The influence of chlorsulfuron on the uptake and utilization of zinc by wheat

Matthew Simon Wheal

Submitted for the degree of Doctor of Philosophy
in the Department of Plant Science,
Faculty of Agriculture and Natural Resource Sciences
The University of Adelaide

December 1996

TABLE OF CONTENTS

SUMMARY

Field experiments testing the sulfonylurea herbicide chlorsulfuron on wheat grown in low-Zn soils have found concentrations of zinc (Zn) in the plants were decreased to deficient levels, and final grain yields were also reduced by the herbicide. Further experiments showed that this response occured when soil Zn levels were low and chlorsulfuron was in close contact with the soil. Experiments in this thesis were conducted to determine the basis for the decreased Zn levels and growth in wheat growing in low-Zn conditions.

The influence of chlorsulfuron on physiological functions of plants requiring Zn was investigated in a series of laboratory experiments, using chelate-buffered nutrient solutions. Preliminary experiments (Chapter 2) were performed to determine concentrations of Zn and chlorsulfuron that would provide adequate and insufficient levels of growth and nutrient concentrations in roots and shoots of the wheat cultivars Excalibur and Gatcher (*Triticum aestivum*) and Durati (*T. turgidum* conv *durum*). Chlorsulfuron decreased root growth after at least 12 d exposure (from 9 to 6 mg/plant) but had no effect on root Zn concentration. At least 12 d exposure to chlorsulfuron was required before shoot growth was significantly reduced. On the other hand, low Zn activity in solution reduced shoot growth (*e.g.* from 35 to 17 mg/plant) but root growth was unaffected or increased relative to Zn-sufficient plants (*e.g.* from 12.8 to 13.3 mg/plant in high Zn plants). Zinc concentrations in shoots and roots were reduced by low Zn activity of solution (from 24 to 16 μg Zn g^{-1} DW). Water contents in roots and shoots were significantly reduced to about 75-80 % of controls roots and shoots by chlorsulfuron treatment.

Because differences in root dry weight between chlorsulfuron-treated and control plants could not be detected earlier than 4 d after addition of chlorsulfuron, an alternative technique measuring root tip extension was developed (Chapter 3). Root extension was decreased to 75 % of control roots after 22 h exposure to relatively low concentrations of chlorsulfuron while high concentrations of chlorsulfuron had measurable effects on root extension within

2-3 h of addition. Addition of Zn to the solution around the root tip decreased root extension of control plants (from 15 mm to 9 mm in 22 h), but increased root extension of chlorsulfuron-treated plants (from 5 mm to 11 mm in 22 h). Neither Zn nor chlorsulfuron added to more mature regions of the root had any influence on root tip extension.

Uptake rates of Zn were measured over 80-90 min periods using ^{65}Zn added to nutrient solutions (Chapter 4). Wheat plants pretreated with chlorsulfuron for 5 d had a significantly lower rate of uptake than control plants (*e.g.* 8 and 15 nmol Zn g^{-1} root FW h^{-1} respectively), while plants treated with chlorsulfuron only during the 90-min uptake period did not differ significantly from untreated plants. Increasing the Zn activity of the solution increased the rate of Zn uptake and decreased the percentage of absorbed Zn that was transported to shoots. Chlorsulfuron pretreatment increased the percentage of Zn transported to shoots from 11 % to 20 % of the total amount of Zn taken up. There was no interaction between Zn activity and chlorsulfuron pretreatment on the transport of Zn to shoots. Plants were capable of recovering from chlorsulfuron exposure after being transferred to nutrient solutions without chlorsulfuron only after 3 d.

Stress responses of wheat plants to Zn deficiency and chlorsulfuron were tested using superoxide radical ($O_2^{\cdot -}$) generation (Chapter 5) and root respiration (Chapter 6) as indicators. In short-term studies, superoxide radical generation was not influenced by Zn activity of solution or chlorsulfuron pretreatment, nor was protein concentration influenced by Zn activity of solution. Increased protein concentration in chlorsulfuron-treated plants (*e.g.* from 1.8 to 2.3 mg g^{-1} in treated plants) was due to a greater decrease in root growth (*e.g.* 433 to 128 mg $plant^{-1}$) than a decrease of protein synthesis.

Respiration of wheat root slices was decreased by low Zn activity in 14-d-old plants, but was increased in 22-d-old plants. Chlorsulfuron had no influence on total respiration rates of root slices, but increased the capacity for respiration via the alternative oxidase by 30 % compared to control plants. Total respiration increased as chlorsulfuron concentration increased (from 70 to 105 nmol O_2

min^{-1} g^{-1} FW); uncoupled respiration decreased as a percentage of total respiration (from +5 % in control plants to -10 % in treated plants), suggesting an alteration in adenylate control of mitochondrial respiration. Differential expression of the alternative oxidase enzyme was not detected by Western blot techniques. Chlorsulfuron appears to increase the capacity for alternative oxidase activity, possibly as a response to herbicide stress.

The experiments in this thesis have shown that chlorsulfuron can reduce plant growth and Zn uptake in nutrient solution. The decreased uptake of Zn can be overcome by addition of Zn to the nutrient solution. While general protein synthesis appeared unaffected by chlorsulfuron treatment, the activity of the alternative oxidase was increased, indicating chlorsulfuron was inducing a stress response. Decreased wheat growth after chlorsulfuron treatment may be exacerbated by adverse effects on physiological functions related to nutrient use. The use of herbicides in the future may have to account for detrimental effects on nutrient uptake in crop plants, especially in conditions in which nutrient availability is already limited.

DECLARATION

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person except where reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library, to be available for loan and photocopying.

Matthew Wheal

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people for their assistance

... to my principal supervisor Dr. Zdenko Rengel for his help and comments throughout the course of my studies even when he was overseas on leave.

... to my joint supervisor Professor Robin Graham for providing additional comments (also when on leave), and encouraging our group in general.

... the Australian Research Council for funding this research with an ARC small grant.

... to Dr Nick Pearson for interesting discussions regarding Zn uptake and efficiency.

... to Nick Robinson and Teresa Fowles for processing the samples for ICP analysis.

... to Neil Bungey for construction of the root growth box.

... to Dr Mike McLaughlin of the CSIRO Division of Soils and Dr Angus Alston of the Department of Soil Science for use of laboratory space and the gamma counter.

... to Dr Qisen Zhang, Dr Marcel Hoefnagel and Professor Joe Wiskich of the Department of Botany for the use of their laboratory and their patient explanations of mitochondrial respiration.

... to the members of our research group for interesting discussions of our research and other topics.

... to my family for supporting me in many ways.

... to Michelle for being there always.

CHAPTER 1

LITERATURE REVIEW

1.1 Zinc

1.1.1 Zinc as an essential plant micronutrient

Zinc (Zn) is classified as a micronutrient as it is essential for growth at relatively low concentrations in living tissues (Marschner, 1995). Zinc was one of the first micronutrient elements to be investigated, when it was found to be essential for growth and reproduction of the fungus *Aspergillus niger* by Raulin in 1869. Improvements in maize (*Zea mays*) growth and curing of chlorosis was achieved by adding Zn to plants (Timiryazev, 1872, in Shkolnik, 1984), indicating the requirement for Zn in higher plants.

If the supply of Zn to plants or animals is inadequate, deficiency conditions will result. Deficiency of Zn has severe effects on a number of metabolic and physiological processes in both plants and animals, which can be treated by supplementing food or nutrient intake with Zn. Zinc deficiency in field crops was demonstrated by Mazé (1914, in Brown *et al.*, 1993). Zinc was subsequently found to be essential for chlorophyll-containing vascular plants (Sommer and Lipman, 1926). The requirement of Zn in the diet for animals was confirmed by Todd *et al.*, (1934). In animals and humans, severe Zn deficiency is uncommon, however marginal Zn deficiency has been detected in a number of countries (Welch, 1993).

1.1.2 Chemical nature of zinc

Zinc is rarely encountered naturally in its elemental form, occurring more often in minerals as a divalent ion (Barak and Helmke, 1993). Zinc has an electronic configuration of $1s^1$, $2s^2$, $2p^6$, $3d^{10}$, $4s^2$, and has only one oxidation state: Zn(II) ($1s^1$, $2s^2$, $2p^6$, $3d^{10}$). Depending on the definitions used, Zn is therefore a transition or post-transition element as it has no unfilled *d* subshells in its structure (see Barak and Helmke, 1993). There are five stable isotopes of Zn, which have an average molecular weight of 65.38. Zinc is more chemically stable than other transition elements (*e.g.* Mn and Fe) as it is not subject to reduction

and oxidation reactions. Zinc is commonly found in cells and enzymes as a stabilising and structural component.

Zinc (II) ions usually form tetrahedral complexes (with four bonds) or octahedral complexes (with six bonds; Uritani, 1975). Aqueous complexes of Zn are generally octahedral (Barak and Helmke, 1993). Pentahedral complexes are also possible in proteins which use Zn as a catalytic component (Brown *et al.*, 1993) and in minerals (Barak and Helmke, 1993).

1.1.3 Zinc in soils

Zinc is the 24th most abundant element in the Earth's crust (Barak and Helmke, 1993). Concentrations of total Zn in soil vary depending on the parent rock; from 10-30 $\mu g\ g^{-1}$ in sandstones and Carboniferous rocks, 40-60 $\mu g\ g^{-1}$ in acid rocks and 60-80 $\mu g\ g^{-1}$ in mafic rocks (Kabata-Pendias and Pendias, 1992). Parent material has a greater influence on soil Zn content than pedogenesis (Aubert and Pinta, 1977).

Zinc generally occurs as a divalent ion in the soil solution and in minerals as ZnS, $ZnSO_4$, ZnO, $ZnFe_2O_4$, $ZnAl_2O_4$, $ZnCO_3$, (sulphides, oxides, carbonates, phosphates; Barak and Helmke, 1993; Kabata-Pendias and Pendias, 1992) and more rarely as silicates (Lindsay, 1972). Zinc is sparingly soluble in soils but relatively soluble compared to other heavy metals (Lindsay, 1972; Kabata-Pendias and Pendias, 1992). Zinc binds to soil components such as clay minerals and metal oxides, creating large pools of Zn that are unavailable to plants (Barrow, 1993). Phyto-availability of Zn is determined by the concentration of Zn in the soil solution ("intensity") and the rate of replenishment from bound forms (Marschner, 1993).

1.1.4 Symptoms of zinc deficiency in plants

Detection of micronutrient deficiencies in plants is often by visual symptoms of above-ground plant parts (*e.g.* Snowball and Robson, 1988), but plant analysis can detect and better quantify deficiency before visual symptoms occur (Jones, 1991). Symptoms of Zn deficiency have been well-defined for both

monocotyledonous and dicotyledonous plants and form the basis for keys to determine micronutrient deficiencies (*e.g.* Bergmann, 1992; Snowball and Robson, 1988; Reuter and Robinson, 1986).

Zinc deficiency symptoms in a wide range of species are described in detail by Bergmann (1992). The major symptoms of Zn deficiency in dicotyledonous plants are reduced growth of meristematic regions and internodes, and malformed leaves, resulting in rosetting of new growth and interveinal chlorosis (Brennan *et al.*, 1993; Marschner, 1995). In monocotyledons, leaves become chlorotic around the midrib, have a water-soaked appearance and develop necrotic regions. Older leaves can eventually wilt and collapse at the necrotic points (Brennan *et al.*, 1993; Snowball and Robson, 1988). Improvements in plant analysis have indicated that Zn-deficient plants do not necessarily have visible symptoms, yet may be deficient to the extent that the yield is reduced by up to 40 % (Carroll and Loneragan, 1968; Riley *et al.*, 1992). Zinc deficiencies are common in early stages of growth of annual plants and can occur transiently during the life of the crop and patchily within fields (Brennan *et al.*, 1993).

The concentration of Zn at which plants become deficient varies comparatively little in contrast to other micronutrients. Grain crops are deficient in Zn when leaf Zn concentrations 45-60 d after sowing are 10-25 mg kg^{-1} DW, legumes are deficient at similar concentrations (10-24 mg kg^{-1}) 42-45 d after sowing, and citrus and stonefruit leaves are deficient at <15 mg kg^{-1} (Reuter and Robinson, 1986). The critical concentration for Zn in mature grain has been suggested to be 15 mg kg^{-1} (Viets, 1966), but may be lower depending on the species *e.g.* 10 mg kg^{-1} in wheat (Riley *et al.*, 1992).

The range of concentrations of Zn for adequate growth are similar for many plant species: 20-100 mg kg^{-1} (DW) for leaves of mature wheat, barley, oats and rice and 20-50 mg kg^{-1} for mature legumes, citrus and stonefruits (Reuter and Robinson, 1986). Zinc concentrations of various organs of a number of crops do not vary considerably in comparison to other elements (14-73 mg Zn kg^{-1} in plants), while grain Zn concentrations ranged from 6-75 mg kg^{-1} (Kabata-Pendias and Pendias, 1992). Native Australian plants appear to grow well with much

lower leaf concentrations *e.g.* 8-9 mg kg^{-1} in waratah (*Telopea*; Reuter and Robinson, 1986), 11 mg kg^{-1} in *Banksia* spp. and 2 mg kg^{-1} in *Hakea victoriae* (Longnecker and Robson, 1993).

1.1.5 Biochemical and physiological roles of zinc

Physiological functions of micronutrients include activation of enzyme reactions and prosthetic groups of metalloenzymes (Römheld and Marschner, 1991). Due to its stable electronic configuration (section 1.1.2), Zn is a common structural feature in biochemistry, as it is less likely to be affected by redox reactions and changing conditions within cells.

Zinc binds tightly to atoms of sulphur, nitrogen and oxygen in amino acids (Barak and Helmke, 1993; Bracey *et al.*, 1994; Rowlett *et al.*, 1994). The main amino acids that Zn binds to in proteins (ligands) are histidine, cysteine, aspartate and glutamate (Vallee and Auld, 1990).

Zinc is used as either a structural or functional component of enzymes and is bound to amino acids within the enzyme (Bracey *et al.*, 1994). Structural Zn atoms are bound by four cysteine residues, forming a tetrahedral complex which helps maintain the local structure of the macromolecule so that other regions of the enzyme can function (Vallee and Auld, 1990). In the known examples of structural Zn enzyme sequences, the sequence of ligands and spaces between ligands is highly conserved for each enzyme (Vallee and Auld, 1990).

Catalytic (functional) Zn atoms are bound by three ligands at various locations along the amino acid sequence. The reaction site generally occurs at the fourth coordination site which is usually occupied by a water molecule (Rowlett *et al.*, 1994). The combination of ligands differs depending upon the enzyme, but most catalytic enzymes bind Zn with histidine and glutamate residues (Vallee and Auld, 1990). The order and spacing of the Zn ligands is highly conserved for each enzyme (Vallee and Auld, 1990). Carbonic anhydrase is an exception however; the plant enzyme appears to have a different set of ligands (Cys-His-Cys) to the animal enzyme (His-His-His) which may be due to separate evolutionary origins of the same functional molecule (Rowlett *et al.*, 1994).

1.1.5.1 Zinc proteins

Many enzymes utilise divalent cations to provide certain functions. These cations are often interchangeable, and can replace others with little change in enzymic activity. Zinc is essential in only five or six enzymes, but is utilised by almost 300 different enzymes from species in all phyla (Vallee and Auld, 1990). Enzymes requiring Zn have been isolated from each of the six major enzyme classes (Barak and Helmke, 1993). The most important enzymes that require Zn as an essential component of their composition are carbonic anhydrase (CA), superoxide dismutase (SOD), DNA-dependent RNA polymerase and alcohol dehydrogenase (ADH; Marschner, 1995).

1.1.5.1.1 Alcohol dehydrogenase

Alcohol dehydrogenase is used to convert pyruvate to ethanol in anaerobic conditions (Marschner, 1995). The rate of this reaction is decreased in Zn-deficient plants but the effects on the plant are unknown (Marschner, 1995). In anaerobic conditions when plants were flooded, rice root ADH activity was much higher in plants supplied with Zn than in untreated controls (Moore and Patrick, 1988). They suggested that metabolic activity was decreased by low Zn concentration in the rice plants. Zinc deficiency may be more prevalent in flooded conditions due to the increased requirement for anaerobic respiration via ADH, decreasing the amount of Zn available for other functions (Brown *et al*, 1993).

The amino acid sequence of ADH has been determined for a number of species and shows a high degree of homology among them (Vallee and Auld, 1990). There are two Zn atoms per molecule of ADH, of which the structural Zn is bound to four Cys ligands and the catalytic component is bound by a Cys-His-Cys combination (Vallee and Auld, 1990).

1.1.5.1.2 Carbonic anhydrase

Carbonic anhydrase (CA) occurs in almost every species and is used to catalyse the reversible hydration of carbon dioxide to bicarbonate (Equation 1.1). In plants, CA may constitute up to 1 % of total leaf protein (Rowlett *et al.*, 1994).

Plant CA is generally confined to the chloroplasts where it may participate in carbon fixation by concentrating CO_2 for the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Bracey *et al.*, 1994). Plant CA is a hexamer with a molecular weight of 168 kDa and contains six Zn atoms. The Zn is integral to the functioning of CA as it binds H_2O or bicarbonate which can react with CO_2 (Rowlett *et al.*, 1994):

$$\begin{aligned} &Zn^{2+} — OH^- + CO_2 \Leftrightarrow Zn^{2+} + HCO_3^- \\ &Zn^{2+} — HCO_3^- + H_2O \Leftrightarrow Zn^{2+} — OH_2 + HCO_3^- \\ &Zn^{2+} — OH_2 \Leftrightarrow Zn^{2+} — OH^- + H^+ \end{aligned} \qquad \text{(Equation 1.1).}$$

Zinc-deficient plants have reduced CA concentrations, but are still able to catalyse this reaction owing to large pools of stored CA apo-enzyme (enzyme without metal cofactor; Marschner, 1995; Rengel, 1995a). Resumption of Zn supply enables rapid recovery of active CA, as the apo-enzyme is otherwise unchanged and is easily reconstituted.

Zinc is required in several other enzymes that are involved in carbohydrate metabolism (Brown *et al.*, 1993). The activity of the enzyme ribulose 1,5-bisphosphate carboxylase (the first step of CO_2 fixation by photosynthesis) is also low in Zn-deficient plants (Brown *et al.*, 1993). Fructose 1, 6-bisphosphatase and aldolase require Zn to catalyse their reactions in the formation of sucrose. In plants grown without Zn, carbohydrate metabolism is reduced to much lower rates than in control plants (Marschner, 1995).

1.1.5.1.3 Superoxide dismutase

Superoxide radicals ($O_2^{\cdot -}$) are highly reactive oxygen molecules that react rapidly with cell components, membrane bilayers, proteins and small molecules, causing peroxidative damage (Cakmak and Marschner, 1988a, 1993; Fridovich, 1986). They are produced as a by-product of oxygen-consuming reactions in the

mitochondria at the ubiquinone-cytochrome b site (Boveris, 1984; Purvis and Shewfelt, 1993; see also section 6.1.3), or by membrane-bound NADPH-oxidases (Cakmak and Marschner, 1988a; Thompson *et al.*, 1987), as well as in a number of other common biological oxidation reactions (Bannister *et al.*, 1987). The superoxide radical is the precursor for several more reactive molecules ($OH\cdot$, H_2O_2 and $^1O_2$) that also cause peroxidative damage to cell components (Fridovich, 1986; Thompson *et al.*, 1987). The role of $O_2\cdot^-$ itself in oxidative reactions has been questioned (Thompson *et al.*, 1987), but has been shown to contribute directly to many different degradative reactions (Fridovich, 1986). A number of abiotic stresses are also known to increase superoxide levels in plants *e.g.* cold (Purvis and Shewfelt, 1993), water stress (Smirnoff, 1993) and salt stress (Hernandez *et al.*, 1993).

Superoxide dismutase (SOD; E.C. 1.15.1.1) catalyses the reaction converting superoxide radicals to hydrogen peroxide (H_2O_2; Fridovich, 1986). Superoxide dismutase occurs in almost all aerobic organisms (Bannister *et al.*, 1987) and has several forms depending on the metal cofactors required; Cu/Zn SOD, Mn SOD, Fe SOD. The Cu/Zn SOD is a 32-kDa homodimeric enzyme with one Zn and one Cu atom per subunit (Bannister *et al.*, 1987). Zinc is a structural component, bound by an aspartate and three histidine residues. The first Zn-binding histidine also binds the functional Cu ion (Carloni *et al.*, 1995).

Cyanide inhibits the activity of Cu/Zn SOD but not Mn and Fe SODs, which has assisted in its identification within plant parts (Bannister *et al.*, 1987). Cu/Zn SOD has been found in the cytosol, chloroplasts and between the membranes of mitochondria (Bannister *et al.*, 1987). The MnSOD enzyme is also found in plants but its occurrence and distribution is much more variable, mostly in the peroxisomes and chloroplasts. Fe SOD is most commonly found in bacteria and rarely seen in plants (Bannister *et al.*, 1987).

Zinc also inhibits NADPH-oxidase activity (Jeffery, 1983), which will suppress oxygen free radical production. Therefore, Zn-deficient plants will produce more $O_2\cdot^-$ and have a decreased ability to dismutate the $O_2\cdot^-$, as the

majority of SOD enzymes in plant cytosol are the Cu/Zn type (Cakmak and Marschner, 1988a).

1.1.5.1.4 DNA-dependent RNA polymerase

Zinc is required by RNA polymerase during cell replication, and when Zn-deficient, cells will not produce sufficient RNA for cell formation and division (Brown *et al.*, 1993). Zinc deficiency can disrupt RNA metabolism (Falchuk *et al.*, 1977), causing increases in DNA and amino acids while RNA and proteins decrease (Falchuk *et al.*, 1976).

The requirement of Zn in RNA polymerase was determined by inhibition of the activity of RNA polymerase from *Euglena gracilis*. Metal chelators (8-hydroxyquinoline-5-sulphonic acid, EDTA, α,α′-bipyridinyl), inhibited the activity of purified RNA polymerase II (Falchuk *et al.*, 1976). A subsequent study showed 1,10 phenanthroline (a metal chelate) completely inhibited activity of RNA polymerase I while 4,7 phenanthroline (the non-chelating form) had no effect, supporting the idea that Zn was essential for RNA polymerase activity (Falchuk *et al.*, 1977).

The presence of Zn also inhibits the activity of ribonuclease, which increases when plants are Zn-deficient (Dwivedi and Takkar, 1974). The earliest causal event of Zn deficiency is reduced RNA levels caused by reduced RNA production, and increased RNase activity breaking down RNA (Brown *et al.*, 1993).

1.1.5.2 Other enzymes

As Zn is required in 300 different enzymes, many reactions or sequences of reactions could be altered by Zn deficiency. One important set of reactions is the aerobic respiration pathway of glycolysis, TCA cycle and electron transport chain which provide energy for cells via the breakdown of sugars (see also Chapter 6). These pathways require many enzymes to catalyse reactions, many of which require metal cofactors, including Zn^{2+} (Paribok, 1973). Important intermediate enzymes of this pathway that involve Zn include aldolase and oxaloacetic acid

dehydrogenase (O'Sullivan, 1970; Shkolnik, 1984). A number of studies have shown that Zn-deficient plants have decreased rates of respiration compared to Zn-adequate plants (*e.g.* barley, (*Hordeum vulgare*; Satsukevich and Vol'-shchuk, 1976; Zakharchishina, 1973); tomato (*Lycopersicon esculentum*; Paribok, 1973); soybean (*Glycine max*; Ohki, 1978; Wu and Xiao, 1992)). Considering the role of Zn in respiration in anaerobic and aerobic conditions, it is surprising that relatively little regarding the role of Zn in respiration has been reported.

