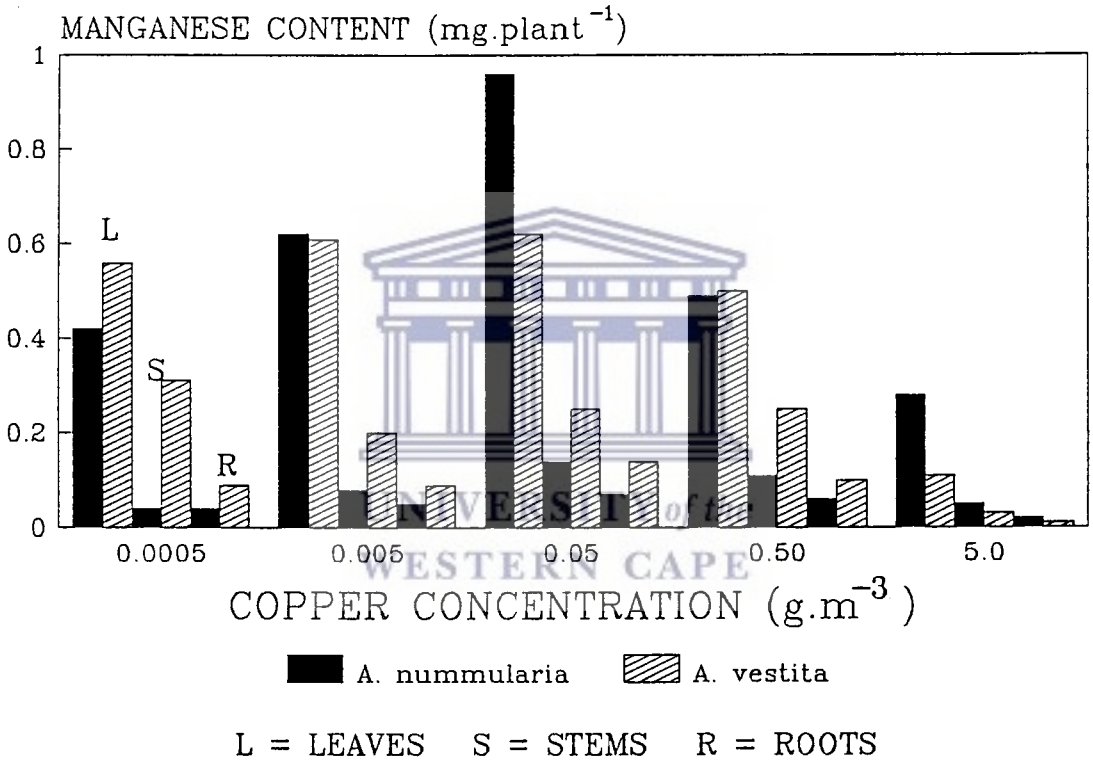

Figure 10: The effect of copper treatment on the manganese content of A. nummularia and A. vestita.

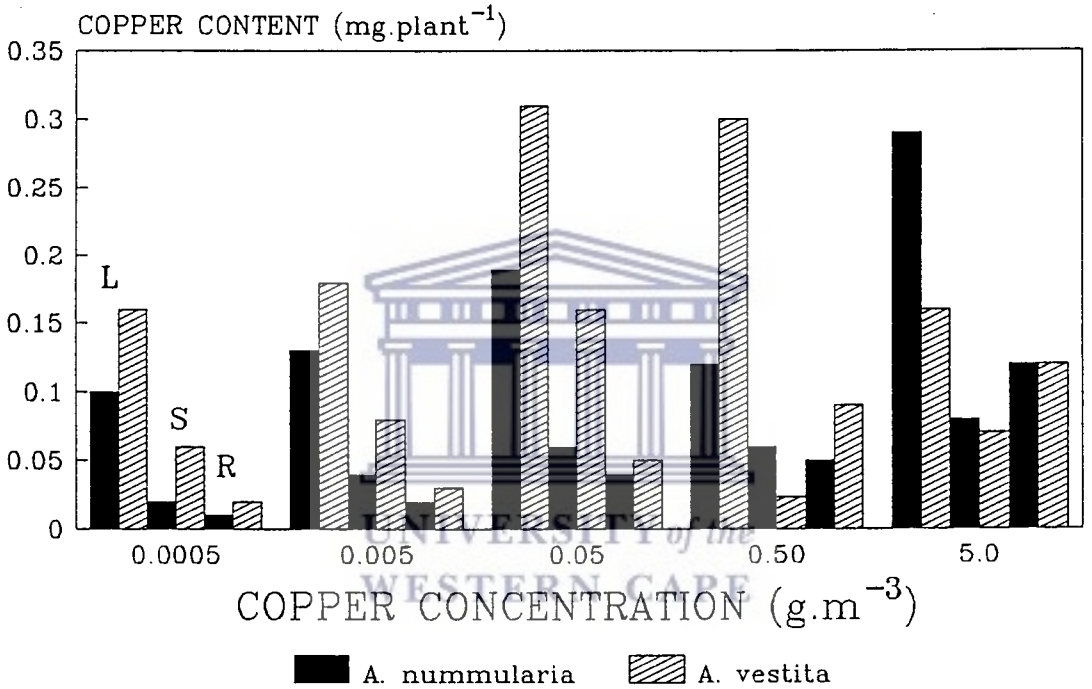

Figure 11: The effect of copper treatment on the copper content of A. nummularia and A. vestita.