1.1.5.3 IAA synthesis

One early discovery of the role of Zn was the demonstration that Zn-deficient plants lack indole-acetic acid (IAA; Skoog, 1940). IAA is a plant hormone responsible for elongation and meristem development (Marschner, 1995). The synthesis of IAA relies upon the intermediate compound tryptophan which is lacking in Zn-deficient plants (Tsui, 1948). Zinc was considered directly required for tryptophan synthesis and indirectly for auxin (IAA) synthesis (Tsui, 1948). A second hypothesis proposed that the precursor of IAA, D-tryptophan (Law, 1987) is converted from L-tryptophan by gibberellic acid (GA), and the level of GA is decreased by Zn-deficiency (Suge *et al.*, 1986). GA may therefore be the compound limited by Zn deficiency rather than IAA (Brown *et al.*, 1993). Regardless of which hormone is decreased first by Zn deficiency, high Zn concentrations increased IAA concentration and chlorophyll concentrations in wheat (Hemantaranjan and Gar, 1984). In Zn-deficient bean (*Phaseolus vulgaris*) plants, the levels of IAA, chlorophyll and protein concentrations were decreased compared to Zn-sufficient plants (Cakmak *et al.*, 1989). Lack of IAA could therefore be responsible for the rosetting and shortened internodes often seen in Zn-deficient dicotyledonous plants (Marschner, 1995).

1.1.5.4 Membranes

A physiologically important role for Zn in plant cells is as a structural component of cell membranes (Chvapil, 1973; Cakmak and Marschner, 1988**b**; Pinton *et al.*, 1994). Zinc helps maintain the integrity of membranes by bridging

sulphydryl (-SH) groups of membrane proteins (Chvapil, 1973). Under Zn deficiency, the -SH groups can form disulphide (S-S) bonds that distort the membrane, leading to destabilization of the membrane and "leakiness" (Cakmak and Marschner, 1988b; Welch and Norvell, 1993). Decreased membrane stability may be the first biochemical change caused by Zn deficiency (Bettger and O'Dell, 1981).

The amount of reactive sulfhydryl groups (*i.e.* -SH ions in the membrane that are able to bind with other groups or with Zn) in wheat root cell membranes was directly related to Zn supply (Rengel, 1995b). Lack of Zn supply in solution for 10 d decreased the amount of reactive -SH groups, and resupply of Zn for 24 h increased reactive -SH groups when Zn concentration was greater in the resupply solution than the preculture solution. Differences between cultivars that differed in Zn efficiency (see section 1.1.6.3) also occurred: Durati (a Zn-inefficient cultivar) had fewer reactive groups than Warigal (efficient cultivar) after 20-d growth, even though total root growth was similar (Rengel, 1995b). Leakage of ions from roots was greater in Zn-deficient than Zn-sufficient plants, and the amount of reactive sulfhydryls was greater in Zn-sufficient plants (Welch and Norvell, 1993). Zinc therefore maintains the membranes by keeping sulfhydryl groups in the reduced state and preventing disulfide formation.

Zinc deficiency was found to increase membrane leakage of ^{32}P and ^{36}Cl from several crop species (Welch *et al.*, 1982). Phosphorus content was increased in Zn-deficient plants even when those plants were smaller than Zn-adequate plants. Zinc-deficient wheat roots were found to retain less ^{32}P, due to greater losses of P from the roots during the "washout" procedure of the experiments. Experiments with ^{36}Cl indicated that Zn-deficient roots retained less Cl^- after the washout period. Chloride-ion retention was less in 5-7 d old plants grown without Zn, while fresh and dry weights were unaffected, indicating that plants may still leak ions without apparent visible symptoms of Zn deficiency. Adequate levels of Zn in roots were insufficient to prevent leakiness as plants transferred to low-Zn solutions still lost some nutrients (Welch *et al.*, 1982). Leakage of solutes is not confined to inorganic ions, as sugars and amino acids were also lost from

Zn-deficient roots to a greater extent than Zn-sufficient roots (Cakmak *et al.*, 1988a).

Free radicals (section 1.1.5.1.3) can also form complexes with Zn-deficient membrane components and further distort membrane structure (Chvapil, 1973). Because superoxide dismutase requires Zn and Cu to function, superoxide levels in Zn-deficient cells are increased and membrane integrity is decreased at the same time. The net result is much reduced cell function in the form of "leaky cells" that lose ions easily from the cell and are more susceptible to infections (Cakmak and Marschner, 1988b).

1.1.5.4 Gene regulation

The role of Zn in the regulation of gene expression was discovered relatively recently (Miller *et al.*, 1985). Correct recognition of the start of a particular gene (the promoter region) is essential for the expression of that gene. Transcription factors (proteins) are specific for binding at the promoter, the most common form being the so-called "zinc-finger" proteins (Rhodes and Klug, 1993). The first such protein to be described in detail was TFIIIA which contains 2-3 mol of Zn per mol and activates transcription of the 5S RNA gene (Rhodes and Klug, 1993; Vallee *et al.*, 1991). TFIIIA contains 9 sets of repeated amino acids with two cysteines and two histidines in the same positions in each repeat. The Zn binds to the cysteines and histidines while the 12-13 amino acids between the centre ligands make a loop that forms the "finger".

A strict definition for Zn finger proteins given by Vallee *et al.*, (1991) requires the following conditions:

i) proteins with one or multiple repeats of about 30 amino acids,

ii) conservation of both two cysteine and two histidine residues and their spacing,

iii) contain two aromatic residues and leucine, and

iv) presumably have a 3-dimensional structure resembling a finger. Other Zn-containing transcription factors form "loops" and "clusters" of the amino acid

chain by altering the Zn-binding sites within the amino acid sequence (Vallee *et al.*, 1991).

DNA recognition is achieved through the different amino acids within the finger region. Combinations of zinc fingers can therefore be made to form a single transcription factor that recognises specific promoters on the DNA (Rhodes and Klug, 1993). The combinations allowed by the different finger sequences and sets of fingers is therefore large and highly specific for each gene sequence (Rhodes and Klug, 1993).

Zinc deficiency prevents proper folding of the zinc finger protein and prevents transcription occurring (Rhodes and Klug, 1993). Lack of Zn in zinc fingers may also contribute to oxygen free radical generation (Sarkar, 1995). Divalent metal ions (Fe, Ni, Co, Cd) can substitute for Zn in zinc finger proteins but can undergo redox reactions to produce oxygen free radicals (section 1.1.5.1.3) in the vicinity of the DNA, thus causing damage (Sarkar, 1995).

1.1.6 Soil-plant relations

1.1.6.1 Extent and degree of zinc deficiency in soils worldwide

Zinc deficiency in crops is common throughout the world (Sillanpää, 1982), including western United States, Central America, southern Europe, India and Bangladesh, northern China and central and western Africa (Takkar and Walker, 1993), and is particularly prevalent in Australia (Welch *et al.*, 1991). The only consistent effort to quantify Zn deficiency across a single country appears to have been in India, where large scale sampling of soils and crops has taken place (Takkar and Walker, 1993).

Zinc deficiency has been reported in most major soil types worldwide. Lindsay (1972) listed the most important factors that contributed to Zn deficiency as:

i) low total content of Zn in soil,

ii) calcareous soils of pH > 7.4,

iii) low soil organic matter (*e.g.* in subsoils),

iv) microbial inactivation of Zn,

v) restricted root zone and cool conditions,

vi) differential ability to take up Zn and utilise Zn between genotypes,

vii) antagonistic effects between Zn and other nutrients.

Soil solution concentrations of Zn^{2+} range from 0.01 μM to 1 μM in calcareous soils (Kochian, 1991). The concentration of freely available Zn in soil solution is low in comparison to the proportion of Zn that is bound to soil components, therefore mass flow of solution to the roots contributes only a small amount of the Zn that is required by the plant, with the remainder supplied by diffusion from bound Zn (Marschner, 1993).

The soil factor with the greatest influence on Zn availability to plants is pH (Marschner, 1993). As pH increases, Zn equilibrium concentration decreases 30-45 times per pH unit. Diffusion coefficients for Zn are up to 50 times lower in calcareous soils than acid soils (Marschner, 1993). Liming of soils was shown to prevent excessive Zn uptake from soils contaminated with Zn-based fungicides (Lee and Craddock, 1969), but liming can induce Zn deficiency in plants growing on soils that are low in Zn (Duguma *et al.*, 1988). Zinc deficiency of plants is common in high pH soils due to the low availability of free Zn^{2+}, whereas in highly leached acid soils, Zn deficiency is common due to the low Zn content (Welch *et al.*, 1991). Plant Zn deficiency occurs in high pH (alkaline) soils, low pH soil with low Zn content, limed acid soils, calcareous soils, sodic soils, both very low and very high organic content soils, sandy soils and soils with high P contents (Takkar and Walker, 1993).

Zinc availability is lower in soils with high organic matter contents due to the increased levels of organic acid chelates (Lindsay, 1972), but is also low in siliceous sands with extremely low organic matter (*e.g.* Nable and Webb, 1993). High organic carbon levels increase bicarbonate levels which increase pH, with consequences for Zn availability as described above (Moraghan and Mascagni, 1991).

Low soil temperatures can increase the occurrence of Zn deficiency in soils low in Zn (Moraghan and Mascagni, 1991). This may be due to decreased translocation of Zn to shoots, lowered availability of Zn in the soil, decreased

mycorrhizal activity or decreased rate of uptake by roots (Moraghan and Mascagni, 1991). Zinc deficiency symptoms often decline as average temperatures increase (Bergmann, 1992), possibly by increasing the rate of root growth and microbial degradation of bound Zn (Moraghan and Mascagni, 1991).

Zinc deficiency of rice (*Oryza sativa*) may be increased by flooded conditions, either through bicarbonate presence in water increasing pH, lack of oxygen in flooded soil or precipitation of Zn (Moraghan and Mascagni, 1991). For the opposite extreme, lack of water in the soil does not appear to increase Zn-deficiency as plants could still take up ^{65}Zn from dry soil, possibly through secreted mucilages (Nambiar, 1976).

Phosphorus in particular is noted for its interactions with Zn. High P soil levels can increase growth, thus decreasing Zn concentrations to deficient levels (Olsen, 1972; Robson and Pitman, 1983). Zinc-deficient wheat plants can accumulate P in shoots while the dry weight of the plant is reduced (Webb and Loneragan, 1988). Greater decrease of whole plant dry weight than P accumulation, and relatively less growth in shoots was responsible for the toxic levels of P (Webb and Loneragan, 1988), rather than increased rate of P uptake (Webb and Loneragan, 1990).

Uptake of Mn can be decreased by high levels of Zn (Kabata-Pendias and Pendias, 1992), although Zn deficiency also increased Mn and Cu uptake in Zn-deficient wheat grown in chelate-buffered nutrient solution (Rengel and Graham, 1996). Copper competes with Zn for uptake in plants, often being taken up in excess when Zn is deficient (Bowen, 1969, 1987).

1.1.6.2 Zinc-deficiency in Australian soils

Crop plants grown in Australian soils are particularly prone to micronutrient deficiencies, with millions of hectares of arable land being subject to decreased levels of one or more trace elements (Donald and Prescott, 1975). Zinc-deficient soils (soils with low levels of plant-available Zn) cover a large part of the cereal growing regions of South Australia (Reuter *et al.*, 1988). Western Australia has the single largest area of Zn-deficient soil in the world (8 million

ha; Donald and Prescott, 1975; Welch *et al.*, 1991). The Ninety-mile Desert in South Australia comprises 12 million ha of low-micronutrient soils including Zn (Lindsay, 1972). Zinc-deficient crops have been recorded in Australia since the 1930s (Donald and Prescott, 1975; Teakle, 1942). Many southern and western Australian soils are low in organic matter, have high pH, and low levels of Zn making them prone to Zn-deficiency (Reuter *et al.*, 1988). These areas comprise a wide range of soil types and textures, mainly calcareous and siliceous sands and loams (Takkar and Walker, 1993).

Contaminants of superphosphate fertilisers have previously added sufficient amounts of Zn (about 400 mg Zn kg^{-1} of fertiliser) to soils which prevented severe Zn deficiency occurring (Donald and Prescott, 1975; Riley *et al.*, 1992). Changes in fertiliser formulations and sources have resulted in less Zn (70 mg kg^{-1} of fertiliser) being added to soils through phosphate fertilization, increasing the frequency of Zn-deficient crops in recent years (Reuter *et al.*, 1988; Riley *et al.*, 1992). Zinc is currently supplied to crops on Zn-deficient soils as separate treatments of Zn compounds. The main forms of inorganic Zn when added as fertiliser are as $ZnSO_4$ or ZnO (Mortvedt and Gilkes, 1993). Partially acidulated ZnO, known as zinc oxysulphate ($xZnO.yZnSO_4$) is also used (Mortvedt and Gilkes, 1993). Synthetic chelates (EDTA, HEDTA, NTA, see also section 1.1.8), release cationic micronutrients over a period of time, which increases their effectiveness, but they are more costly to produce (Mortvedt and Gilkes, 1993). Foliar sprays of Zn compounds are able to cure symptoms of Zn deficiency rapidly in fruit trees and other crops (Bergmann, 1992), but this may not always be a sufficient strategy to prevent yield losses, as plants may not exhibit visual Zn deficiency symptoms while still suffering from Zn deficiency (Reuter *et al.*, 1988).

1.1.6.3 Plant tolerance to Zn-deficient soils

Plants vary in their ability to grow in Zn-deficient soils. Cultivars of a species that are able to grow and yield more than average cultivars on Zn-deficient soils are termed "zinc-efficient" (Graham *et al.*, 1992; Graham and Rengel, 1993). Lack of efficiency traits is one factor contributing to Zn deficiency (see section 1.1.6.1).

Cereal plants are less susceptible to Zn deficiency than other species *e.g.* fruit trees (Viets *et al.*, 1954). An early report of Zn deficiency in apple (*Malus domestica*) trees (Mulder, 1950) noted that some cultivars were more susceptible to Zn deficiency than others. Susceptibility to Zn-deficiency varies widely in cereals: rye (*Secale cereale*) was found in the 1930s to grow better than oats (*Avena sativa*) in micronutrient-poor soils in South Australia (Donald and Prescott, 1975). Wheat is the least tolerant species of grain crop to Zn deficiency.

Mechanisms contributing Zn efficiency to different cultivars are not yet understood; more than one process may be operating in a single genotype (Graham, 1984; Rengel and Graham, 1995a, 1996). Potential mechanisms that improve Zn efficiency include increased root growth, changes in root morphology, increased uptake rate of Zn, variable utilization of Zn within cells and contribution by mycorrhizas. Increased rate of root growth would allow roots to occupy a greater volume of soil and absorb more Zn than a slower growing cultivar. Increased root surface area would allow a greater soil volume to be explored, while increased rate of uptake of Zn may be another mechanism by which cultivars vary in their efficiency (Dong *et al.*, 1995a).

One important mechanism of Zn efficiency possessed by graminaceous species is the production of phytosiderophores. Evolved as a response to iron deficiency, phytosiderophores are non-proteinogenic amino acids exuded by roots that chelate Fe(III) and improve iron uptake (known as Strategy II iron-acquisition; Marschner, 1995). Phytosiderophores improve Fe uptake by mobilizing Fe from insoluble complexes in the soil, and diffusing towards the roots where they are taken up as either in complexed form or as dissociated Fe(III) (Römheld and Marschner, 1986). Phytosiderophores also have affinity for other divalent metal ions, and under conditions of Zn-deficiency, can be released in order to mobilise Zn from adsorption sites in soils (von Wirén *et al.*, 1996; Zhang *et al.*, 1989a). Rates of release of phytosiderophores are similar in Fe-deficient and Zn-deficient wheat plants (Marschner and Römheld, 1994). Factors that influence the rate of phytosiderophore release include factors that influence Zn deficiency, including plant age, severity of deficiency, temperature and light intensity

(Römheld and Marschner, 1990; von Wirén *et al.*, 1994). Cultivars of wheat that varied in their Zn efficiency produced different amounts of phytosiderophores when grown in Zn-deficient soil (Cakmak *et al.*, 1996).

1.1.7 Plant uptake of zinc

Zinc is absorbed by roots from solution as the free Zn^{2+} ion (Kochian, 1993). As the pH of soil increases to high levels, the presence of $Zn(OH)^{+}$ increases and may also be absorbed by plants to a small extent (Bergmann, 1992). Zinc is transported to roots primarily by diffusion as the concentration of Zn in soil solution is insufficient to supply requirements via mass flow (Marschner, 1993; Moraghan and Mascagni, 1991). Conditions that decrease diffusion of Zn to plant roots will therefore limit Zn availability.

Zinc can diffuse towards roots in chelated forms (artificial chelates; section 1.1.8, or phytosiderophores; section 1.1.6.3); however, the Zn usually dissociates outside the root and the complexed forms are not taken up across the root cell membrane (Halvorson and Lindsay, 1977). Recent results have indicated that Zn may be taken up as Zn-phytosiderophore complexes (von Wirén *et al.*, 1996), while (Reid *et al.*, 1996) suspected the low Zn activities used in chelate-buffered solutions were insufficient to supply plants with levels of Zn adequate for growth and suggested that chelated Zn may be taken up across the cell membrane.

There is debate as to whether the uptake of Zn is under metabolic control or not (Kochian, 1991, 1993). Active transport requires the expenditure of energy to transport ions against an electrochemical gradient (Ussing, 1949, in Kochian, 1991). Passive transport of ions does not require expenditure of energy, but still requires the generation of a membrane potential. Root cells commonly generate potentials of -120 mV which can theoretically support a 10^4-fold accumulation of a divalent cation (Kochian, 1991). Soil solution concentrations of 0.01 μM Zn^{2+} would provide an internal concentration of 100 μM Zn^{2+}. As internal concentrations of Zn are well below this, it is obvious that Zn can be taken up by passive uptake alone (Clarkson and Lüttge, 1989; Kochian, 1991, 1993).

Zinc uptake may still be dependent on metabolism, as low temperature and metabolic inhibitors can decrease Zn uptake (Bowen, 1969; Giordano *et al.*, 1974; Schmid *et al.*, 1965). Dinitrophenol (DNP) was used to depolarize (uncouple) the membrane, which lowers the electrochemical gradient and decreases the passive flux of cations (Clarkson and Lüttge, 1989), and decreased the rate of Zn uptake in barley (Schmid *et al.*, 1965) and rice (Giordano *et al.*, 1974). Zinc influx did not alter when membrane potentials were clamped, suggesting the mechanism is electroneutral, possibly by exchanging two protons for one Zn^{2+} ion, and regulated by ATP (Reid *et al.*, 1996).

Passive uptake of Zn may be mediated by ion channels and/or pumps (Kochian, 1991). There may be more than one type of channel involved in Zn uptake as there appears to be both high- and low-affinity uptake of Zn depending on the concentration of Zn available in solution (Reid *et al.*, 1996). The radii of the hydrated cations of Cu and Zn are similar (Kochian, 1991), and uptake of Zn was competitively inhibited by Cu, indicating that Zn and Cu are likely to be taken up by the same transporter (Bowen, 1969, 1987; Brar and Sekhon, 1976). Plants can take up Cu in place of Zn when Zn availability is low (*e.g.* Dong *et al.*, 1995b; Rengel and Graham, 1995b).

Studies of Zn uptake mechanisms have to account for cell-wall binding of Zn, as this can constitute a considerable amount of the Zn absorbed from the solution by the roots that has not crossed the cell membrane (Reid *et al.*, 1996). Once this component is removed (by desorption of ions using solutions such as $LaCl_3$ or other suitable solution; Reid *et al.*, 1996), the amount of Zn taken up across the membrane can be calculated.

1.1.8 Solution cultures

Until recently, hydroponic solutions used for micronutrient studies have been subject to problems with variable concentrations of micronutrients over time and contamination (Bell *et al.*, 1991; Yang *et al.*, 1994). Supplying plants with low activities of micronutrients in conventional nutrient solution (*e.g.* 1-10 n*M* $ZnSO_4$), can result in variable supply of Zn to plants over time as the plants

deplete the solution of the very low levels of micronutrients, before the desired concentration is restored by changing the solution. Large-volume flowing solution culture techniques have been used to reduce the variability in nutrient supply with time (*e.g.* Loneragan *et al.*, 1979), but are still subject to contamination problems (Bell *et al.*, 1991).

The recent development of chelate-buffered nutrient solutions (Bell *et al.*, 1991; Chaney, 1988; Norvell, 1991) and the calculation of equilibrium constants has enabled the use of nutrient solutions with known concentrations of free micronutrient ions (Welch and Norvell, 1993). By chelating Zn with artificial chelates such as HEDTA, the free Zn^{2+} activity in the solution is tightly regulated and allows much lower activities of Zn to be achieved (Parker, 1993; Norvell and Welch, 1993; Yang *et al.*, 1994). An excess of chelate buffers the free ions and reduces free activity of micronutrients by several orders of magnitude in comparison to conventional solutions (Norvell and Welch, 1993). One considerable advantage of chelate-buffered solutions is that the activity of an individual ion can be varied independently of the activity of other ions in the solution (Norvell and Welch, 1993). The free Zn^{2+} activity of a 0.1 μ*M* solution of ZnHEDTA buffered with 25 μ*M* K_3HEDTA was calculated to be 2 p*M* (using GEOCHEM: Sposito and Mattigod, 1980) and yielded Zn-deficient wheat plants within 22 d (Rengel and Graham, 1995a). Adequate levels of Zn for growth of barley were provided by 3 μ*M* ZnHEDTA with 50 μ*M* K_3HEDTA excess (Welch and Norvell, 1993).

1.2 Herbicides

1.2.1 Weeds and herbicides

Weeds can be defined as "any plant competing with cultivated plants" (Stephens, 1982) or on a wider scale, as plants that "interfere with human activities and as a result are undesirable" (Ross and Lembi, 1985). The second definition includes crop plants that carry over into different crops in following years. Weeds reduce crop yield through direct competition with crop plants or interference with harvesting, reduce crop quality by contamination with weed

seeds, and increase the costs of transport and handling (Ross and Lembi, 1985). Weed control is an integral requirement of agricultural practice.

Modern weed management aims to incorporate all appropriate methods of weed control. Before the introduction of herbicides in the middle of the 20th century, weed control involved extensive use of crop rotation and cultivation to prevent weed growth during early growth of the crops (Lockhart and Wiseman, 1988). Hand-hoeing is still a major method of weed control in non-industrial countries (Stephens, 1982). Ploughing physically disturbs the soil and disrupts weed growth by covering weeds with soil or damaging their roots (Pratley and Rowell, 1987). However, the physical action of ploughing increases the potential for soil erosion by wind or water and cannot be used after sowing without damaging the crop as well. Excessive cultivation of Australian farm soils to control weed growth has led to erosion and loss of topsoils by wind (Amor and de Jong, 1983). Increasing awareness of soil erosion problems in farmlands has led to the widespread use of minimum-tillage practices and much greater use of herbicides to control weeds.

Modern herbicide use began in the late 19th century with the discovery that copper sulphate-lime mixture (Bordeaux mixture) selectively controlled Charlock (*Sinapsis arvensis*) in cereals (Stephens, 1982). A limited number of inorganic chemicals was developed until the 1940s, when DNOC (4, 6-dinitro-orthocresol) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were developed (Stephens, 1982). Acceptance of herbicides was slowed by the lack of suitable spraying equipment until World War II, when food production efforts were increased. The range of weed control agents has increased since the 1940s, allowing greater selectivity for weeds and lower rates of application of herbicides. These advantages have also reduced the need for excessive soil cultivation when preparing soils for cropping (Pratley and Rowell, 1987). In Australia there are currently about 60 different herbicides available from 20 classes of chemicals.

1.2.1 Sulfonylureas

1.2.1.1 Structure

Sulfonylureas have been used since the 1940s for the treatment of humans suffering from non-insulin-dependent diabetes mellitus (NIDDM; Gerich, 1989). Herbicidal properties of sulfonylureas were first discovered in 1966 (Beyer *et al.*, 1988), however little work was continued until researchers at DuPont in the USA discovered compounds in 1975 with high levels of herbicidal activity (Beyer *et al.*, 1988).

Herbicidal sulfonylureas consist of a sulfonyl bridge with two carbon rings (an aryl ring and a heterocycle ring) and various substituted components on each ring (Fig. 1.1). Attached to the aryl ring is a single chain or group "R". The heterocycle ring contains either 2 or 3 nitrogen atoms and two substituents "X" and "Y". The R, X and Y chains can vary in composition, however, smaller components generally have higher biological activity than larger ones (Beyer *et al.*, 1988).

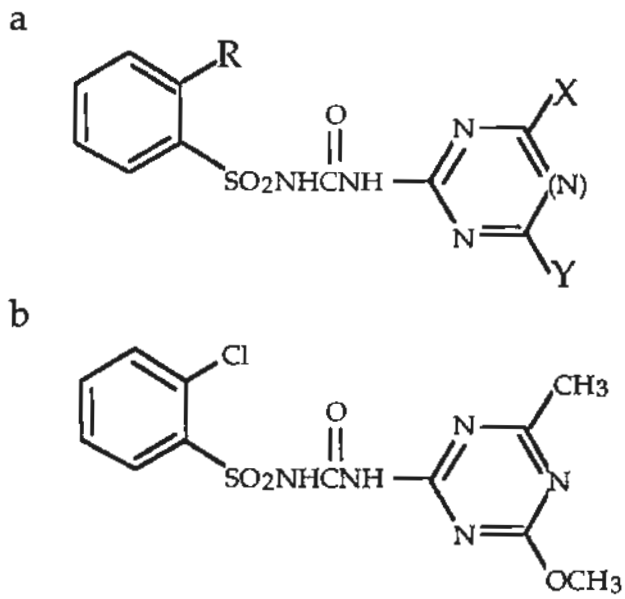

Figure 1.1a. General structure of sulfonylurea herbicides. R, X and Y indicate variable side chains that can be altered for differential activity. (N) indicates variable nitrogen component. Fig. 1.1b Chlorsulfuron structure (molecular weight 357.78).

1.2.1.2 History, development and commercial release of sulfonylureas

The discovery of sulfonylureas was a major advance in herbicide technology as the products were more potent than any previous classes of herbicides and their mode of action (the method and site of biochemical activity) was quite different to that of other herbicides (Brown, 1990). The mode and site of action (section 1.2.1.3) were discovered and explained before widespread development of sulfonylureas occurred (Stidham, 1991). A second class of chemicals with the same site of action (imidazolinones) was discovered independently by researchers at American Cyanamid and has also been developed further (Stidham, 1991).

Development of herbicidal sulfonylureas increased rapidly after their discovery, with 230 patents for compounds in June 1987 (Beyer *et al.*, 1988) and 375 patents in May 1989 with DuPont holding approximately 75 % of these (Brown, 1990). The rapid creation of new chemicals is due to the general structure of the chemicals which allows a wide range of formulations. In 1988, there were six sulfonylurea chemicals sold either as individual chemicals or in mixtures with other chemicals, increasing to at least 20 sulfonylurea herbicides available worldwide in 1996.

Chlorsulfuron (Fig. 1.1b; 2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl)benzenesulfonamide (Beyer *et al.*, 1988; Ray, 1982) was the first sulfonylurea available commercially, released by DuPont in 1982. In Australia, five sulfonylurea herbicides are registered for use; chlorsulfuron (sold as Glean), metsulfuron-methyl (Ally), triasulfuron (Logran) and bensulfuron (Londax), which are available as single chemicals while thifensulfuron is mixed with metsulfuron-methyl (Harmony M) (Parsons, 1992; Chambers, 1995).

1.2.1.3 Mode and site of action of sulfonylureas

The primary herbicidal mode of action of sulfonylureas is inhibition of cell division (Ray, 1982), while the biochemical site of action is inhibition of acetolactate synthase (ALS, E.C. 4.1.3.18, also known as acetohydroxacid synthase, AHAS; Beyer *et al.*, 1988). Sulfonylureas have low oral, dermal and inhalation toxicities to mammals (Beyer *et al.*, 1988), primarily because animals lack the ALS

enzyme, therefore there having no target site for the herbicides to act upon (Brown, 1990).

The mode of action of sulfonylureas was established by Ray (1982) with experiments that showed that chlorsulfuron decreased root growth, probably by inhibiting cell division or cell enlargement. Cell division was significantly reduced by chlorsulfuron (Ray, 1982), but mitosis was not affected directly. Lipid synthesis in soybean cells was inhibited up to 90 % of untreated cells depending on dosage (Hatzios and Howe, 1982). Other processes shown to be affected by chlorsulfuron treatments were protein synthesis, photosynthesis and RNA synthesis, but these were inferred to be secondary effects to the primary inhibition (Hatzios and Howe, 1982).

The metabolic site of action of sulfonylureas was determined in studies with bacteria (LaRossa and Schloss, 1984). Inhibition of *Salmonella typhimurium* growth in the presence of L-valine and sulfometuron-methyl was reversed by addition of isoleucine but not other amino acids. This result suggested that sulfometuron-methyl inhibited acetolactate synthase (LaRossa and Schloss, 1984). ALS is the primary step in the enzyme pathways responsible for production of branched-chain amino acids valine, leucine and isoleucine (LaRossa and Schloss, 1984). ALS synthesises valine and leucine from pyruvate and isoleucine from α-ketobutyrate via a second pathway (Shaner and Singh, 1993). The site of action of sulfonylurea herbicides was confirmed in plants when inhibition of pea (*Pisum sativum*) root growth by 28 n*M* chlorsulfuron was reversed by addition of 100 µ*M* of the amino acids valine, leucine, isoleucine and alanine (Ray, 1984). In addition, purified ALS from pea plants was found to be strongly inhibited by 28 and 84 n*M* chlorsulfuron (Ray, 1984). ALS from several species was inhibited to half-maximal rates by concentrations of chlorsulfuron that ranged from 18 to 36 n*M* (Ray, 1984). When chlorsulfuron was applied to wheat and maize, amino acid levels were increased except for the branched chain amino acids (Royuela *et al.*, 1991). Recycling of amino acids from protein catabolism can increase amino acid levels (Rhodes *et al.*, 1987). The link between inhibition of cell division and

decreased production of the branched chain amino acids by sulfonylureas is unknown (Clayton and Reynolds, 1991).

Accumulation of intermediates in the branched-chain amino acid synthesis chain was suspected as a cause of growth decline (Rhodes *et al.*, 1987), but further studies indicated α-ketobutyrate could be added to roots with no effect on shoot elongation (Shaner and Singh, 1993). However, Landstein *et al.* (1995) suggested that addition of α-ketobutyrate does not accurately represent the consequence of ALS inhibition, as the concentration of another metabolite, ketoisovalerate, is lowered in ALS-inhibited plants (Landstein *et al.*, 1995). Competition between α-ketobutyrate and ketoisovalerate for other enzymes may result in misincorporation in these pathways, which may be toxic (Landstein *et al.*, 1995). Levels of α-ketobutyrate declined 3 h after addition of sulfometuron-methyl, suggesting metabolism of α-ketobutyrate took place (Landstein *et al.*, 1995).

1.2.1.4 Chlorsulfuron applications

Chlorsulfuron is widely used against broadleaf weeds in cereal crops in Australia (Parsons, 1992). However, use of chlorsulfuron has declined recently as newer sulfonylurea herbicides have been released, and problems with weed resistance (Hall *et al.*, 1994) and residual carryover have increased (Ferris *et al.*, 1992).

Common weeds targeted for control by chlorsulfuron in Australia include perennial and annual ryegrasses (*Lolium perenne* and *L. rigidum*), wireweed, deadnettle, shepherd's purse, capeweed, Indian hedge mustard, prickly lettuce, Patterson's curse, docks and mustards (Parsons, 1992). Chlorsulfuron can be applied to soils either before or after emergence of crop seedlings. Application is generally at low concentrations (4-26 g active ingredient (a.i.) ha^{-1} in cereal crops, 13-158 g a.i. ha^{-1} in non-crop situations) (Beyer *et al.*, 1988). In Australia, chlorsulfuron is generally applied at 15-30 g a.i. ha^{-1} without incorporation into soil (Parsons, 1992).

1.2.1.5 Chlorsulfuron in soils and plants

As chlorsulfuron is a weak acid, pH influences the state of chlorsulfuron in the soil where the rate of breakdown is decreased in acid soils. Chlorsulfuron in high pH soils had half-lives of 10-12 weeks while in acid soils had half-lives of 2-3 weeks (Fredrickson and Shea, 1986). As soil temperature increases, the half-life of active chlorsulfuron is reduced *e.g.* from 33 weeks at 10°C to 9 weeks at 40°C (Zimdahl *et al.*, 1978 in Beyer *et al.*, 1988). Soil moisture also influences degradation, with wetter soils degrading chlorsulfuron faster than drier soils (Walker and Brown, 1983) in (Beyer *et al.*, 1988). Wet conditions increased the severity of chlorsulfuron damage in barley (Lemerle, 1993).

The selectivity of chlorsulfuron relies on differential susceptibility of species. Tolerant species are inhibited by concentrations of chlorsulfuron up to 1000 times greater than susceptible species (*e.g.* Royuela *et al.*, 1991). Tolerant plants metabolise chlorsulfuron to inactive forms faster than susceptible plants (Sweetser *et al.*, 1982). Tolerance to herbicides can depend on the rate of uptake or absorption of the herbicide, translocation within the plant, or the rate of metabolism to inactive forms (Bowran and Blacklow, 1987; Dastgheib *et al.*, 1994; Lemerle *et al.*, 1986). Most of the variation in tolerance to chlorsulfuron of Australian wheats was due to differences in the rate of chlorsulfuron metabolism rather than different absorption rates (Dastgheib *et al.*, 1994).

Tolerance to chlorsulfuron varies with crop species and cultivar. Wheat is the most tolerant species of crop plant that chlorsulfuron is used on (Lemerle *et al.*, 1986; Beyer *et al.*, 1988). Barley was less tolerant to chlorsulfuron than wheat when grown in solution culture (Foley, 1985). Barley root FW was reduced by chlorsulfuron after 1- to 3-d of exposure while wheat appeared unaffected (Foley, 1985). Shoot DW of some cultivars of wheat was reduced after spraying with recommended rates of chlorsulfuron but had recovered by 62 d after spraying (Dastgheib *et al.*, 1994). Root DW of barley and wheat was affected more than shoot DW in each crop, especially when chlorsulfuron was applied to the soil (Lemerle and Cousens, 1993). With increasing use of herbicides, especially in minimum-tillage farming practices, the incidence of crop plants sprayed with

herbicides suffering decreased root growth resulting in reduced ability to absorb nutrients will increase.

1.2.2 Herbicide-micronutrient interactions

Herbicides are generally assumed to improve crop yield by decreasing competition between crop plants and weeds. Damage to crop plants can occur if the herbicide is used inappropriately, causing yield losses that may be greater than if the herbicide was not used. Inhibition of root growth by herbicides will limit the ability of plants to absorb water and nutrients; however, the effects of herbicide usage on crop plant nutrient uptake may be more complicated than simply reducing nutrient absorption (Hance, 1981). Herbicides are used in conjunction with reduced-tillage practices, which disturb the soil less than conventional ploughing. In some cases, changes in fertiliser applications are required to maintain yields. Changes in weed populations (that compete for nutrients) may also contribute to changes in nutrient uptake by crop plants. Herbicides may change soil chemistry, reduce root growth or directly interfere with nutrient uptake by root cells, all of which will decrease net nutrient uptake of plants (Hance, 1981).

Few reports of decreased micronutrient uptake in cereals due to herbicide application exist. Hance (1981) reviewed pesticide and nutrient interaction studies, but most cases were concerned with the macronutrients N, P, K and S. Fertilizers have reduced the negative impacts of herbicides on crop nutrition *e.g.* N and P changes in crops when sprayed with 2,4-D and dicamba were lessened by fertilizing (Gruzdev *et al.*, 1976, in Hance, 1981). In other cases, nutrient uptake was reduced by herbicides *e.g.* K uptake by wheat in solution culture was inhibited by 2,4-D when pH was below 4 (Zsoldos *et al.*, 1978). Leaf P, K and Mn of maize in solution culture was decreased by 2,4-D and atrazine while leaf N was increased (Gill and Burley, 1970). Potential for reduced micronutrient uptake in field crops due to herbicide use exists in USA, but has not been reported (R. Chaney, USDA-ARS, pers. comm.).

1.2.3 Zinc and sulfonylureas

The first report of a sulfonylurea herbicide affecting plant nutrient concentration was by Bowran *et al.* (1987), who noted that chlorsulfuron (applied as Glean herbicide) and another herbicide, diclofop-methyl (an aryloxyphenoxypropionate herbicide), reduced plant height, induced leaf chlorosis, and lowered Zn and Cu concentrations in three cultivars of wheat. Grain yield was significantly reduced (20-25 % of untreated controls) by diclofop-methyl, but not significantly influenced by chlorsulfuron, being reduced by approximately 8.5 % (Bowran *et al.*, 1987). Zinc concentrations in shoots of chlorsulfuron-treated wheat were reduced to below adequate levels, while shoot concentrations of Zn in untreated plants remained adequate.

When Glean herbicide and pure chlorsulfuron were applied at the same rate (14 µg chlorsulfuron/3 kg soil), both treatments significantly reduced shoot DW, root DW, root length and Zn concentration in comparison to untreated control plants, and there was no significant difference in the responses to Glean and chlorsulfuron (McLay and Robson, 1992). It was concluded that the effects were due to the chlorsulfuron rather than the additives in Glean (McLay and Robson, 1992). These effects were greater at low soil Zn levels than at high Zn levels (McLay and Robson, 1992; Osborne and Robson, 1992).

The response of wheat to chlorsulfuron was examined in glasshouse experiments by Robson and Snowball (1990). The severity of copper deficiency in plants grown with low levels of Cu in the soil was increased when plants were treated with chlorsulfuron. Copper deficiency in plants occurred when chlorsulfuron was incorporated in the soil, but did not occur when sprayed onto leaves only. Root weight was reduced by chlorsulfuron at all levels of Cu supply except the highest. Copper concentration in shoots was reduced by all concentrations of chlorsulfuron at low soil Cu treatments and by the highest chlorsulfuron concentrations at high soil Cu treatment (Robson and Snowball, 1990).

When Zn and chlorsulfuron were added to soil in a split-root experiment, severe Zn-deficiency symptoms occurred only when chlorsulfuron and Zn were

applied in the same compartment (Robson and Snowball, 1990). Shoot and root dry weight and tillering were decreased when Zn was incorporated into the soil together with chlorsulfuron. Reduced micronutrient uptake appeared unrelated to reduction of root or shoot weight (Robson and Snowball, 1990). This finding suggested that Zn uptake reductions was not a simple consequence of reduced root growth. The most severe incidences of Zn deficiency occurred when Glean was incorporated into soil rather than sprayed onto leaves (Robson and Snowball, 1990).

Experiments with varying levels of other nutrients showed that chlorsulfuron reduced phosphorus concentrations in wheat leaves and induced P deficiency at all rates of P supply (Osborne *et al.*, 1993). Root DW and length were decreased by chlorsulfuron, especially at low P supply. Decreased P contents in shoots occurred at low chlorsulfuron levels, even though root length was not reduced, indicating that P uptake also was not limited by root mass reductions. Lower N content of shoots was due to decreased growth following chlorsulfuron application rather than decreased uptake (Osborne *et al.*, 1993).

In solution culture, Zn concentrations of shoots and youngest emerged leaf blades of wheat were not affected by chlorsulfuron (McLay and Robson, 1992). Root and shoot weights were decreased by chlorsulfuron to a greater extent in low Zn concentrations than in high Zn concentrations. Chlorsulfuron reduced root length at all Zn levels in solution (McLay and Robson, 1992). The lack of effect of chlorsulfuron on shoot Zn concentration in solution-cultured plants was attributed to the circulation of solution allowing the roots to access Zn, while in soils Zn uptake is limited by the rate of diffusion to roots (McLay and Robson, 1992). Chlorsulfuron may have inhibited root growth and therefore decreased the ability of plants growing in soil to absorb Zn.

Reduction of wheat root and shoot growth due to chlorsulfuron was evident from 2 to 6 weeks after sowing but plants began to show signs of recovery by 8 weeks (Osborne and Robson, 1992). Plants in high-Zn soil showed no symptoms of Zn deficiency at any stage, while plants in low-Zn soil developed Zn deficiency symptoms after 3 weeks. Shoot Zn concentration was decreased by chlorsulfuron

at low and high Zn supply, compared to plants grown at high Zn supply without chlorsulfuron. Chlorsulfuron reduced tillering, root and shoot weight in low-Zn conditions only (Osborne and Robson, 1992). The deleterious effects of chlorsulfuron on root growth and Zn uptake occurred simultaneously therefore Osborne and Robson (1992) were unable to determine if chlorsulfuron reduced root growth and subsequently limited Zn uptake, or whether effects were independent of each other.

In an experiment conducted using Zn-deficient soil from the south-east of South Australia, chlorsulfuron reduced shoot DW in three cultivars of wheat at 3 and 5 wks after sowing, although there was a greater effect at high Zn supply (Dong *et al.*, 1995b). Plants grown with low Zn supply showed little effect of chlorsulfuron on shoot DW as growth was already severely inhibited by Zn deficiency. Root diameter was increased by chlorsulfuron treatment because the proportion of fine roots (diameter $\leq$ 0.2 mm) was significantly reduced. Total root length was also reduced by chlorsulfuron at each Zn level and differed between genotypes (Dong *et al.*, 1995b). Zinc concentration was reduced by both chlorsulfuron and low Zn treatment similarly to the effect on shoot DW.

As chlorsulfuron persists for long periods in alkaline soils (Fredrickson and Shea, 1986), and Zn deficiency is common in alkaline soils (Lindsay, 1972), the interaction between chlorsulfuron and Zn-deficiency is more likely to occur in these soils. Knowledge regarding the mechanisms of decreased Zn uptake due to chlorsulfuron treatment would assist in preventing further occurrences by avoiding those conditions.

Root FW reductions due to chlorsulfuron have not been reported, apart from Robson and Snowball (1990) who found that wheat grown in Cu- and Zn-deficient soils in split root experiments had reduced FW of tops when chlorsulfuron was combined with the deficient element. The water usage of compartments with chlorsulfuron and Cu together was less than when chlorsulfuron and the elements were in separate compartments.

1.3 Objectives of this study

The experiments conducted by Robson and coworkers described the effects of applying chlorsulfuron to wheat grown in low Zn and Cu soils on yield and nutrient concentration. The mechanisms by which chlorsulfuron decreases uptake of Zn and other diffusion-limited nutrients were not determined fully in these experiments. Explanations for the decreased uptake were a decrease in root surface area by altering root shape (decreasing root length and increasing root thickness; Osborne and Robson, 1993) or a possible decrease of water flow to roots (Robson and Snowball, 1990). Altered cell membrane electrical potentials were also suggested as a cause of decreased ability to take up Zn (Osborne and Robson, 1993).

This study was carried out in order to describe the effects of chlorsulfuron application to Zn-deficient wheat plants differing in Zn efficiency, and to determine the causes of these effects. A range of experiments was performed to examine the following:

- The effect of chlorsulfuron application on Zn concentrations of roots and shoots of wheat grown in chelate-buffered nutrient solution with low Zn concentrations.
- Water relations of low Zn concentration plants when treated with chlorsulfuron.
- Root tip extension of wheat when supplied with chlorsulfuron.
- The rate of Zn uptake of wheat cultivars varying in Zn efficiency when supplied with chlorsulfuron in differing combinations.
- Protein content (and composition) of wheat plants when grown in low Zn concentrations and treated with chlorsulfuron.
- Superoxide radical production in wheat roots when grown in various concentrations of Zn and chlorsulfuron.
- Respiration rates of wheat plants supplied with chlorsulfuron when grown in low and high Zn concentrations.

By examining these factors, it should be possible to determine whether chlorsulfuron is inducing Zn deficiencies in wheat by direct means (interfering

with Zn uptake from solution so that plants are prevented from absorbing Zn) or indirect means (by decreasing growth or metabolism so that the requirement for Zn uptake is decreased), or altering Zn use within the plants.

1.4 References

Amor, R. L. and DeJong, R. W. (1983) Changing weed problems in cereal cropping in Victoria since 1920. *The Journal of the Australian Institute of Agricultural Science* 49, 139-149.

Aubert, H., and Pinta, M. (1977). Trace elements in soils. Elsevier, Amsterdam, Netherlands.

Bannister, J. V., Bannister, W. H. and Rotilio, G. (1987). Aspects of the structure, function and applications of superoxide dismutase. *CRC Critical Reviews in Biochemistry* 22, 111-180.

.Barak, P. and Helmke, P. A. (1993). The chemistry of zinc. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 1-14.

Barrow, N. J. (1993). Mechanisms of reaction of zinc with soil and soil components. In Robson, A. D. Ed. *Zinc in Soils and Plants*. Kluwer Academic Publishers.

Bell, P. F., Chaney, R. L. and Angle, J. S. (1991). Free metal activity and total metal concentrations as indices of micronutrient availability to barley [*Hordeum vulgare* (L.) 'Klages']. *Plant and Soil.* 130, 51-62.

Bergmann, W. (1992). Nutritional disorders of plants: Development, visual and analytical diagnosis. Gustav Fischer Verlag Jena. Stuttgart, Germany.

Bettger, W. J. and O'Dell, B. L. (1981). A critical physiological role of Zn in the structure and function of biomembranes. *Life Sciences* 28, 1425-1438.

Beyer, E. M., Duffy, M. J., Hay, J. V. and Schlueter, D. D. (1988). Sulfonylureas. In Kearney, P. C., Kaufman, D. D. Eds. *Herbicides: Chemistry, degradation and mode of action.* Marcel Dekker. pp. 117-189.

Boveris, A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods in Enzymology* 105, 429-435.

Bowen, J. E. (1969). Absorption of Cu, Zn and Mn by sugarcane leaf tissue. *Plant Physiology* 44, 253-261.

Bowen, J. E. (1987). Physiology of genotypic differences in zinc and copper uptake in rice and tomato. *Plant and Soil* 99, 115-125.

Bowran, D. G. and Blacklow, W. M. (1987). Sensitivities of spring wheat cultivars to chlorsulfuron measured as inhibitions of leaf elongation rates and there were genotype × environment interactions. *Australian Journal of Agricultural Research* 38, 253-262.

Bowran, D. G., Porritt, S. and Madin, R. (1987). Chlorsulfuron and diclofop-methyl effects on the copper and zinc status and yield of wheat. In Lemerle, D., Leys, A. R. Eds. *Proceedings of the Eighth Australian Weeds Conference, 1987.* pp. 404-407.

Bracey, M. H., Christiansen, J., Tovar, P., Cramer, S. P. and Bartlett, S. G. (1994). Spinach carbonic anhydrase - investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis, and EXAFS. *Biochemistry* 33, 13126-13131.

Brar, M. S. and Sekhon, G. S. (1976). Interaction of zinc with other micronutrient cations: I. Effect of copper on zinc^{65} absorption by wheat seedlings and its translocation within the plants. *Plant and Soil* 45, 137-143.

Brennan, R. F., Armour, J. D. and Reuter, D. J. (1993). Diagnosis of zinc deficiency. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 167-181.

Brown, H. M. (1990). Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. *Pesticide Science* 29, 263-281.

Brown, P. H., Cakmak, I. and Zhang, Q. (1993). Form and function of zinc in plants. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 93-106.

Cakmak, I. and Marschner, H. (1988a). Enhanced superoxide radical production in roots of zinc-deficient plants. *Journal of Experimental Botany* 39, 1449-1460.

Cakmak, I. and Marschner, H. (1988b). Increase in membrane permeability and exudation in roots of Zn deficient plants. *Journal of Plant Physiology* 132, 356-361.

Cakmak, I. and Marschner, H. (1993). Effect of zinc nutritional status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves. *Plant and Soil* 156, 127-130.

Cakmak, I., Marschner, H. and Bangerth, F. (1989). Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolaris vulgaris* L.). *Journal of Experimental Botany* 40, 405-412.

Cakmak, I., Sari, N., Marschner, H., Kalayci, M., Yilmaz, A., Eker, S. and Gulut, K. Y. (1996). Dry matter production and distribution of zinc in bread and durum wheat genotypes differing in zinc efficiency. *Plant and Soil* 180, 173-181.

Carloni, P., Blochl, P. E. and Parrinello, M. (1995). Electronic structure of the Cu, Zn superoxide dismutase active site and its interactions with the substrate. *Journal of Physical Chemistry* 99, 1338-1348.

Carroll, M. D. and Loneragan, J. F. (1968). Response of plant species to concentrations of zinc in solution. I. Growth and zinc content of plants. *Australian Journal of Agricultural Research* 19, 859-868.

Chambers, A. (1995). Field Crop Herbicide Information, 4th edn. Victorian Department of Agriculture, Rutherglen Research Institute, Victoria.

Chaney, R. (1988). Metal speciation and interactions among trace elements affect trace element transfer in agricultural and environmental food chains. In Kramer, J. R. and Allen, H. E. Eds. *Metal speciation: Theory, analysis and application.* Lewis Publisher, Inc. pp. 219-260.

Chvapil, M. (1973). New aspects in the biological role of zinc: A stabilizer of macromolecules and biological membranes. *Life Sciences* 13, 1041-1049.

Clarkson, D. T. and Lüttge, U. (1989). Divalent cations, transport and compartmentation. *Progress in Botany* 51, 93-112.

Clayton, D. S. and Reynolds, T. L. (1991). Chlorsulfuron effects on protein synthesis and accumulation in cultured root tips of *Pisum sativum* L. *Journal of Plant Physiology* 137, 337-341.

Dastgheib, F., Field, R. J. and Namjou, S. (1994). The mechanism of differential response of wheat cultivars to chlorsulfuron. *Weed Research* 34, 299-308.

Donald, C. M. and Prescott, J. A. (1975). Trace elements in Australian crop and pasture production, 1924-1974. In Nicholas, D. J. D. and Egan, A. R. Eds. *Trace Elements in Soil-Plant-Animal Systems.* Academic Press. pp. 7-37.

Dong, B., Rengel, Z. and Graham, R. D. (1995a). Root morphology of wheat genotypes differing in zinc efficiency. *Journal of Plant Nutrition* 18, 2761-2773.

Dong, B., Rengel, Z. and Graham, R. D. (1995b). Effects of herbicide chlorsulfuron on growth and nutrient uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant and Soil* 173, 275-282.

Duguma, B., Kang, B. T. and Okeli, D. U. U. (1988). Effect of liming and phosphorus application on performance of *Leucaena leucoce-phala* in acid soils. *Plant and Soil* 110, 57-61.

Dwivedi, R. S. and Takkar, P. N. (1974). Ribonuclease activity as an index of hidden hunger of zinc in crops. *Plant and Soil* 40, 445-451.

Falchuk, K. H., Ulpino, L., Mazus, B. and Vallee, B. L. (1976). *Euglena gracilis* DNA dependent RNA polymerase II: A zinc metalloenzyme. *Biochemistry* 15, 4468-4475.

Falchuk, K. H., Ulpino, L., Mazus, B. and Vallee, B. L. (1977). RNA polymerase I: A zinc metalloenzyme. *Biochemical and Biophysical Reseacrh Communications* 74, 1206-1212.

Ferris, I. G., Pederson, R. N., Schwinghamer, M. W., and Haigh, B. M. (1992). Sulfonylurea herbicides in cereal farming systems - Detection, persistence and impact. In *Proceedings of national workshop on subsoil constraints to root growth and high soil water and nutrient use by plants.* Vol. I, Tanunda, S.A.

Foley, M. E. (1985). Response differences of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) to chlorsulfuron. *Weed Science* 34, 17-21.

Fredrickson, D. R. and Shea, P. J. (1986). Effect of soil pH on degradation, movement, and plant uptake of chlorsulfuron. *Weed Science* 34, 328-332.

Fridovich, I. (1986). Biological effects of the superoxide radical. *Archives of Biochemistry and Biophysics* 247, 1-11.

Gerich, J. E. (1989). Oral hypoglycemic agents. *The New England Journal of Medicine* 321, 1231-1245.

Gill, H. S. and Burley, J. W. A. (1970). Effect of atrazine, amitrole, 2,4-D and eptam on the early growth and mineral absorption by maize. *Indian Journal of Agricultural Science* 40, 587-.

Giordano, P. M., Noggle, J. C. and Mortvedt, J. J. (1974). Zinc uptake by rice, as affected by metabolic inhibitors and competing cations. *Plant and Soil* 41, 637-646.

Graham, R. D. (1984). Breeding for nutritional characteristics in cereals. *Advances in Plant Nutrition* 1, 57-102.

Graham, R. D., Ascher, J. S. and Hynes, S. C. (1992). Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant and Soil* 146, 241-250.

Graham, R. D. and Rengel, Z. (1993). Genotypic variation in zinc uptake and utilization by plants. In Robson, A. D. Ed. *Zinc in soils and plants*. Kluwer Academic Publishers. pp. 107-118.

Hall, L. M., Tardif, F. J. and Powles, S. B. (1994). Mechanisms of cross and multiple herbicide resistance in *Alopecurus myosuroides* and *Lolium rigidum*. *Phytoprotection* 75, 17-23.

Halvorson, A. D. and Lindsay, W. L. (1977). The critical Zn^{2+} concentration for corn and the nonabsorption of chelated zinc. *Soil science Society of America Journal* 41, 531-534

Hance, R. J. (1981). Effect of pesticides on plant nutrition. *Residue Reviews* 78, 13-41.

Hatzios, K. K. and Howe, C. M. (1982). Influence of the herbicides hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pesticide Biochemistry and Physiology* 17, 207-214.

Hemantaranjan, A. and Gar. O. K. (1984). Effect of zinc fertilization on the senescence of wheat varieties. *Indian Journal of Plant Physiology* 27, 239-246.

Hernandez, J. A., Almansa, M. S., Delrio, L. and Sevilla, F. (1993). Effect of salinity on metalloenzymes of oxygen metabolism in two leguminous plants. *Journal of Plant Nutrition* 16, 2539-2554.

Jeffery, E. H. (1983). The effect of zinc on NADPH oxidation and mono-oxygenase activity in rat hepatic microsomes. *Molecular Pharmacology* 23, 467-471.

Jones, J. B. (1991). Plant tissue analysis. In Mortvedt, J. J., Cox, F. R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in agriculture. 2nd Edn.* Soil Science Society of America. pp. 477-522.

Kabata-Pendias, A., and Pendias, H. (1992). Trace elements in soils and plants. 2nd Edition. CRC Press, Boca Raton, USA.

Kochian, L. V. (1991). Mechanisms of micronutrient uptake and translocation in plants. In Mortvedt, J. J., Cox, F. R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition*. Soil Science Society of America. pp. 229-296.

Kochian, L. V. (1993). Zinc absorption from hydroponic solutions by plant roots. In Robson, A. D. Ed. *Zinc in soils and plants*. Kluwer Academic Publishers. pp. 45-58.

Landstein, D., Epelbaum, S., Arad, S., Barak, Z. and Chipman, D. M. (1995). Metabolic response of *Chlorella emersonii* to the herbicide sulfometuron methyl. *Planta* 197, 219-224.

LaRossa, R. A. and Schloss, J. V. (1984). The herbicide sulfometuron methyl is bacteriostatic due to inhibition of acetolactate synthase. *Journal of Biological Chemistry* 259, 8753-8757.

Law, D. M. (1987). Gibberellin enhanced indole-3-acetic acid biosynthesis: D-tryptophan as the precursor of indole-3-acetic acid. *Physiologia Plantarum* 70, 626-632.

Lee, C. R. and Craddock, G. R. (1969). Factors affecting plant growth in high-zinc medium. II. Influence of soil treatments on growth of soybeans on strongly acid soil containing zinc from peach sprays. *Agronomy Journal* 61, 565-567.

Lemerle, D. (1993). Influence of rainfall and temperature on sensitivity of barley (*Hordeum vulgare*) to chlorsulfuron. *Australian Journal of Agricultural Research* 44, 23-32.

Lemerle, D. and Cousens, R. D. (1993). Responses of spring barley (*Hordeum vulgare* L.) and spring wheat (*Triticum aestivum* L.) to foliar or root entry of chlorsulfuron. *Weed Research* 33, 335-344.

Lemerle, D., Kidd, C. R. and Read, B. R. (1986). Tolerances of barley cultivars to post-emergence herbicides. *Australian Journal of Experimental Agriculture* 26, 383-392.

Lindsay, W. L. (1972). Zinc in soils and plant nutrition. *Advances in Agronomy* 24, 147-186.

Lockhart, J. A. R. and Wiseman, A. J. L. (1988). Introduction to Crop Husbandry: Including Grasslands. 6th Edn. Pergamon, Oxford, England.

Loneragan, J. F., Grove, T. S., Robson, A. D. and Snowball, K. (1979). Phosphorus toxicity as a factor in zinc-phosphorus interactions in plants. *Soil Science Society of America Journal* 43, 966-972.

Longnecker, N. E. and Robson, A. D. (1993). Distribution and transport of zinc in plants. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 79-92.

Marschner, H. (1993). Zinc uptake from soils. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 59-78.

Marschner, H. (1995). Mineral Nutrition of Higher Plants, 2nd Edn. Academic Press, London.

Marschner, H. and Römheld, V. (1994). Strategies of plants for acquisition of iron. *Plant and Soil* 165, 261-274.

McLay, L. D. and Robson, A. D. (1992). The effect of chlorsulfuron and diclofop-methyl on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 43, 59-65.

Miller, J., McLachlan, A. D. and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus laevis* oocytes. *European Molecular Biology Organization Journal* 4, 1609-1614.

Moore, P. A. and Patrick, W. H. (1988). Effect of zinc deficiency on alcohol dehydrogenase activity and nutrient uptake in rice. *Agronomy Journal* 80, 882-885.

Moraghan, J. T. and Mascagni, H. J. (1991). Environmental and soil factors affecting micronutrient deficiencies and toxicities. In Mortvedt, J. J., Cox, F.

R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in Agriculture, 2nd Edn.* Soil Science Society of America. pp. 371-425.

Mortvedt, J. J. and Gilkes, R. J. (1993). Zinc fertilizers. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 33-44.

Mulder, D. (1950). Zinc deficiency of fruit trees in Europe. In *Symposium organized by the International Union of Biological Sciences.* Chronica Botanica Company.

Nable, R. O. and Webb M. J. (1993). Further evidence that zinc is required throughout the root zone for optimal plant growth and development. *Plant and Soil* 150, 247-253.

Nambiar, E. K. S. (1976). Uptake of Zn^{65} from dry soil by plants. *Plant and Soil* 44, 267-271.

Norvell, W. A. (1991). Reactions of metal chelates in soils and nutrient solutions. In Mortvedt, J. J., Cox, F. R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition.* Soil Science Society of America. pp. 187-228.

Norvell, W. A. and Welch, R. M. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: I. Zinc ion requirements. *Plant Physiology* 101, 619-625.

Ohki, K. (1978). Zinc concentration in soybean as related to growth, photosynthesis and carbonic anhydrase activity. *Crop Science* 18, 79-82.

Olsen, S. R. (1972). Micronutrient interactions. In Mortvedt, J. J., Giordano, P. M. and Lindsay, W. L. Eds. *Micronutrients in agriculture.* Soil Science Society of America. pp. 243-264.

Osborne, L. D. and Robson, A. D. (1992). Duration of zinc uptake inhibition by chlorsulfuron in wheat. *Australian Journal of Agricultural Research* 43, 1169-1174.

Osborne, L. D. and Robson, A. D. (1993). What is the significance of herbicide nutrient interactions on wheat? *Plant and Soil* 156, 529-532.

Osborne, L. D., Robson, A. D. and Bowran, D. G. (1993). The impact of chlorsulfuron and diclofop-methyl on nutrient uptake by wheat. *Australian Journal of Agricultural Research* 44, 1757-1766.

O'Sullivan, M. (1970). Aldolase activity in plants as an indicator of zinc deficiency. *Journal of Scientific Field Agriculture* 21, 607-609.

Paribok, T. A. (1973). The effect of varying the zinc supply on tomato respiration. *Fiziologiya Rastenii* 20, 158-164.

Parker, D. R. (1993). Novel nutrient solutions for zinc nutrition research: buffering free $zinc^{2+}$ with synthetic chelators and P with hydroxyapatite. *Plant and Soil* 155/156, 461-464.

Parsons, J. M. (1992). Australian weed control handbook, 9th edn. Inkata Press, Sydney.

Pinton, R., Cakmak, I. and Marschner, H. (1994). Zinc deficiency enhanced NAD(P)H dependent superoxide radical production in plasma membrane vesicles isolated from roots of bean plants. *Journal of Experimental Botany* 45, 45-50.

Pratley, J. E. and Rowell, D. L. (1987). From the first fleet - Evolution of Australian farming systems. In Cornish, P. S. and Pratley, J. E. Eds. *Tillage: New directions in Australian agriculture.* Inkata Press. pp. 2-10.

Purvis, A. C. and Shewfelt, R. L. (1993). Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* 88, 712-718.

Raulin, M. J. (1869). Etudes chimiques sur la vègètation. *Annals Sciences Naturale: Botanique, Biologique et Vègètale (Ser. 5).* 11, 93-299.

Ray, T. B. (1982). The mode of action for chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 10-17.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Reid, R. J., Brookes, J. D., Tester, M. A. and Smith, F. A. (1996). The mechanism of zinc uptake in plants - characterisation of the low-affinity system. *Planta* 198, 39-45.

Rengel, Z. (1995a). Carbonic anhydrase activity in leaves of wheat genotypes differing in Zn efficiency. *Journal of Plant Physiology* 147, 251-256.

Rengel, Z. (1995b). Sulfhydryl groups in root-cell plasma membranes of wheat genotypes differing in Zn efficiency. *Physiologia Plantarum* 95, 604-612.

Rengel, Z. and Graham, R. D. (1995a). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. I. Growth *Plant and Soil* 176, 307-316.

Rengel, Z. and Graham, R. D. (1995b). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake *Plant and Soil* 176, 317-324.

Rengel, Z. and Graham, R. D. (1996). Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. *Journal of Experimental Botany* 47, 217-226.

Reuter, D. J., Cartwright, B., Judson, G. J., McFarlane, J. D., Maschmedt, D. J. and Robinson, J. B. (1988). Trace elements in South Australian Agriculture. *Department of Agriculture South Australia Technical Report* No. 139,

Reuter, D. J., and Robinson, J. B. (1986). Plant analysis: An interpretation manual. Inkata Press, Melbourne.

Rhodes, D., Hogan, A. L., Deal, L., Jamieson, G. C. and Haworth, P. (1987). Amino acid meatbolism of *Lemna minor* L: Responses to chlorsulfuron. *Plant Physiology* 84, 775-780.

Rhodes, D. and Klug, A. (1993). Zinc fingers. *Scientific American* 2, 56-65.

Riley, M. M., Gartrell, J. W., Brennan, R. F., Hamblin, J. and Coates, P. (1992). Zinc deficiency in wheat and lupins in Western Australia is affected by the source of phosphatic fertilizers. *Australian Journal of Experimental Agriculture* 32, 455-463.

Robson, A. D. and Pitman, M.G. (1983). Interactions between nutrients in higher plants. In Lauchli, A. and Bieleski, R. L. Eds. *Encyclopedia of Plant Physiology. New-Series.* Volume 15A. *Inorganic Plant Nutrition.* pp 147-180. Springer-Verlag. Berlin.

Robson, A. D. and Snowball, K. (1990). The effect of chlorsulfuron on the uptake and utilization of copper and zinc in wheat. *Australian Journal of Agricultural Research* 41, 19-28.

Römheld, V. and Marschner, H. (1986). Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. *Plant Physiology* 80, 175-180.

Römheld, V. and Marschner, H. (1991). Function of micronutrients in plants. In Mortvedt, J. J., Cox, F. R., Shuman, L. M., Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition.* Soil Science Society of America. pp. 297-328.

Römheld, V. and Marschner, H. (1990). Genotypical differences among graminaceous species in release of phytosiderophores and uptake of iron-phytosiderophores. *Plant and Soil* 123, 147-153.

Ross, M. A., and Lembi, C. A. (1985). Applied Weed Science. Macmillan Publishing, New York, NY.

Rowlett, R. S., Chance, M. R., Wirt, M. D., Sidelinger, D. E., Royal, J. R., Woodroffe, M., Wang, Y. F. A., Saha, R. P. and Lam, M. G. (1994). Kinetic and structural characterization of spinach carbonic anhydrase. *Biochemistry* 33, 13967-13976.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Sarkar, B. (1995). Metal replacement in DNA-binding zinc finger proteins and its relevance to mutagenicity and carcinogenicity through free radical generation. *Nutrition* 11, 646-649.

Satsukevich, V. B. and Vol'-shchuk, O. V. (1976). Physiological and biochemical indices of barley given additional application of trace elements. *Vestsi Akademii Navuk BSSR, Biyalagichnykh Navuk* 1, 31-36.

Schmid, W. E., Haag, H. P. and Epstein, E. (1965). Absorption of zinc by excised barley roots. *Physiologia Plantarum* 18, 860-869.

Shaner, D. L. and Singh, B. K. (1993). Phytotoxicity of acetohydroxyacid synthase inhibitors is not due to accumulation of 2-ketobutyrate and/or 2-aminobutyrate. *Plant Physiology* 103, 1221-1226.

Shkolnik, M. Y. (1984). Trace elements in plants. Elsevier, Amsterdam.

Sillanpää, M. (1982). Micronutrients and the nutrient status of soils: A global study. Food and Agriculture Organization of the United Nations, Rome.

Skoog, F. (1940). Relationship between zinc and auxin in the growth of higher plants. *American Journal of Botany* 27, 939-950.

Smirnoff, N. (1993). Tansley Review No. 52: The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* 125, 27-58.

Snowball, K., and Robson, A. D. (1988). Symptoms of Nutrient Deficiencies. University of Western Australia Press, Nedlands, Western Australia.

Sommer, A. L. and Lipman, C. B. (1926). Evidence on the indispensible nature of zinc and boron for higher green plants. *Plant Physiology* 1, 231-249.

Stephens, R. J. (1982). Theory and Practice of Weed Control. Macmillan Press, London, Great Britain.

Stidham, M. A. (1991). Herbicides that inhibit acetohydroxyacid synthase. *Weed Science* 39, 428-434.

Suge, H. Takahashi, H., Arita, S. and Takaki, H. (1986). Gibberellin relationships in zinc-deficienct plants. *Plant Cell Physiology* 27, 1010-1012.

Sweetser, P. B., Schow, G. S. and Hutchison, J. M. (1982). Metabolism of chlorsulfuron by plants: Biological basis for selectivity of a new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 18-23

Takkar, P. N. and Walker, C. D. (1993). Diagnosis of zinc deficiency. In A. D. Robson Eds. *Zinc in Soils and Plants.* Kluwer Academic Publishers. pp. 151-156.

Teakle, L. J. H. (1942). Experiments with micro-elements for the growth of crops in Western Australia. VI. Further results from the use of copper-containing fertilisers in the wheatbelt. *Journal of the Department of Agriculture of Western Australia, 2nd Series* 19, 242-253.

Thompson, J. E., Legge, R. L. and Barber, R. F. (1987). The role of free radicals in senescence and wounding. *New Phytologist* 105, 317-344.

Todd, W. R., Elvehjem, C. A. and Hart, E. B. (1934). Zinc in the nutrtion of the rat. *American Journal of Physiology* 107, 146-156.

Tsui, C. (1948). The role of zinc in auxin synthesis in tomato plant. *American Journal of Botany* 35, 172-179.

Uritani, I. (1975). Physiology of copper and zinc. In Okajima, H., Uritani, I., Kun-Huang, H. Eds. *The significance of minor elements on plant physiology.* Food and Fertilizer Technology Centre. pp. 30-60.

Vallee, B. L. and Auld, D. S. (1990). Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29, 5648-5659.

Vallee, B. L., Coleman, J. E. and Auld, D. S. (1991). Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proceedings of the National Academy of Sciences of the United States of America* 88, 999-1003.

Viets, F. G. (1966). Zinc deficiency in the soil plant system. In Prasad, A. S. Ed(s). *Zinc metabolism.* C. C. Thomas. pp. 99-127.

Viets, F. G., Boawn, L. C. and Crawford, C. L. (1954). Zinc contents and deficiency symptoms of 26 crops grown on a deficient soil. *Soil Science* 78, 305-316.

von Wirén, N., Mori, S., Marschner, H. and Römheld, V. (1994). Iron inefficiency in the maize mutant ys1 (*Zea mays* L cv. yellow-stripe) is caused by a defect in uptake of iron phytosiderophores. *Plant Physiology* 106, 71-77.

von Wirén, N., Marschner, H. and Römheld, V. (1996). Roots of iron-efficient maize also absorb phytosiderophore-chelated zinc. *Plant Physiology* 111, 1119-1125.

Webb, M. J. and Loneragan, J. F. (1988). Effect of zinc deficiency on growth, phosphorus concentration, and phosphorus toxicity of wheat plants. *Soil Science Society of America Journal* 52, 1676-1680.

Webb, M. J. and Loneragan, J. F. (1990). Zinc translocation to wheat roots and its implications for a phosphorus/zinc interaction in wheat plants. *Journal of Plant Nutrition* 13, 1499-1512.

Welch, R. M. (1993). Zinc concentrations and forms in plants for humans and animals. In Robson, A. D. Ed. *Zinc in soils and plants.* Kluwer Academic Publishers. pp. 183-195.

Welch, R. M., Allaway, W. H., House, W. A. and Kubota, J. (1991). Geographic distribution of trace element problems. In Mortvedt, J. J., Cox, F. R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition*. Soil Science Society of America. pp. 31-57.

Welch, R. M. and Norvell, W. A. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* 101, 627-631.

Welch, R. M., Webb, M. J. and Loneragan, J. F. (1982). Zinc in membrane function and its role in phosphorus toxicity. In Scaife, A. Ed. *Proceedings of the Ninth plant nutrition colloquium, Warwickshire, England.* Commonwealth Agricultural Bureaux. pp. 710-715.

Wu, M. C. and Xiao, C. Z. (1992). The physiological effect of zinc on soyabeans. *Acta Agriculturae Boreali Sinica* 7, 73-77.

Yang, X., Römheld, V., Marschner, H. and Chaney, R. L. (1994). Application of chelator-buffered nutrient solution technique in studies on zinc nutrition in rice plant (*Oryza sativa* L.). *Plant and Soil* 163, 85-94.

Zakharchishina, V. O. (1973). Effect of 2, 4-dinitrophenol on respiration of mitochondria in barley grown under zinc deficiency conditions. *Visnik Kharkivskogo Universitetu, Biologiya* 89, 77-80.

Zhang, F., Römheld, V. and Marschner, H. (1989). Effect of zinc deficiency in wheat on the release of zinc and iron mobilizing root exudates. *Zeitschrift für Pflanzenernährung und Bodenkunde* 152, 205-210.

Zsoldos, F. and Haunold, E. (1978). Potassium influx and efflux of 2,4-D and MCPA treated plants. *Plant and Soil* 49, 219-224.

CHAPTER 2

PRELIMINARY EXPERIMENTS WITH SOLUTION CULTURE TECHNIQUES

2.1 Introduction

The application of the herbicide chlorsulfuron at recommended rates to wheat plants grown in low-Zn soils was suspected to induce both zinc and copper deficiencies, reducing grain yields at maturity (Bowran *et al.*, 1987). Follow-up studies determined the responses of wheat to chlorsulfuron when grown in low-Zn conditions and found that chlorsulfuron-treated plants became Zn-deficient (section 1.2.3; McLay and Robson, 1992; Robson and Snowball, 1989, 1990), but did not determine the mechanisms involved in decreasing Zn concentrations. Nutrient uptake was reduced by concentrations of chlorsulfuron which had no effect on root growth, suggesting effects were more complex than reduction of root mass alone (Osborne and Robson, 1992). Experiments by Dong *et al.*, (1995) showed overall root length was reduced by chlorsulfuron treatment, while Zn uptake rates on a root surface area basis were unaffected.

In order to investigate the effects of chlorsulfuron on Zn uptake in wheat, experiments that establish the growth and nutritional status of wheat plants under controlled conditions are required. Solution culture of plants allows easy manipulation of the root environment for experimental purposes (Rengel and Graham, 1996). Chelate-buffered nutrient solutions allow very low, controlled activity of micronutrients in solutions (see section 1.1.8). By using an excess of chelate to lower the activity of free micronutrient cations, the activities of micronutrient cations can be maintained at levels similar to those that are available for plant uptake in soils (Norvell, 1991; Norvell and Welch, 1993; Welch and Norvell, 1993).

Growing plants in solutions with different levels of Zn availability enables observation of growth responses of wheat with differing zinc efficiencies (section 1.1.6.3; Rengel and Graham, 1995a, b, 1996). As chlorsulfuron is suspected to decrease Zn uptake by wheat plants (section 1.2.3), cultivars of wheat that vary in their Zn efficiency can be examined to determine whether chlorsulfuron

influences Zn-efficient plants differently to Zn-inefficient plants (see also Dong *et al.*, 1995).

The main objectives of the following experiments were to determine conditions that would provide a suitable level of Zn deficiency response for different wheat genotypes, using chelate-buffered nutrient solution and to determine the effect of chlorsulfuron on the growth and nutrient levels of wheat plants.

2.2 Materials and methods

2.2.1 Seed

Seed of breadwheat cultivars Excalibur (*Triticum aestivum*; Zn-efficient) and Gatcher (*T. aestivum*; Zn-inefficient) and durum wheat cultivar Durati (*T. turgidum* L. conv. *durum* (Desf.) MacKey; Zn-inefficient) were collected from trials conducted on zinc-deficient field sites; all cultivars had a similarly low level of zinc in seed. Average zinc content (± SE) of Excalibur and Gatcher was 320 ± 30 ng Zn/grain and of Durati was 320 ± 40 ng Zn/grain.

By using the rate of growth of the third leaf to quantify wheat tolerance to chlorsulfuron (Bowran and Blacklow, 1987), Gatcher was the cultivar most tolerant to chlorsulfuron when Zn was available, while Excalibur was most tolerant under Zn-deficient conditions (Dong *et al.*, 1995). Cultivar Durati was the least tolerant to chlorsulfuron when Zn was available, and grew less than the other cultivars in Zn-deficient conditions, but was not decreased by a greater amount when chlorsulfuron was applied (Dong *et al.*, 1995).

2.2.2 Germination of seeds

Seeds were surface-sterilised by soaking in double-deionised water (18 MΩ.cm resistivity; Milli-Q® water purification system; DD water) for 30 min, washing in 70 % (v/v) ethanol for 1 min, soaking in 1.4 % (v/v) sodium hypochlorite for 5 min, then rinsing in 10 changes of DD water and pre-germinated for 36 h in an aerated solution of 5 mg L^{-1} Bayleton fungicide. Seeds were rinsed in 5 changes of DD water before sowing. Germinated seeds were

placed into 10-mL polyethylene cups with the bases replaced with 5-mm polyethylene mesh. Nine seeds per cup were used and covered with black polythene beads to exclude light from solution. The cups were placed into holes cut into tightly fitting lids of 2-L black plastic pots (24 plants per pot).

2.2.3 Nutrient solutions

Pots were filled with chelate-buffered nutrient solutions (Norvell and Welch, 1993; Parker, 1993), made with DD water and analytical grade compounds. Pots were aerated continuously. The final concentrations (m*M*) of nutrients supplied in solution to plants were: 2.0 $Ca(NO_3)_2$; 0.5 $MgSO_4$; 1.5 KNO_3; 0.1 KCl; 0.01 H_3BO_3; 0.0001 Na_2MoO_4. Metal micronutrients were supplied as chelates of HEDTA (Norvell and Welch, 1993) at the following concentrations (μ*M*): 0.5 Cu; 1 Mn; 0.1 Ni; 100, Fe. ZnHEDTA concentrations were varied in different experiments to determine optimum free Zn activities for adequacy and deficiency. The free Zn^{2+} activity in nutrient solutions was calculated using GEOCHEM-PC (Sposito and Mattigod, 1980). ZnHEDTA concentrations and free Zn activities are described in detail for each experiment later in this chapter. In addition to metal micronutrient chelates, 25 μ*M* K_3HEDTA was added to keep ionic activities of free micronutrient cations at very low levels. The solution was buffered at pH 6.0 with 1 m*M* MES.

Plants were grown in half-strength solution on days 1-5. The solution was replaced with full-strength solution on days 6, 10, 14, 16, 18 and 20. Zinc was added to nutrient solutions as ZnHEDTA at the full strength of the concentration required, for the duration of each experiment. To avoid Zn-deficiency-induced P-toxicity (Parker, 1993; Welch *et al.*, 1982), $NH_4H_2PO_4$ was provided at an initial concentration of 5 μ*M* until day 6, and added to pots each day thereafter in amounts of 1 μ*M* d^{-1} (d 7-10), 2 μ*M* d^{-1} (d 11-13), 3 μ*M* d^{-1} (d 14-17), 4 μ*M* d^{-1} (d 18-20), 5 μ*M* d^{-1} (d 21-24).

2.2.4 Growth chamber

Plants were kept in a growth chamber with a 15/10°C, 10/14 h light/dark cycle and a photosynthetically active irradiance of 300 μmol m^{-2} s^{-1} at plant tops. Light was provided by high density metal-halide lamps.

2.2.5 Chlorsulfuron application

Because the effect of chlorsulfuron on growth and Zn concentration of wheat is not significantly different to that of the commercial formulation of Glean herbicide which contains chlorsulfuron (McLay and Robson, 1992), chlorsulfuron was applied by adding stock solution of 20 mg L^{-1} (26.6 mg Glean herbicide L^{-1}) directly to the nutrient solution immediately after nutrient solution replacement.

2.2.6 Harvests

Plants were harvested for growth and nutrient analysis by removing them from pots and washing plastic beads and meshes from the roots with deionised water. Whole plants were rinsed in two separate containers of DD water, roots were then washed in 1 m*M* $LaCl_3$ for 1 min to remove ions from the apoplastic region of roots (Reid *et al.*, 1996). Roots were rinsed in a final container of DD water before being blotted dry with paper tissues. Plants were separated into roots and shoots and seed coat remains were discarded. Plant parts were wrapped in powder paper, placed in labelled envelopes and dried at 80°C for at least 48 h before being weighed and digested for nutrient analysis.

2.2.7 Nutrient analysis

Plant material was analysed for mineral nutrient concentrations using an inductively coupled plasma (ICP) emission spectrometer (model ARL 3580, Analytical Research Laboratories, Switzerland). Plant parts were prepared for ICP analysis based on previously described procedures (*e.g.* Rengel and Graham, 1995a, b). In brief, samples were digested in 70 % (v/v) HNO_3, then heated to

126 °C until most of the acid had evaporated (6-8 h). Residues were then resuspended in 1 % (v/v) nitric acid added to make up a final volume of 5 or 10 mL depending on original dry weight of material (< 0.3 g or ≥ 0.3 g DW). The liquid was left to settle overnight before decanting for ICP analysis. A set of standard plant samples of known Zn and other element concentrations was analysed at the same time to ensure the procedure was accurate.

2.2.8 Experimental treatments and designs

Fully randomised, factorial designs were employed for each experiment.

Experiment 1

To determine the concentration of chlorsulfuron at which wheat plants become Zn deficient, plants were exposed to a range of chlorsulfuron concentrations at an adequate and a deficient level of Zn in solution. Gatcher wheat plants were grown for 22 d in nutrient solution containing 0.2 or 4 μ*M* ZnHEDTA (4.2 and 83 p*M* free Zn^{2+} respectively). Five chlorsulfuron treatments including a control (0, 0.4, 4.0, 40 and 400 μg L^{-1}) were applied from d 10 to d 22 for a total exposure time of 12 d. The concentrations of chlorsulfuron applied to pots were equivalent to 1.1-1110 n*M*. These concentrations were based on previous experiments with wheat and other species exposed to chlorsulfuron (*e.g.* Ray 1984, Royuela *et al.*, 1991). A total of 2 Zn activities × 5 chlorsulfuron levels = 10 treatments were created. There were three replicates of each treatment.

Experiment 2

The response of different wheat cultivars to chlorsulfuron exposed for different periods of time was tested in the second experiment. Cultivars Excalibur, Gatcher and Durati were grown in nutrient solution containing 0.2 μ*M* ZnHEDTA (4.2 p*M* free Zn^{2+}) and supplied with 0 or 40 μg chlorsulfuron L^{-1} from d 11 after planting. Plants were harvested from each pot on d 13, d 15 and d 18 giving exposure times of 2-, 4- and 7-d. A total of 3 cultivars × 2 chlorsulfuron levels × 3 exposure times = 18 treatments were used with three replicates of each treatment.

Experiment 3

The influence of Zn availability on plant response to chlorsulfuron was examined by growing Gatcher wheat for 21 d in solutions containing 0.05, 0.1 or 4 μ*M* ZnHEDTA(1.0, 2.1, and 83 p*M* free Zn^{2+}). Chlorsulfuron treatments (0 or 40 μg L^{-1}) were applied from d 8. Plants were harvested on d 8, d 14 and d 21, giving exposure times of 0, 6 and 13 d. A total of 3 Zn activities × 2 chlorsulfuron × 3 exposure times = 18 treatments was used with four replicates of each treatment.

2.3 Results

2.3.1 Root and shoot dry weights

Root DW of wheat was not significantly altered by Zn activity of nutrient solution when Zn activity was varied in Experiments 1 and 3 (Fig. 2.1, Table 2.2) or by any first-order interactions involving solution Zn activity. Root DW increased with plant age (Tables 2.1, 2.2). Cultivar root DW increased in the order of Durati > Excalibur > Gatcher (Table 2.1).

The response of root DW to chlorsulfuron varied with time of exposure as it was not influenced by 7-d exposure to 40 μg chlorsulfuron L^{-1} (Table 2.1), but was significantly decreased by 12-d exposure to 40 and 400 μg chlorsulfuron L^{-1} (Fig. 2.1). There was a chlorsulfuron × exposure time interaction in which plants were unaffected by 0- and 6-d exposure to 40 μg chlorsulfuron L^{-1} but were significantly decreased by 13-d exposure (Table 2.2).

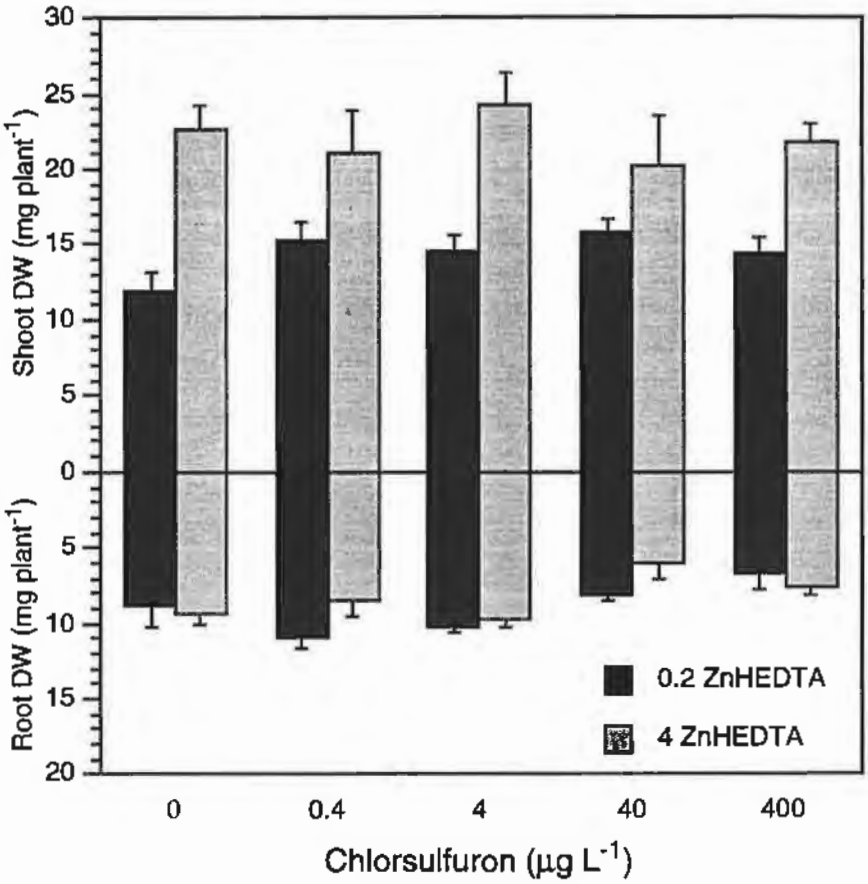

Figure 2.1 Mean (± SE, n=3) shoot and root DW (mg plant^{-1}) of Gatcher wheat grown for 22 days in nutrient solution containing 0.2 or 4 µM ZnHEDTA (Exp 1). Chlorsulfuron was added to nutrient solution at d 10 for a total exposure time of 12 d.

Probabilities of treatment significance for shoot DW: zinc $P \leq 0.01$, chlorsulfuron $P \leq 0.85$, zinc × chlorsulfuron ≤ 0.43.

Probabilities of of treatment significance for root DW: zinc $P \leq 0.16$, chlorsulfuron $P \leq 0.01$, zinc × chlorsulfuron ≤ 0.24.

Table 2.1 Mean (± SE, n=4) root and shoot DW (mg plant^{-1}) of three cultivars of wheat grown for 18 days in nutrient solution containing 0.2 μ*M* ZnHEDTA (Exp 2). Chlorsulfuron (CS; 0 or 40 μg L^{-1}) was added to nutrient solution from d 11, therefore plants were exposed to chlorsulfuron for 2-, 4 -and 7-d. Probability (P) values refer to significance of main effects as interactions were not significant.

		Root DW		
		Exposure time		
Cultivar	Chlorsulfuron	2 d	4 d	7 d
Durati	0 CS	3.7 ± 0.9	3.8 ± 0.4	7.3 ± 3.8
	40 CS	3.1 ± 0.4	3.0 ± 1.0	7.4 ± 2.5
Excalibur	0 CS	4.3 ± 0.5	5.9 ± 0.6	8.1 ± 2.0
	40 CS	3.9 ± 0.1	4.3 ± 0.8	8.4 ± 0.1
Gatcher	0 CS	9.5 ± 0.3	9.9 ± 0.5	10.8 ± 1.3
	40 CS	7.7 ± 1.7	7.9 ± 0.9	8.5 ± 0.4
Cultivar P ≤ 0.01				
Chlorsulfuron P ≤ 0.33				
Exposure time P ≤ 0.01				

		Shoot DW		
		Exposure time		
Cultivar	Chlorsulfuron	2 d	4 d	7 d
Durati	0 CS	10.1 ± 1.7	10.4 ± 0.4	19.5 ± 6.3
	40 CS	15.5 ± 2.1	8.8 ± 2.7	21.7 ± 6.0
Excalibur	0 CS	10.6 ± 1.4	13.5 ± 0.8	15.9 ± 3.1
	40 CS	13.3 ± 3.3	10.0 ± 1.0	22.7 ± 1.3
Gatcher	0 CS	22.2 ± 1.2	22.7 ± 1.2	22.3 ± 1.1
	40 CS	20.7 ± 1.9	22.4 ± 2.9	22.0 ± 1.0
Cultivar P ≤ 0.01				
Chlorsulfuron P ≤ 0.41				
Exposure time P ≤ 0.01				

Table 2.2 Mean (± SE, n= 4) root and shoot DW (mg plant^{-1}) of Gatcher wheat grown in nutrient solution for 21 days in nutrient solution containing 0.05, 0.1 or 4 μ*M* ZnHEDTA (Exp 3). Chlorsulfuron (CS; 0 or 40 μg L^{-1}) was added to nutrient solution from d 8, therefore plants were exposed to chlorsulfuron for 0-, 6 -and 13-d. ZnHEDTA = zinc concentration of solution (μ*M*). Probability (P) values refer to significant main effects and interactions only.

		Root DW		
		Exposure time		
ZnHEDTA	Chlorsulfuron	0 d	6 d	13 d
0.05	0 CS	2.5 ± 0.2	6.1 ± 0.5	12.8 ± 1.1
	40 CS	2.6 ± 0.2	5.1 ± 0.4	8.2 ± 0.5
0.1	0 CS	2.2 ± 0.1	5.9 ± 0.6	11.9 ± 1.4
	40 CS	2.3 ± 0.2	5.1 ± 0.5	7.2 ± 0.5
4.0	0 CS	2.3 ± 0.2	5.2 ± 0.3	13.3 ± 0.7
	40 CS	2.5 ± 0.2	5.1 ± 0.4	6.7 ± 0.2
ZnHEDTA	P ≤ 0.33			
Chlorsulfuron × Exposure time	P ≤ 0.01			

		Shoot DW		
		Exposure time		
ZnHEDTA	Chlorsulfuron	0 d	6 d	13 d
0.05	0 CS	4.3 ± 0.2	11.0 ± 0.6	17.4 ± 1.6
	40 CS	4.6 ± 0.3	13.3 ± 0.4	22.6 ± 0.8
0.1	0 CS	4.1 ± 0.2	11.4 ± 0.9	19.3 ± 1.0
	40 CS	4.4 ± 0.4	11.8 ± 0.8	21.1 ± 2.3
4.0	0 CS	4.5 ± 0.3	16.3 ± 0.5	33.7 ± 1.2
	40 CS	4.5 ± 0.4	16.1 ± 0.5	35.3 ± 1.8
Chlorsulfuron	P ≤ 0.01			
ZnHEDTA × Exposure time	P ≤ 0.01			

Shoot DW generally was decreased in low Zn-activity solutions (Fig. 2.1, Table 2.2). Shoot DW increased with plant age (Table 2.1). There was a significant Zn × exposure time interaction (Table 2.2); shoot DW of plants grown at 4 μ*M* ZnHEDTA did not differ significantly from the two low-Zn treatments at 0 d exposure but was significantly greater at the later harvests.

Chlorsulfuron did not significantly influence shoot DW except for Exp 3 when Gatcher shoot DW was slightly increased by 40 μg chlorsulfuron L^{-1} (Table

2.2). There were no significant interactions between chlorsulfuron and other treatments in any experiment.

The shoot:root ratio of Durati was increased considerably by chlorsulfuron, while the other cultivars were unaffected (Table 2.3). Shoot:root ratio in each cultivar did not differ significantly from each other, however, control plants increased the shoot:root ratio in the order Excalibur < Durati < Gatcher.

Table 2.3 Shoot: root ratio (mean ± SE, n = 3) of three cultivars of wheat grown for 18 d in nutrient solution containing 0.2 μM ZnHEDTA. Chlorsulfuron (CS, 0 or 40 μg L^{-1}) was applied from d 11 (total exposure time = 7 d). Probability (P) values refer to significance of main effects and interaction.

	0 CS	40 CS
Durati	1.96 ± 0.26	3.14 ± 0.43
Excalibur	1.57 ± 0.08	1.47 ± 0.23
Gatcher	2.71 ± 0.13	2.62 ± 0.26
cultivar	P ≤ 0.01	
CS	P ≤ 0.13	
cultivar × CS	P ≤ 0.06	

2.3.2 Zinc concentrations

Zinc concentration in roots was significantly decreased by low solution Zn activity (Fig. 2.2, Table 2.5), in some cases reaching deficient levels (Table 2.5). Chlorsulfuron did not influence root Zn concentration (Fig. 2.2, Tables 2.4, 2.5). The concentration of Zn in roots decreased significantly with plant age in Exp 2 (Table 2.4) however, this may be due to a growth "dilution" effect, where increases in root DW with plant age (Table 2.1) decrease the Zn concentration. To verify this, Zn content in roots was calculated and found to increase with plant age (Table 2.6). Chlorsulfuron reduced Zn content in both roots and shoots at each harvest (Table 2.6).

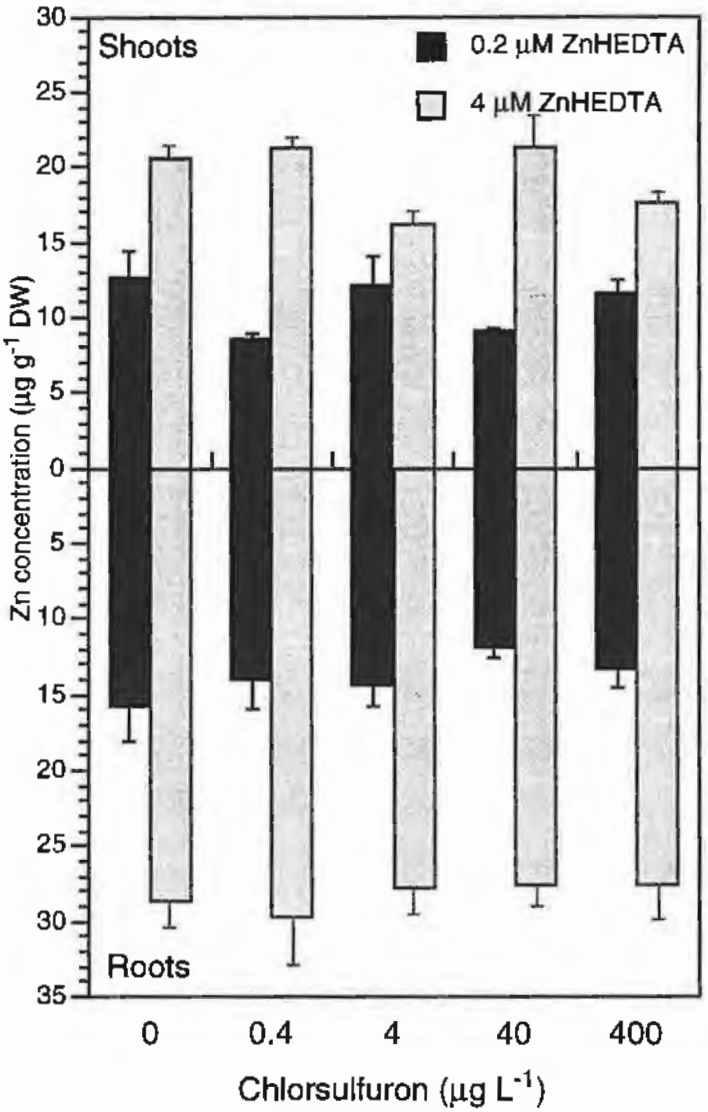

Figure 2.2 Means (± SE, n= 3) root and shoot Zn concentration (µg g^{-1}) of Gatcher wheat grown for 22 days in nutrient solution containing 0.2 or 4 µM ZnHEDTA (Exp. 1). Chlorsulfuron was added to nutrient solution at d 10 for total exposure time of 12 d.

Probabilities of treatment significance for shoot Zn concentration: zinc $P \leq 0.01$, chlorsulfuron $P \leq 0.36$, zinc × chlorsulfuron ≤ 0.01.

Probabilities of treatment significance for root Zn concentration: zinc $P \leq 0.01$, chlorsulfuron $P \leq 0.20$, zinc × chlorsulfuron ≤ 0.28.

Table 2.4 Mean (± SE, n=4) of Zn concentration (μg g^{-1}) for main effect treatments imposed on three cultivars of wheat grown in nutrient solution (Exp 2). Chlorsulfuron (CS; 40 μg L^{-1}) was added to nutrient solution from d 11, therefore plants were exposed to chlorsulfuron for 2-, 4 -and 7-d. P values refer to significance of treatment effects. Probability (P) values refer to significance of main effects and interactions.

		Root Zn concentration		
		Exposure time		
Cultivar	Chlorsulfuron	2 d	4 d	7 d
Durati	0 CS	20.4 ± 3.7	16.9 ± 1.2	13.5 ± 0.5
	40 CS	14.6 ± 0.9	16.2 ± 0.9	16.1 ± 1.7
Excalibur	0 CS	14.8 ± 3.0	15.3 ± 1.7	14.8 ± 0.7
	40 CS	14.8 ± 1.2	14.1 ± 0.2	12.2 ± 0.4
Gatcher	0 CS	13.8 ± 1.5	13.0 ± 0.6	13.8 ± 2.2
	40 CS	15.1 ± 0.8	13.8 ± 0.6	12.8 ± 1.5
Cultivar	P ≤ 0.03			
Chlorsulfuron	P ≤ 0.32			
Exposure time	P ≤ 0.19			

		Shoot Zn concentration		
		Exposure time		
Cultivar	Chlorsulfuron	2 d	4 d	7 d
Durati	0 CS	20.6 ± 2.4	13.8 ± 0.7	9.8 ± 0.4
	40 CS	14.3 ± 1.8	12.1 ± 0.8	8.8 ± 0.4
Excalibur	0 CS	18.7 ± 2.7	15.3 ± 0.5	10.5 ± 0.2
	40 CS	14.1 ± 0.7	13.0 ± 0.5	9.7 ± 0.5
Gatcher	0 CS	15.0 ± 0.9	15.6 ± 1.5	10.9 ± 0.7
	40 CS	14.1 ± 0.7	12.8 ± 0.2	9.8 ± 0.2
Cultivar	P ≤ 0.71			
Chlorsulfuron	P ≤ 0.01			
Exposure time	P ≤ 0.01			

Table 2.5 Means (± SE, n= 4) of root and shoot Zn concentration (μg g^{-1}) in Gatcher wheat grown in nutrient solution for 21 d in three concentrations of ZnHEDTA (Exp 3). Chlorsulfuron (CS; 0 or 40 μg L^{-1}) was added from d 8. Probability (P) values refer to significance of main effects and interactions.

		Root Zn concentration		
		Exposure time		
ZnHEDTA	Chlorsulfuron	0 d	6 d	13 d
0.05	0 CS	35.5 ± 4.9	25.3 ± 2.9	16.0 ± 1.7
	40 CS	24.0 ± 2.4	23.5 ± 2.5	16.5 ± 2.6
0.1	0 CS	27.3 ± 0.9	23.5 ± 3.2	22.0 ± 2.5
	40 CS	23.8 ± 2.7	26.8 ± 5.2	18.0 ± 1.5
4.0	0 CS	28.0 ± 0.1	38.5 ± 7.6	23.8 ± 1.6
	40 CS	32.3 ± 2.8	36.0 ± 8.6	23.7 ± 0.9
ZnHEDTA	P ≤ 0.03			
Chlorsulfuron	P ≤ 0.77			
Exposure time	P ≤ 0.58			
		Shoot Zn concentration		
		Exposure time		
ZnHEDTA	Chlorsulfuron	0 d	6 d	13 d
0.05	0 CS	53.6 ± 11.0	33.6 ± 1.5	17.4 ± 2.4
	40 CS	39.3 ± 4.6	22.9 ± 2.3	18.2 ± 2.8
0.1	0 CS	35.0 ± 4.1	38.2 ± 6.4	15.5 ± 1.9
	40 CS	34.9 ± 2.3	22.8 ± 1.2	19.7 ± 2.0
4.0	0 CS	29.9 ± 1.5	34.4 ± 3.8	23.5 ± 1.3
	40 CS	34.2 ± 2.0	26.6 ± 1.6	11.9 ± 0.9
ZnHEDTA	P ≤ 0.17			
Chlorsulfuron	P ≤ 0.01			
Exposure time	P ≤ 0.01			

Table 2.6 Mean (± SE, n = 8) root and shoot Zn content (ng $plant^{-1}$) of Gatcher wheat at three harvests (Exp 3). Data were pooled over Zn activity treatments.

	Root Zn content		Shoot Zn content	
Exposure time	0 CS	40 CS	0 CS	40 CS
0 d	70 ± 6	64 ± 4	203 ± 34	163 ± 13
6 d	161 ± 14	146 ± 19	457 ± 42	334 ± 26
13 d	256 ± 19	139 ± 10	466 ± 75	419 ± 33

Shoot Zn concentration was significantly reduced by low Zn activity in solution (Fig. 2.2, Table 2.5), and decreased with plant age (Tables 2.4, 2.5). Shoot DW increases with plant age (Table 2.1) may have enhanced this Zn concentration response through a "dilution" effect. Zinc content of shoots decreased with plant age and chlorsulfuron treatment (Table 2.6). Chlorsulfuron had no effect on shoot Zn concentration at either level of Zn activity in Exp 1 (Fig. 2.2) but 40 μg chlorsulfuron L^{-1} decreased shoot Zn concentration in Experiments 2 and 3 (Tables 2.4, 2.5). Shoot Zn content was decreased by 40 and 400 μg chlorsulfuron L^{-1} when plants were grown in 4 μ*M* ZnHEDTA solutions but not in 0.2 μ*M* ZnHEDTA solutions (Table 2.7).

Table 2.7 Means (± SE, n= 3) of root and shoot Zn content (ng per plant) in Gatcher wheat grown in nutrient solution for 22 d (Experiment 1). Chlorsulfuron treatment (CS; μg L^{-1}) was added from d 10. Probability (P) values refer to significance of main effects and interactions.

		Chlorsulfuron (μg L^{-1})				
	ZnHEDTA	0 CS	0.4 CS	4 CS	40 CS	400 CS
Shoots	0.2 μ*M*	147 ± 16	129 ± 7	175 ± 21	142 ± 6	166 ± 16
	4 μ*M*	468 ± 45	448 ± 60	388 ± 27	416 ± 42	381 ± 15
ZnHEDTA	P ≤ 0.01					
Chlorsulfuron	P ≤ 0.83					
Interaction	P ≤ 0.25					
Roots	0.2 μ*M*	143 ± 51	156 ± 33	146 ± 12	96 ± 4	86 ± 4
	4 μ*M*	283 ± 9	251 ± 16	243 ± 24	178 ± 23	194 ± 15
ZnHEDTA	P ≤ 0.01					
Chlorsulfuron	P ≤ 0.01					
Interaction	P ≤ 0.71					

2.3.3 Nutrient concentrations in roots and shoots

In addition to Zn, many other elements were analysed simultaneously using the ICP spectrometer. As there are results for both roots and shoots for 12 elements from three experiments, the possibility that a type I statistical error is committed (accepting a false null hypothesis) is high (Zar, 1984). Responses that appear significant may be due to chance rather than represent true responses to

the imposed treatments. To overcome this, only those elements which responded consistently to the treatments in all experiments are presented here.

Of the 12 elements analysed, Zn, Cu, Mn and P yielded significant results in all experiments. The major influences on these elements were Zn activity and chlorsulfuron treatment. Low solution Zn activity increased the Mn concentrations in both roots and shoots and increased shoot concentrations of Cu and P (Table 2.8). Chlorsulfuron treatment decreased Mn and Cu concentrations in the shoots and decreased Mn and P concentrations in the roots (Table 2.8). Concentrations of Mn, Cu and P did not exceed the range for adequate nutrition of any element. Manganese concentrations in control (0 chlorsulfuron) shoots increased with plant age, but decreased markedly when treated with chlorsulfuron (Exp 2 data not shown).

Table 2.8 Mean (± SE, n=3) shoot and root concentrations (mg kg^{-1}) of P, Cu and Mn in wheat from experiments 1, 2 and 3. Element concentrations were influenced significantly ($P \leq 0.05$) by Zn activity of solution or by chlorsulfuron concentration. Identical letters in each row indicate values that do not differ significantly ($P \leq 0.05$, Fisher's LSD). "-" indicates not determined

	Zinc concentration (μ*M* ZnHEDTA)			
	0.05 ZnHEDTA	0.1 ZnHEDTA	0.2 ZnHEDTA	4 ZnHEDTA
Shoot (P) Exp 1	-	-	5530 ± 160^{b}	4450 ± 200^{a}
Shoot (P) Exp 3	5460 ± 280^{b}	5600 ± 285^{b}	-	5000 ± 400^{a}
Shoot (Cu) Exp 1	-	-	6.4 ± 0.2^{b}	5.1 ± 0.1^{a}
Shoot (Mn) Exp 1	-	-	72 ± 5^{b}	48 ± 2^{a}
Shoot (Mn) Exp 3	52 ± 2^{b}	56 ± 2^{b}	-	41 ± 1^{a}
Root (Cu) Exp 3	6.3 ± 0.9^{b}	7.4 ± 1.0^{b}	-	4.5 ± 0.3^{a}

	Chlorsulfuron (μg L^{-1})				
	0 CS	0.4 CS	4 CS	40 CS	400 CS
Root (P) Exp 1	3580 ± 145^{a}	3490 ± 205^{ab}	3115 ± 100^{bc}	2845 ± 145^{cd}	2500 ± 100^{d}
Root (P) Exp 2	2900 ± 115^{a}	-	-	2640 ± 90^{b}	-
Root (Mn) Exp 1	58 ± 8^{a}	58 ± 2^{a}	57 ± 5^{a}	36 ± 6^{b}	33 ± 4^{b}
Root (Mn) Exp 2	47 ± 4^{a}	-	-	37 ± 3^{b}	-
Root (Mn) Exp 3	35 ± 3^{a}	-	-	22 ± 1^{b}	-
Shoot (Mn) Exp 1	71 ± 10^{a}	69 ± 5^{a}	63 ± 8^{a}	52 ± 6^{b}	45 ± 4^{b}
Shoot (Mn) Exp 3	54 ± 2^{a}	-	-	45 ± 2^{b}	-

Chlorsulfuron had a significant influence on the water content of Gatcher wheat roots, especially in plants grown in 4 μ*M* ZnHEDTA solutions (Table 2.9). However, there was no significant interaction between Zn and chlorsulfuron treatments. At the two highest concentrations of chlorsulfuron, both root DW (Fig. 2.1) and the water content were decreased, indicating that root FW was decreased by chlorsulfuron treatment. Low Zn activity also significantly decreased the root water content.

Table 2.9. Mean (± SE, n=3) water content of roots of Gatcher wheat grown for 22 d in nutrient solution. Chlorsulfuron (CS, μg L^{-1}) was applied from d 10 for a total exposure time of 12 d. P values refer to significance of treatment effects. Probability (P) values refer to significance of main effects and interaction.

	Root water content				
	g H_20 g DW^{-1}				
	Chlorsulfuron				
ZnHEDTA	0 CS	0.4 CS	4 CS	40 CS	400 CS
0.2 μ*M*	14.3 ± 1.7	13.5 ± 2.3	18.1 ± 3.1	11.5 ± 0.7	14.0 ± 1.5
4 μ*M*	18.9 ± 4.8	23.9 ± 5.7	24.5 ± 2.8	14.4 ± 1.9	11.7 ± 1.7
ZnHEDTA	P ≤ 0.03				
Chlorsulfuron	P ≤ 0.04				
Interaction	P ≤ 0.33				

The water content of roots was much greater than that of shoots (Table 2.10). Water content of roots and shoots decreased with plant age, and decreased in plants treated with 40 μg chlorsulfuron L^{-1} more than in control plants. After 13-d exposure, water content of chlorsulfuron-treated roots was reduced to 74 % of control roots while shoot water content was reduced to 79 % of controls. Low Zn activity of solution decreased root water content but had no effect on shoot water content (Table 2.10).

Table 2.10. Mean (± SE, n= 3) water content of roots and shoots of Gatcher wheat sampled at d 8, d 14, and d 21. Chlorsulfuron (CS, in μg L^{-1}) was applied from d 8 creating exposure times of 0-, 6- and 13-d. Zinc main effect means were pooled over exposure times and chlorsulfuron treatments (n=6). Probability (P) values refer to significance of main effects and interactions.

	Root water content		Shoot water content	
	g H_20 g DW^{-1}		g H_20 g DW^{-1}	
Exposure	Chlorsulfuron		Chlorsulfuron	
time	0 CS	40 CS	0 CS	40 CS
0 d	18.7 ± 0.5	18.5 ± 0.4	10.1 ± 0.2	10.1 ± 0.2
6 d	17.3 ± 0.6	15.5 ± 0.7	9.3 ± 0.2	8.5 ± 0.1
13 d	15.9 ± 0.4	11.6 ± 0.5	8.2 ± 0.4	6.2 ± 0.2
CS × Exposure time	P ≤ 0.01		P ≤ 0.01	
ZnHEDTA				
0.05	15.6 ± 0.6		8.9 ± 0.3	
0.1	15.3 ± 0.6		8.7 ± 0.3	
4.0	17.8 ± 0.6		8.6 ± 0.4	
ZnHEDTA	P ≤ 0.01		P ≤ 0.36	

2.4 Discussion

2.4.1 Effect of zinc activity in solution

Chelate-buffered nutrient solution provides a reliable method of growing plants with controlled, low activities of micronutrient cations (Norvell and Welch, 1993; Rengel and Graham, 1995a, b; Welch and Norvell, 1993). The growth of the wheat cultivars tested indicate low Zn activity in solution increased the root DW and decreased the shoot DW in a similar manner to previous results with the same wheat cultivars (*e.g.* Rengel and Graham, 1995a, b). Low solution Zn activity decreased Zn concentrations in roots and shoots, in some cases to levels indicative of severe deficiency (*e.g.* Table 2.2; Reuter and Robinson, 1986). Zinc concentration of shoots was reduced after growing in low-Zn solution compared to the sufficient-Zn solution (effect greatest in older plants). The concentration of Zn observed in plants grown in low Zn solutions was below the critical concentration for growth of 21 $\mu g\ g^{-1}$ DW derived for plants grown in chelate-buffered solutions (Rengel and Graham, 1995a). Experiments by McLay and Robson (1992) showed Zn concentration of the youngest emerged blade of plants grown in solution culture were reduced by low Zn activity to similar concentrations (10.9-12.6 $\mu g\ g^{-1}$ DW) as obtained in the experiments described here (Table 2.4).

Increased root growth and decreased shoot growth are suggested to be a response to zinc deficiency (Rengel and Graham, 1995a). This response is often seen in solution-cultured plants in Zn-deficient conditions (Cumbus, 1985; Rengel and Graham, 1995a, b; Robson and Snowball, 1990; Welch *et al.*, 1982), and results in a change in the shoot:root ratio as seen in other studies (Rengel and Graham, 1995a). By decreasing the shoot:root DW ratio when Zn-deficient, a relatively greater amount of root is present for absorption of zinc from solution, although the rate of Zn uptake per unit of root length or surface area may be lower than in plants adequately supplied with Zn (Rengel and Graham, 1995a). Lowered Zn concentrations decreased the shoot:root ratio of wheat in both soil and solution culture (McLay and Robson, 1992); in contrast, Zn activity of

solution had no effect on shoot:root ratio in the cultivars tested here. Zinc-efficient cultivars are less likely to alter this ratio as they can obtain sufficient zinc to maintain shoot growth without having to alter root growth (Rengel and Graham, 1995a). Cakmak *et al.* (1996) suggested that the shoot:root ratio changes because Zn-inefficient cultivars are more susceptible to light-induced peroxidative damage of shoots under Zn deficiency.

2.4.2 Effect of chlorsulfuron

In broad-acre farming, chlorsulfuron is applied as a pre-emergence herbicide (*i.e.* before the crop has germinated), therefore it is present in the soil and is taken up primarily by the roots of germinating wheat plants (Beyer *et al.*, 1988; Robson and Snowball, 1989). Root uptake of chlorsulfuron has more severe effects on growth than shoot uptake (Dastgheib *et al.*, 1994; Lemerle and Cousens, 1993a). Roots are often damaged to a greater extent than shoots, especially when chlorsulfuron is applied via the roots (Lemerle and Cousens, 1993a, b). The greater sensitivity of root DW to chlorsulfuron compared to shoot DW (Fig. 2.1 has been observed in other experiments when chlorsulfuron was applied directly via the roots (Dong *et al.*, 1995; McLay and Robson, 1992). Root DW of chlorsulfuron-treated plants was significantly decreased after 3 and 5 weeks of exposure in soil (Dong *et al.*, 1995) and 4 weeks in solution culture (McLay and Robson, 1992). Growth rates of shoots in chlorsulfuron-treated plants were lower than control plants (Tables 2.1, 2.2)).

There was no interaction between solution Zn activity and chlorsulfuron on shoot DW in this study as has been observed elsewhere (McLay and Robson, 1992; Osborne and Robson, 1992). Plants in the experiments presented here were younger than those of McLay and Robson (1992) and Osborne and Robson, (1993), therefore may not have grown for long enough to develop the same level of response.

The greater shoot:root ratio of chlorsulfuron-treated plants was due to both an increase in shoot growth and a decrease in root growth (Table 2.3). The shoot:root ratio of maize plants increased from 2.0 in control plants to 4.0 in

plants exposed to 0.1 μg chlorsulfuron L^{-1} as root growth was decreased more than shoot growth (Ray, 1982). Wheat is more tolerant of chlorsulfuron than other grain crops (Beyer *et al.*, 1988), therefore should have a smaller response in shoot:root ratio. Shoot:root ratios of wheat plants were not influenced by chlorsulfuron after 3 or 5 weeks of exposure to 5 μg chlorsulfuron per kg of soil (Dong *et al.*, 1995).

Shoot Zn content of chlorsulfuron-treated plants grown in Zn-adequate solutions was decreased, but was unaffected in Zn-deficient solutions (Table 2.7). Similarly, root DW was reduced by chlorsulfuron to a greater extent when plants received adequate supplies of Zn as Zn-deficient roots were already stressed and their growth could not be decreased further (Dong *et al.*, 1995). No other interactions between solution Zn activity and chlorsulfuron treatment were observed.

The decrease in shoot Zn concentration in chlorsulfuron-treated plants was most probably due to decreased translocation to shoots, as Zn concentrations in roots were not affected by chlorsulfuron (*e.g.* Table 2.5) and shoot DW continued to increase, although more slowly in chlorsulfuron-treated plants. Similarly, shoot Zn concentrations were decreased to critical levels by concentrations of chlorsulfuron that had no significant influence on root DW (McLay and Robson, 1992, Robson and Snowball, 1990). The cause of these decreases in shoot Zn was not determined as root growth and Zn uptake were decreased simultaneously, making it difficult to separate root growth decreases that influenced nutrition from entirely independent effects (Osborne and Robson, 1993).

One possible cause of decreased Zn concentrations in shoots that has not been investigated is the potential binding of Zn by sulfonylureas. Sulfonylureas can bind divalent cations effectively in model situations (Hatzidimitriou *et al.*, 1990). Herbicidal sulfonylureas may bind the freely available fraction of Zn in the soil (*e.g.* DTPA-extractable Zn), thus reducing the Zn available for uptake by plants. This may be less important in solutions as the bound form may still be available to roots. Further studies may indicate the extent of this response in soils.

2.4.3 Responses of other elements to chlorsulfuron and Zn

Of the 12 elements that were analysed by ICP, Zn, Mn, Cu and P showed consistent responses to the chlorsulfuron and solution Zn activity treatments. Chlorsulfuron decreased the concentration of each nutrient in comparison to control plants, while low Zn activity increased the nutrient concentrations (Table 2.8). The other treatments of time of exposure (plant age) and cultivar also yielded significant differences for some elements; these could be expected to occur as nutrient concentrations do not remain static as plants grow and are unlikely to be the same for different cultivars.

The nutrients most affected by chlorsulfuron in soil-grown plants appeared to be those transported to the roots by diffusion, while those that were least affected are those transported to roots by mass flow (McLay and Robson 1992; Osborne *et al.*, 1993). In the solution culture experiments presented here, it appears that uptake of the same nutrients (Zn, Cu, P) is altered by chlorsulfuron, but not to the same extent as in soil, suggesting that chlorsulfuron is directly interfering with the uptake of these nutrients, rather than the reducing access of the roots to nutrients within the soil.

Phosphorus was the macro-element most affected by chlorsulfuron (McLay and Robson, 1992; Osborne *et al.*, 1993). Chlorsulfuron decreased P concentrations in roots but had no influence on the shoot concentrations (Table 2.8). Phosphorus concentrations were significantly reduced by chlorsulfuron as was shoot DW (Osborne *et al.*, 1993). In a field experiment, P and K concentrations in shoots of chlorsulfuron-treated plants were decreased to deficient levels. In contrast, N concentrations in shoots were increased by chlorsulfuron, but this was due mostly to the corresponding decreased shoot growth (Osborne *et al.*, 1993). These results indicate that chlorsulfuron can inhibit uptake of nutrients without having an effect on root growth.

Interactions between Zn and P, including Zn-deficiency-induced P toxicity have been described previously (Webb and Loneragan, 1990; Welch *et al.*, 1982) and various methods have been used to prevent excessive P uptake in studies of

Zn deficiency (*e.g.* Parker, 1993). Phosphorus concentrations were increased in plants grown in low-Zn activity solutions (Table 2.8) although concentrations in shoots did not reach toxic levels.

Low Zn activity increased Cu uptake in shoots (Table 2.8), which was also observed by Dong *et al.* (1995). Zinc and Cu compete for uptake (Bowen 1969; Schmidt *et al.*, 1965) therefore, when Zn availability is low, Cu is taken up in its place (Bowen, 1969). However, Cu concentrations did not reach toxic levels in any treatment.

Chlorsulfuron treatment decreased Zn, Cu and Mn concentrations in shoots (Table 2.8) to levels similar to those of field-grown plants treated with 15-30 g chlorsulfuron ha^{-1} (Osborne *et al.*, 1993). Decreased uptake of Cu and Mn in chlorsulfuron-treated plants was observed by Dong *et al.* (1995).

In contrast to results of Dong *et al.* (1995), neither Zn nor Cu concentrations in roots were influenced by chlorsulfuron treatment at any stage in the experiments presented here. This effect was also noted by McLay and Robson (1992), who attributed it to constant circulation of the nutrient solution around the roots, enabling the roots to access the entire supply of Zn in the solution. Uptake of Zn and Cu is limited in soils by the rate of diffusion to the roots.

2.4.4 Water content

The reduced water content of plants grown in solution culture (Table 2.9) suggests that chlorsulfuron not only decreases root growth, but also interferes with water content in the roots. Water usage by chlorsulfuron-treated plants in a split root-experiment was reduced in pots which had been treated with chlorsulfuron (Robson and Snowball, 1990), but was not investigated further.

Zinc-deficient plants can become water-stressed, even when grown in nutrient solution (Sharma *et al.*, 1994). Zinc helps to maintain the water holding capacity of plants, possibly by controlling transpiration and stomatal opening (Sharma *et al.*, 1995). Zinc-deficient plants have lower water potentials than Zn-adequate plants, indicating an increase in water stress. Resupply of Zn restored diffusive resistance and transpiration to control rates, but had no influence of

water potential for at least 24 h, indicating that water potential was not directly responsible for decreased water holding capacity in leaves (Sharma *et al.*, 1995). Plants in low-Zn activity solutions had lower water contents than plants in adequate-Zn solutions (Table 2.10).

2.4.5 Conclusions

The chelate-buffered nutrient solution was capable of controlling the supply of Zn to plants from deficiency to adequacy. Zinc concentrations of 0.2 and 4 μ*M* were appropriate for deficiency and adequacy of Zn nutrition respectively. Plants developed Zn deficiency in 0.2 μ*M* ZnHEDTA solution and had sufficient Zn in 4 μ*M* ZnHEDTA solution to sustain growth. Cultivars that varied in their Zn efficiency were not distinguished by differences between their root or shoot DW, contrasting with the results of Rengel and Graham (1995a). The shoot:root ratio was not significantly different between cultivars, but could be ranked in a similar order to that of Rengel and Graham, (1995a), which suggests that Zn efficiency can be expressed in solution culture, especially as plant age increases.

The experiments described in this chapter indicated that 40 μg chlorsulfuron L^{-1} was sufficient to decrease growth of wheat (Experiment 1), however, time of exposure to chlorsulfuron was important, as 7-d exposure was insufficient for growth decreases, but shoot growth was decreased by 12-d exposure.

The fact that nutrient concentrations in tissues were still decreased by chlorsulfuron treatment even though the plants were grown in circulated nutrient solution suggests that factors other than chlorsulfuron-reduced root surface area (Dong *et al.*, 1995) are responsible for the observed induced nutrient deficiency symptoms. Decreased water content of roots and shoots in chlorsulfuron-treated plants compared to untreated plants also indicates impaired water relations which may alter nutrient requirements. The results in this chapter indicate that further study of the influence of chlorsulfuron on Zn uptake in wheat is worthwhile as the observed decrease in Zn concentration is not a simple response to decreased root growth.

2.5 References

Beyer, E. M., Duffy, M. J., Hay, J. V., and Schlueter, D. D. (1988). Sulfonylureas. In Kearney, P. C., and Kaufman, D. D. Eds. *Herbicides: Chemistry, degradation and mode of action.* Marcel Dekker. pp. 117-189.

Bowen, J. E. (1969). Absorption of Cu, Zn and Mn by sugarcane leaf tissue. *Plant Physiology* 44, 253-261.

Bowran, D. G. and Blacklow, W. M. (1987). Sensitivities of spring wheat cultivars to chlorsulfuron were measured as inhibitions of leaf elongation rates and there were genotype × environment interactions. *Australian Journal of Agricultural Research.* 38, 253-262.

Bowran, D. G., Porritt, S., and Madin, R. (1987). Chlorsulfuron and diclofop-methyl effects on the copper and zinc status and yield of wheat. In Lemerle, D., and Leys, A. R. Eds. *Proceedings of the Eighth Australian Weeds Conference, 1987.* pp. 404-407.

Cumbus, I. P. (1985). Development of wheat roots under zinc deficiency. *Plant and Soil* 83, 313-316.

Dastgheib, F., Field, R. J. and Namjou, S. (1994). The mechanism of differential response of wheat cultivars to chlorsulfuron. *Weed Research* 34, 299-308.

Dong, B., Rengel, Z. and Graham, R. D. (1995). Effects of herbicide chlorsulfuron on growth and nutrient uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant and Soil* 173, 275-282.

Hatzidimitriou, A. G., Kessissoglou, D. P., Manoussakis, G. E., Kourounakis, P. N. and Economidis, G. (1990). Solid and solution behavior of sulphonylurea complexes with ions of IIA group metals. Molecular modeling of $K[Zn(ClCH_4SO_2NCONHC_3H_7)_3]$ and action of zinc-sulphonylurea complexes as hypoglycemic agents. *Journal of Inorganic Biochemistry* 39, 263-276.

Lemerle, D. and Cousens, R. D. (1993a). Influence of growth stage on spring barley (*Hordeum vulgare*) tolerance to chlorsulfuron. *Weed Science* 41, 127-132.

Lemerle, D. and Cousens, R. D. (1993b). Responses of spring barley (*Hordeum vulgare* L.) and spring wheat (*Triticum aestivum* L.) to foliar or root entry of chlorsulfuron. *Weed Research* 33, 335-344.

McLay, L. D. and Robson, A. D. (1992). The effect of chlorsulfuron and diclofop-methyl on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 43, 59-65.

Norvell, W. A. (1991). Reactions of metal chelates in soils and nutrient solutions. In Mortvedt, J. J., Cox, F. R., Shuman, L. M., and Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition*. Soil Science Society of America. pp. 187-228.

Norvell, W. A. and Welch, R. M. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: I. Zinc ion requirements. *Plant Physiology* 101, 619-625.

Osborne, L. D. and Robson, A. D. (1992). Duration of zinc uptake inhibition by chlorsulfuron in wheat. *Australian Journal of Agricultural Research* 43, 1169-1174.

Osborne, L. D. and Robson, A. D. (1993). What is the significance of herbicide nutrient interactions on wheat? *Plant and Soil* 156, 529-532.

Osborne, L. D., Robson, A. D. and Bowran, D. G. (1993). The impact of chlorsulfuron and diclofop-methyl on nutrient uptake by wheat. *Australian Journal of Agricultural Research* 44, 1757-1766.

Parker, D. R. (1993). Novel nutrient solutions for zinc nutrition research: buffering free $zinc^{2+}$ with synthetic chelators and P with hydroxyapatite. *Plant and Soil* 155/156, 461-464.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Reid, R. J., Brookes, J. D., Tester, M. A. and Smith, F. A. (1996). The mechanism of zinc uptake in plants - characterisation of the low-affinity system. *Planta* 198, 39-45.

Rengel, Z. and Graham, R. D. (1995a). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. I. Growth. *Plant and Soil* 176, 307-316.

Rengel, Z. and Graham, R. D. (1995b). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake. *Plant and Soil* 176, 317-324.

Rengel, Z. and Graham, R. D. (1996). Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. *Journal of Experimental Botany* 47, 217-226.

Reuter, D. J., and Robinson, J. B. (1986). Plant analysis: An interpretation manual. Inkata Press, Melbourne.

Robson, A. D. and Snowball, K. (1989). The effect of 2-(4-2',4'-dichlorophenoxy-phenoxy)-methyl propanoate on the uptake and utilization on zinc by wheat. *Australian Journal of Agricultural Research* 40, 981-990.

Robson, A. D. and Snowball, K. (1990). The effect of chlorsulfuron on the uptake and utilization of copper and zinc in wheat. *Australian Journal of Agricultural Research* 41, 19-28.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Sharma, P. N., Kumar, N. and Bisht, S. S. (1994). Effect of zinc deficiency on chlorophyll content, photosynthesis and water relations of cauliflower plants. *Photosynthetica* 30, 353-359.

Sharma, P. N., Tripathi, A. and Bisht, S. S. (1995). Zinc requirement for stomatal opening in cauliflower. *Plant Physiology* 107, 751-756.

Sposito, G. and Mattigod, S. V. (1980). GEOCHEM: A computer program for the calculation of chemical equilbria in soil solutions and other natural water systems. Kearney Foundation of Soil Science, University of California, Riverside.

Webb, M. J. and Loneragan, J. F. (1990). Zinc translocation to wheat roots and its implications for a phosphorus/zinc interaction in wheat plants. *Journal of Plant Nutrition* 13, 1499-1512.

Welch, R. M. and Norvell, W. A. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* 101, 627-631.

Welch, R. M., Webb, M. J., and Loneragan, J. F. (1982). Zinc in membrane function and its role in phosphorus toxicity. In Scaife, A. Ed(s). *Proceedings of the Ninth plant nutrition colloquium, Warwickshire, England.* Commonwealth Agricultural Bureaux. pp. 710-715.

Zar, J. H. (1984). Biostatistical analysis, 2nd Edn. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

CHAPTER 3

CHLORSULFURON REDUCES GROWTH OF ZINC-DEFICIENT WHEAT ROOT TIPS IN SOLUTION CULTURE[1]

3.0 Abstract

Root tip extension was used as a measure of wheat root growth during exposure to the sulfonylurea herbicide chlorsulfuron. Plants previously grown in low-zinc (0.2 μ*M* ZnHEDTA) solutions were placed in a perspex chamber with nutrient solution either side of a partition separating the root tip from the rest of the plant. The root tip was exposed to concentrations of 30, 120 and 400 μg chlorsulfuron L^{-1} and observed for 22 h. Increasing the zinc concentration of the solution around the root tip from 0.2 to 4 μ*M* ZnHEDTA did not alter root tip growth in the absence of chlorsulfuron (≈13 mm/22h). Significant decreases in root growth after 22 h (≤ 35 % of control root tip extension) were obtained with concentrations of ≥ 120 μg chlorsulfuron L^{-1}. Increasing zinc concentration from 0.2 to 20 μ*M* ZnHEDTA in the nutrient solution around the root tip enabled chlorsulfuron-treated plants to grow at the same rate as control plants (≈9 mm/22h). Adding Zn and chlorsulfuron to the more mature root parts above the root tip partition did not significantly influence root tip growth. The conclusion is that chlorsulfuron inhibits wheat root growth and that increased Zn supply alleviates or prevents deleterious effects of chlorsulfuron.

3.1 Introduction

There is increasing reliance on herbicides for weed control as minimum tillage practices are employed to minimise soil degradation (Reuter *et al.*, 1988). Herbicides that do not affect non-target species (plants or animals) and have greater efficacy are preferable. Sulfonylurea herbicides are atractive in this situation due to their low application rates (10-25 g ha^{-1}, Parsons, 1992), high target specificity and low mammalian toxicity (Beyer *et al.*, 1988).

---

[1]This chapter has been used as the basis for a manuscript that has been prepared for submission to the journal *Annals of Botany*. The formatting of the text and figures has been altered to match the rest of this thesis.

At the same time, Zn deficiency is a common problem in the soils of wheat growing regions of southern and western Australia (Reuter *et al.*, 1988). Use of plant cultivars that are able to grow well in these soils (zinc-efficient varieties, Graham *et al.*, 1992) is often recommended or required in order to obtain economic yields.

The use of the sulfonylurea herbicide chlorsulfuron (Glean) was first found to aggravate zinc deficiency in wheat crops growing on low-Zn soils in Western Australia (Bowran *et al.*, 1987). Application at recommended rates reduced growth and tillering but not final yield of several cultivars varying in tolerance to chlorsulfuron. Subsequent experiments showed chlorsulfuron decreased root and shoot dry weights of wheat at low soil-Zn concentrations (McLay and Robson, 1992; Robson and Snowball, 1989, 1990). Zinc concentration of leaves was also decreased in plants treated with chlorsulfuron (McLay and Robson, 1992; Robson and Snowball, 1990). These effects diminished with time as the herbicide degraded in soil (Osborne and Robson, 1992).

A decrease in Zn concentration in wheat shoots during chlorsulfuron treatment may be due to reduced root growth, limiting the root surface area available for absorbing Zn from the soil. Wheat root length and surface area were reduced by Zn deficiency and by chlorsulfuron addition (Dong *et al.*, 1995; McLay and Robson, 1992). Low root surface area would reduce uptake of diffusion-limited nutrients particularly, leading to decreased tissue Zn concentrations and decreased plant size (Osborne *et al.*, 1993). If plants have insufficient Zn at early stages of growth, they may be unable to reach maturity or it may be delayed significantly, reducing yields (Nable and Webb, 1993).

The experiments presented in this chapter were conducted to investigate the influences of Zn availability and chlorsulfuron treatment on wheat root tip extension in solution culture. The response of wheat grown in low-Zn nutrient solution to addition of Zn to the nutrient solution was tested to determine whether root tip extension increased or decreased. Addition of chlorsulfuron to the solution was tested to determine the degree of response of root tip extension.

3.2 Materials and methods

3.2.1 Seed

Wheat cultivar Gatcher was used in this experiment. Gatcher is a Zn inefficient cultivar: that is, it suffers significant growth reduction under conditions of Zn deficiency (Graham *et al.*, 1992; Nable and Webb, 1993). Seeds were collected from a trial conducted on Zn-deficient field sites and had a low level of Zn in seed. Average Zn content (± SE) of seed was 320 ± 30 ng Zn/grain.

3.2.2 Germination procedure

Seeds were surface-sterilised by soaking in double-deionised water (DD water; 18 MΩ.cm resistance; Milli-Q® water purification system) for 30 min, washing in 70 % (v/v) ethanol for 1 min, soaking in 1.4 % (v/v) sodium hypochlorite for 5 min, then rinsing in 10 changes of DD water followed by pre-germination for 36 h in an aerated solution of 5 mg L^{-1} Bayleton® fungicide. Individual seeds were then transferred to 1.5 mL Eppendorf tubes with the bases removed and placed into holes cut in the lids of 2-L black plastic pots (24 plants/pot).

3.2.3 Growth conditions

Pots were filled with chelate-buffered nutrient solutions (Parker, 1993; Norvell and Welch, 1993), made with DD water and analytical grade compounds. Pots were aerated continuously. The final concentrations (in m*M*) of nutrients supplied in solution to plants were: 2.0 $Ca(NO_3)_2$; 0.5 $MgSO_4$; 1.5 KNO_3; 0.1 KCl; 0.01 H_3BO_3; 0.0001 Na_2MoO_4. Metal nutrients were supplied as chelates of HEDTA (Norvell and Welch, 1993) at the following concentrations (μ*M*): 0.2 Zn; 0.5 Cu; 1 Mn; 0.1 Ni; 100, Fe. In addition, 25 μ*M* K_3HEDTA was added to keep ionic activities of free micronutrient cations at very low levels. The activity of Zn^{2+} in the growth solution was 4.2 p*M* (calculated using GEOCHEM; Sposito and Mattigod, 1980). The solution was buffered at pH 6.0 with 1 m*M* MES. To avoid Zn-deficiency-induced P toxicity (Parker, 1993; Welch *et al.*, 1982), $NH_4H_2PO_4$ was added at 1 μ*M* day^{-1} after d 6.

Plants were kept in a growth chamber with a 15/10 °C, 10/14 h light/dark cycle and a photosynthetically active irradiance of 300 μmol m^{-2} s^{-1} at plant tops. Plants were grown in half-strength nutrient solution for the first six days then changed to full-strength solution on days 6, 10, 14, and 16. Freshly made solution was used when measurements were made in the compartmented root-growth box.

3.2.4 Root tip extension measurements

Measurements of root tip extension (RTE; increases in root length with time) were made using a compartmented perspex chamber similar in design to that used by Huang *et al.* (1993). The internal dimensions of the chamber were 150 × 40 × 30 mm. Tip extension of one main root was measured for each plant by placing the root in the chamber so that the root tip could be isolated by a partition created by placing a 40 × 30 × 2.5 mm perspex block approximately half-way along the chamber. The partition was notched to allow the root to remain intact and then sealed with vacuum grease. Leaks were detected by changes in water level on each side of the chamber and repaired before measurements commenced. Approximately 5 mm of root were allowed to protrude into the "tip" side of the chamber. Length of the root tip was measured by placing a sheet of overhead projector transparency with 1 mm lines inside the chamber and measuring from the partition. Extension was calculated by subtracting the starting length from the lengths obtained at subsequent times. Root length was measured immediately after setting up, every hour for 7 h, then at 22 h.

Several experiments were conducted, in which the conditions of the solution on the "root-tip" side of the partition were altered. All experiments used 0.2 μ*M* ZnHEDTA on the "shoot" side of the partition unless specified otherwise. One plant root per replicate was used in each case.

Experiment 3.1 measured RTE of plants varying in age from 10 to 24 d. Zinc concentration in the "root-tip" compartment was 0.2 or 4 μ*M* ZnHEDTA and chlorsulfuron was applied at 0 or 30 μg L^{-1} (83 n*M* chlorsulfuron) after roots were placed in the box. A full factorial design of 2 Zn × 2 chlorsulfuron with 3

replicates per treatment and complete randomisation of treatments was employed. The results from this experiment determined the optimal age of plants for use in the remaining experiments.

The second experiment (3.2) measured the effect of four concentrations of chlorsulfuron (0, 30, 120 and 400 μg chlorsulfuron L^{-1}) on RTE of plants grown in 0.2 μ*M* ZnHEDTA for 12 d. Four replicates per chlorsulfuron treatment were used. Experiment 3.3 examined the effect of ZnHEDTA concentration (0.2, 2, 20 μ*M*) and exposure to 0 or 120 μg chlorsulfuron L^{-1} on RTE of plants grown for 12 d in 0.2 μ*M* ZnHEDTA solution (3 Zn × 2 chlorsulfuron treatments with four replicates per treatment). Experiment 3.4 examined the effect of adding 120 μg chlorsulfuron L^{-1}, 2 μ*M* ZnHEDTA or both to the solution above the root tip (3 treatments × 4 replicates).

Average root tip extension after 22 h was analysed by analysis of variance and Fisher's LSD. Means were considered significantly different at $P \leq 0.05$.

3.3 Results

There was a tendency for chlorsulfuron to decrease root tip extension of 10 to 24-d-old plants (Fig. 3.1, 12-d and 20-d results shown). After 22 h, 30 µg chlorsulfuron L^{-1} decreased RTE of 18-d-old plants by up to 50 % (data not shown). Variability of results increased in older plants and effects due to chlorsulfuron became less distinct. No differences in root growth could be seen until at least 7 h after addition of chlorsulfuron in plants from either age (Fig. 3.1).

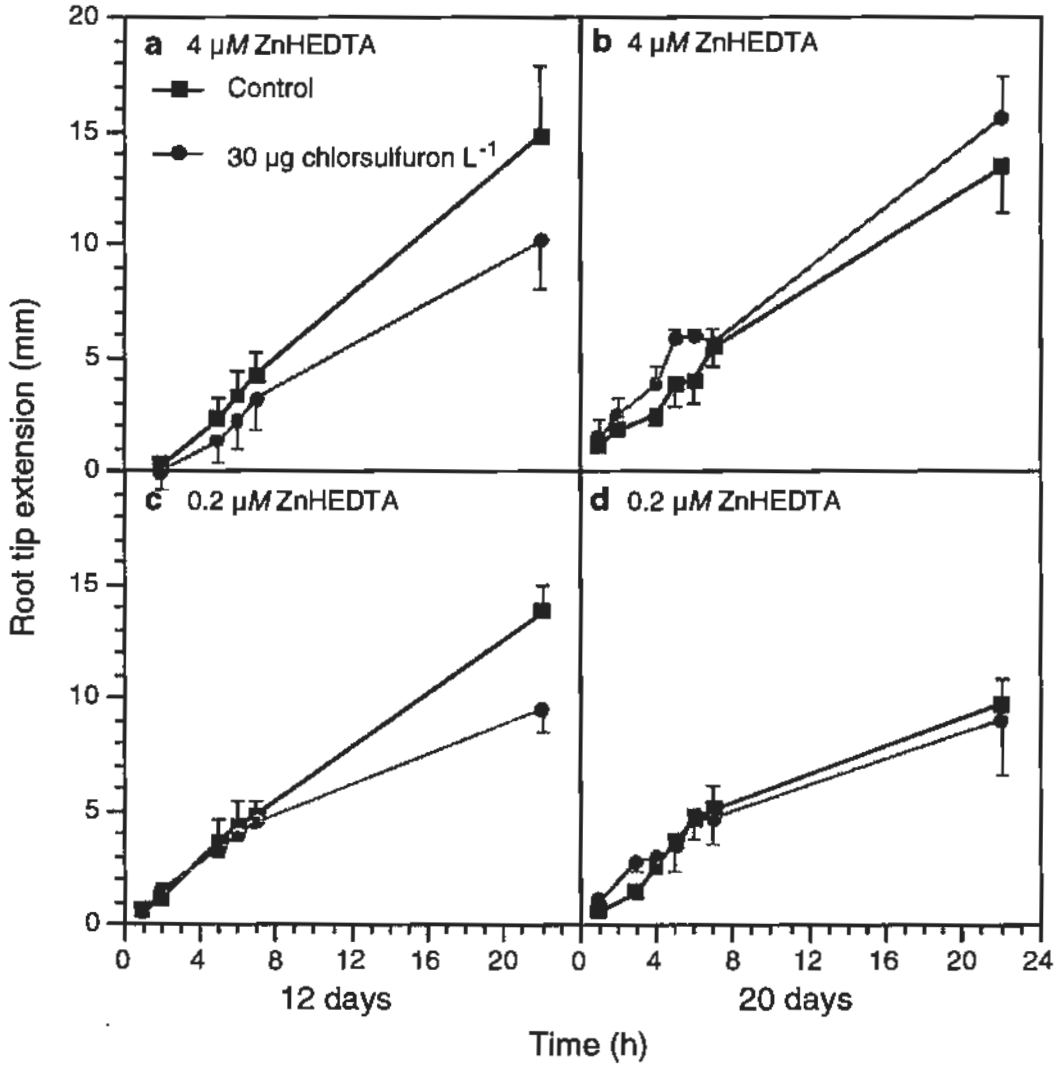

Figure 3.1. Root tip extension (mm) of Gatcher wheat in solutions containing 4 µM ZnHEDTA (a, b) or 0.2 µM ZnHEDTA (c, d) at 12 d (a, c) or at 20 d (b, d) after sowing. All plants were pre-grown in solution containing 0.2 µM ZnHEDTA. Chlorsulfuron (30 µg L^{-1}) was applied to root tips only after plants were placed in the compartmented root-growth box (time = 0). Data points are mean ± SE, n = 3.

Analyses of final RTE showed that there was no zinc × chlorsulfuron interaction in either 12- or 20-d-old plants. Root tip extension in low- and high-Zn solutions was significantly ($P \leq 0.04$) reduced by 30 µg chlorsulfuron L^{-1} at 12 d but not at 20 d ($P \leq 0.70$). Increasing the Zn concentration around the root tip significantly increased root extension of 20-d-old plants ($P \leq 0.02$) but not that of 12-d-old plants ($P \leq 0.19$). The remaining experiments used 12-d-old plants.

RTE was decreased proportionally more as the chlorsulfuron concentration increased (Fig. 3.2). The 30 µg chlorsulfuron L^{-1} treatment had no significant effect on RTE compared to the control treatment during the first 7 h of measurements, but after 22 h, had decreased RTE to 75 % of the control roots. The higher rates of 120 and 400 µg chlorsulfuron L^{-1} decreased RTE rapidly and effects could be detected within 2-3 hours of application (Fig. 3.2). After 22 h, 120 and 400 µg chlorsulfuron L^{-1} had decreased RTE to 35 % and 25 % respectively, of the control roots.

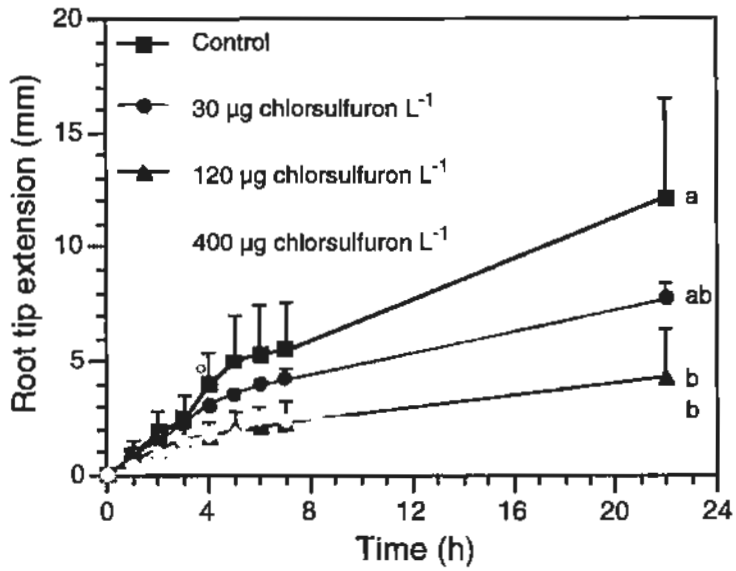

Figure 3.2. Root tip extension of Gatcher wheat in solution containing 0.2 µM ZnHEDTA and exposed to 0 (Control), 30, 120 or 400 µg chlorsulfuron L^{-1} for 22 h. Plants were pre-grown in nutrient solution containing 0.2 µM ZnHEDTA for 12 d. Chlorsulfuron was applied to root tips only after plants were placed in the compartmented root-growth box. Identical letters indicate treatments that do not differ significantly from each other (Fisher's LSD test, $\alpha \leq 0.05$). Data points are mean ± SE, n = 4.

Analysis of variance and Fisher's LSD indicated a significant difference between the RTE of the control treatment and 120 and 400 μg chlorsulfuron L^{-1} treatments (Fig. 3.2). The 30 μg chlorsulfuron L^{-1} treatment was grouped with the control root treatment and with the two highest chlorsulfuron concentration treatments (Fig. 3.2).

Increasing the ZnHEDTA concentration of the solution around the root tip generally improved the RTE of chlorsulfuron-treated plants compared to control plants (Fig. 3.3a-c). Without chlorsulfuron, root tips grown in 0.2 μ*M* ZnHEDTA solutions extended more than root tips in higher Zn activity solutions. Addition of chlorsulfuron decreased the RTE of roots in 0.2 μ*M* ZnHEDTA by the greatest amount (56 %). At the intermediate Zn concentration (2 μ*M* ZnHEDTA), the RTE of chlorsulfuron-treated plants was decreased by 38 % compared to control plants (Fig. 3.3b). The highest concentration of Zn (20 μ*M* ZnHEDTA) allowed chlorsulfuron-treated roots to extend as much as control roots (Fig. 3.3c). Chlorsulfuron-treated roots supplied with 20 μ*M* ZnHEDTA increased RTE compared to the chlorsulfuron-treated roots at lower Zn concentrations.

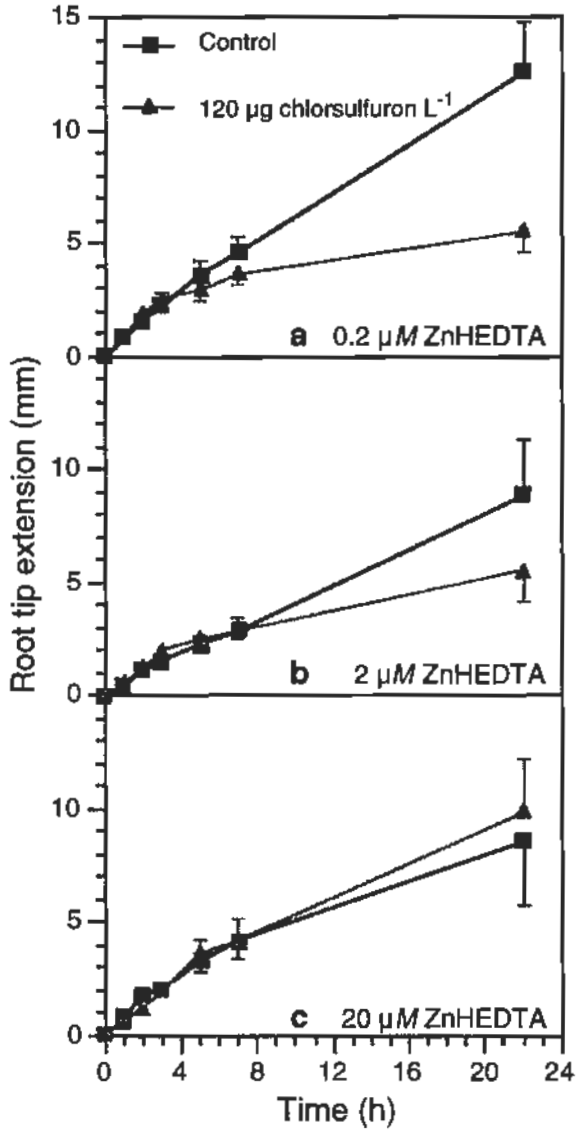

Figure 3.3. Root tip extension of Gatcher wheat in solutions containing 0.2 (a), 2 (b) and 20 µ*M* ZnHEDTA (c). Plants were pre-grown in nutrient solution containing 0.2 µ*M* ZnHEDTA for 12 d. Chlorsulfuron (120 µg L^{-1}) and zinc treatments were applied to root tips only after plants were placed in the compartmented root-growth box. Data points are mean ± SE, n = 4.

Adding chlorsulfuron to the more mature parts of the root system (above the root tip partition) did not significantly influence RTE compared to control roots grown in 0.2 μ*M* ZnHEDTA solution (Fig. 3.4). RTE was not altered when increased Zn concentration (2 μ*M* ZnHEDTA) and 120 μg chlorsulfuron L^{-1} were added to the solution above the partition. There was no significant difference between the three treatments ($P \leq 0.63$).

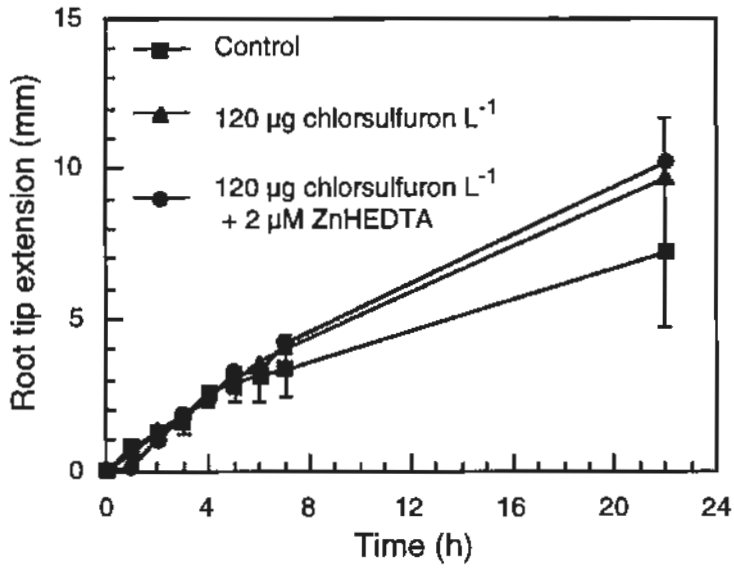

Figure 3.4. Root tip extension of Gatcher wheat in solution containing 0.2 μ*M* ZnHEDTA on the root-tip side of the partition. Chlorsulfuron (120 μg L^{-1}) and 2 μ*M* ZnHEDTA were added to roots above the root-tip partition after plants were placed in the compartmented root-growth box. Plants had been pre-grown in nutrient solution containing 0.2 μ*M* ZnHEDTA for 12 d. Data points are mean ± SE, n = 4.

3.4 Discussion

The primary site of action of chlorsulfuron in susceptible species is inhibition of acetolactate synthase (ALS, E.C. 4.1.3.18, also known as acetohydroxyacid synthase, AHAS, Beyer *et al.*, 1988). ALS is the primary step in the enzyme pathway responsible for production of branched-chain amino acids (LaRossa and Schloss, 1984). Chlorsulfuron treatment reduces branched chain amino acids levels and can reduce protein synthesis (Landstein *et al.*, 1996; Royuela *et al.*, 1991).

The mode of action of chlorsulfuron is inhibition of cell division, causing decreased growth in meristematic regions of roots and shoots (Ray, 1982, 1984). Chlorsulfuron reduced extension of pea (*Pisum sativum*) root sections after 4-d exposure to 2.8-28 n*M* chlorsulfuron (1-10 μg L^{-1}; Ray, 1984). Maize (*Zea mays*) seedling root fresh weights were reduced by 2.8-2800 n*M* (1-1000 μg L^{-1}) chlorsulfuron after 2 d (Ray, 1982). Similarly, maize root growth was inhibited by 4- and 10-d exposure to 100 n*M* (36 μg L^{-1}) chlorsulfuron (Royuela *et al.*, 1991). The higher concentrations of chlorsulfuron used in this study (83-1120 n*M*) and lower inhibition of root tip extension indicate the greater tolerance of wheat to chlorsulfuron than pea and maize plants.

Wheat plants often suffer reductions in root growth (DW and length) when grown in chlorsulfuron-treated soil (Robson and Snowball, 1990). The effects of chlorsulfuron on wheat root growth and nutrient status were greater when plants were grown in low-Zn soils (*e.g.* Dong *et al.*, 1995; McLay and Robson, 1992; Osborne and Robson, 1992). Chlorsulfuron also decreased root weight and root length in solution-cultured wheat plants (McLay and Robson, 1992; see also chapter 4). Sulfonylurea herbicides reduce root growth in a number of species; sulfometuron-methyl reduced root growth of barley plants in fields with low micronutrient status (Pederson *et al.*, 1994), metsulfuron-methyl reduced wheat root lengths in pot experiments in 4 days (Flaburiari and Kristen, 1996) and bensulfuron decreased root length of rice after 6 days (Yim and Bayer, 1996).

In the present study, inhibition of wheat root tip extension appears to occur relatively rapidly (within 22 h and as little as 2-3 h) when exposed to

chlorsulfuron. In older plants, the effects of chlorsulfuron on RTE became less pronounced (Fig. 3.1), presumably due to greater stores of Zn and other reserves (*e.g.*, nutrients, amino acids, carbohydrates) that could be used as chlorsulfuron inhibited acetolactate synthase activity in the roots. Twenty-days-old plants showed no significant differences in RTE when treated with chlorsulfuron for 22 h in low or high Zn solutions (Fig. 3.1b, 3.1d). Twelve-day-old plants responded to chlorsulfuron treatments with the least amount of variability in results, and were therefore used for the remaining experiments.

In this study, root extension of Gatcher wheat was decreased by higher Zn supply (Fig. 3.3). An overall increase in root length could occur if root tip growth was promoted by Zn-deficient conditions. Wheat root growth responses to Zn deficiency can vary considerably, however Zn-deficient wheat in nutrient solution can increase root DW compared to adequately supplied plants (Rengel and Graham, 1995b). In other cases, shoot:root ratio (see Chapter 2) decreased as root mass increased under Zn-deficiency *e.g.* 12- to 19-day-old wheat plants in Zn-deficient nutrient solution had reduced shoot DW and unchanged root DW in comparison to adequate-Zn control plants (Webb and Loneragan, 1988). Root DW of mature plants grown in Zn-deficient subsoil also did not change compared to adequate-Zn subsoil (Nable and Webb, 1993). Other cultivars, including the one used in the present experiments, grown in Zn-deficient soils, experienced reduced root dry weight compared to adequate-Zn control plants (Dong *et al.*, 1995; Rengel and Graham, 1995a; Robson and Snowball, 1989, 1990).

Zinc-deficient plants can increase root length while having reduced root mass in order to maximise root surface area for Zn uptake, *e.g.* wheat plants in Zn-deficient soil increased root length per unit of root weight compared to Zn-supplemented soil over 21-28 days of growth (Osborne and Robson, 1992). Roots extended less when transferred to solution with increased Zn (Fig. 3.3), as the limiting resource was more available, and roots did not have to extend as much to increase the nutrient gathering capacity to obtain Zn (Dong *et al.*, 1995). Prolonged growth in low Zn solution would eventually reduce root growth as

nutrient supplies ran out and roots became Zn-deficient. However, this state is unlikely to be reached since nutrient solutions were replaced at regular intervals.

The concentrations of Zn applied to the root tip solution appear much greater than normal soil solution Zn^{2+} concentrations of 0.01-1 μM (Kochian, 1991), however the free Zn activity of the HEDTA-chelated solutions is considerably less than the stated concentrations, *e.g.* a 0.2 μM ZnHEDTA solution has approximately 4 pM free Zn, and 4 μM ZnHEDTA solution approximately 83 pM. Even at 20 μM ZnHEDTA (Fig. 3.3c), the free Zn activity would be around 400 pM which is still much lower than normal soil solution Zn activity. The lower activity of Zn in chelated solutions is sustained by the large buffered Zn concentration, allowing a constant supply of Zn over time (Marschner, 1993). Zinc fertilization of wheat treated with chlorsulfuron may not need to be excessive to allow plants to overcome inhibition of root tip extension.

Chlorsulfuron-treated roots grew as well as control roots in high-Zn concentration solution and grew more than chlorsulfuron-treated roots in low-Zn concentrations (Fig. 3.3). In low-Zn solution, chlorsulfuron-treated roots grew significantly less than control roots (*e.g.* Fig. 3.1c, 3.3a). Zinc supplementation increased root tip extension of chlorsulfuron-treated roots at the highest level of Zn. Recovery of root growth by addition of Zn to chlorsulfuron-treated of roots was not examined in the present study, but Zn uptake rates of chlorsulfuron-treated plants can recover after transferring plants to chlorsulfuron-free solutions for several days (see Chapter 4). Deleterious effects of chlorsulfuron on root growth were overcome by increasing the Zn supply in soil or degradation of chlorsulfuron with time (Osborne and Robson, 1992).

In the present study, the presence of chlorsulfuron appeared detrimental to root growth (RTE) except in solutions with relatively high concentrations of Zn, suggesting that Zn supplementation may prevent the inhibition of root growth. Zinc may have improved the membrane structure of the roots by maintaining the sulfhydryl groups in the root-cell plasma membrane in the reduced form (Rengel, 1995; Welch and Norvell, 1993), or decreasing membrane permeability to chlorsulfuron. Zinc supplementation may have also improved general

physiological status of the plants, allowing them to better tolerate the effects of chlorsulfuron. If chlorsulfuron can chelate Zn in the external solution as suggested by Hatzidimitriou *et al.*, (1990), the Zn will be less available for plant uptake, and increased Zn activity will be required for the same level of root tip extension as low-Zn plants without chlorsulfuron treatment..

Root tip extension was not significantly altered by addition of Zn above the root tip (Fig. 3.4), suggesting that Zn was not translocated toward the root tip in sufficient quantities to supply the root tip requirements. Zinc can be translocated via the phloem towards root tips of low-Zn plants (Pearson and Rengel, 1995), even though the amount translocated may not be sufficient for all requirements (Webb and Loneragan, 1990).

Since Zn is transported through soil toward roots primarily by diffusion (Kochian, 1993), chlorsulfuron-induced root growth inhibition will result in less root surface area available for uptake of Zn from soils, and consequently, reduced Zn levels within plants (Dong *et al.*, 1995; McLay and Robson, 1992). These effects will deleteriously affect wheat growth, especially on soils low in plant-available Zn, as those that abound in southern and western parts of Australia.

3.5 References

Beyer, E. M., Duffy, M. J., Hay, J. V. and Schlueter, D. D. (1988). Sulfonylureas. In Kearney, P. C., Kaufman, D. D. Eds. *Herbicides: Chemistry, degradation and mode of action.* Marcel Dekker. pp. 117-189.

Bowran, D. G., Porritt, S. and Madin, R. (1987). Chlorsulfuron and diclofop-methyl effects on the copper and zinc status and yield of wheat. In Lemerle, D., Leys, A. R. Eds. *Proceedings of the Eighth Australian Weeds Conference, 1987.* pp. 404-407.

Dong, B., Rengel, Z. and Graham, R. D. (1995). Effects of herbicide chlorsulfuron on growth and nutrient uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant and Soil* 173, 275-282.

Flaburiari, A. and Kristen, U. (1996). The influence of chlorsulfuron and metsulfuron methyl on root growth and on the ultrastructure of root tips of germinating maize seeds. *Plant and Soil* 180, 19-28.

Graham, R. D., Ascher, J. S. and Hynes, S. C. (1992). Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant and Soil* 146, 241-250.

Hatzidimitriou, A. G., Kessissoglou, D. P., Manoussakis, G. E., Kourounakis, P. N. and Economidis, G. (1990). Solid and solution behavior of sulphonylurea complexes with ions of IIA group metals. Molecular modeling of $K[Zn(ClCH_4SO_2NCONHC_3H_7)_3]$ and action of zinc-sulphonylurea complexes as hypoglycemic agents. *Journal of Inorganic Biochemistry* 39, 263-276.

Huang. J. W., Grunes, D. L. and Kochian, L. V. (1993). Aluminum effects on calcium ($^{45}Ca^{2+}$) translocation in aluminum-tolerant and aluminum-sensitive wheat (*Triticum aestivum* L.) cultivars. *Plant Physiology* 102: 85-93.

Kochian, L. V. (1991). Mechanisms of micronutrient uptake and translocation in plants. In Mortvedt, J. J., Cox, F. R., Shuman, L. M. and Welch, R. M. Eds. *Micronutrients in agriculture, 2nd edition.* Soil Science Society of America. pp. 229-296.

Kochian, L. V. (1993). Zinc absorption from hydroponic solutions by plant roots. In Robson, A. D. Ed. *Zinc in soils and plants*. Kluwer Academic Publishers. pp. 45-58.

Landstein, D., Epelbaum, S., Arad, S., Barak, Z. and Chipman, D. M. (1995). Metabolic response of *Chlorella emersonii* to the herbicide sulfometuron methyl. *Planta* 197: 219-224.

LaRossa, R. A. and Schloss, J. V. (1984). The herbicide sulfometuron methyl is bacteriostatic due to inhibition of acetolactate synthase. *Journal of Biological Chemistry* 259, 8753-8757.

Marschner, H. (1993). Zinc uptake from soils. In Robson, A. D. Ed. *Zinc in soils and plants*. Kluwer Academic Publishers. pp. 59-78.

McLay, L. D. and Robson, A. D. (1992). The effect of chlorsulfuron and diclofop-methyl on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 43, 59-65.

Nable, R. O. and Webb M. J. (1993). Further evidence that zinc is required throughout the root zone for optimal plant growth and development. *Plant and Soil* 150, 247-253.

Norvell, W. A. and Welch, R. M. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: I. Zinc ion requirements. *Plant Physiology* 101, 619-625.

Osborne, L. D. and Robson, A. D. (1992). Duration of zinc uptake inhibition by chlorsulfuron in wheat. *Australian Journal of Agricultural Research* 43, 1169-1174.

Osborne, L. D., Robson, A. D. and Bowran, D. G. (1993). The impact of chlorsulfuron and diclofop-methyl on nutrient uptake by wheat. *Australian Journal of Agricultural Research* 44, 1757-1766.

Parker, D. R. (1993). Novel nutrient solutions for zinc nutrition research: buffering free $zinc^{2+}$ with synthetic chelators and P with hydroxyapatite. *Plant and Soil* 155/156, 461-464.

Parsons, J. M. (1992). Australian weed control handbook, 9th edn. Inkata Press, Sydney.

Pearson, J. N. and Rengel, Z. (1995). Uptake and distribution of ^{65}Zn and ^{54}Mn in wheat grown at sufficient and deficient levels of Zn and Mn. I. During vegetative growth. *Journal of Experimental Botany* 46, 833-839.

Pederson, R. N., Black, I. D., Dyson, C. B. and Hannam, R. J. (1994). Effects of the herbicide metsulfuron-methyl on root length, nutrient levels, grain protein, and yield of barley. *Australian Journal of Experimental Agriculture* 34, 499-504.

Ray, T. B. (1982). The mode of action for chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 10-17.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Rengel, Z. (1995a). Carbonic anhydrase activity in leaves of wheat genotypes differing in Zn efficiency. *Journal of Plant Physiology* 147, 251-256.

Rengel, Z. (1995b). Sulfhydryl groups in root-cell plasma membranes of wheat genotypes differing in Zn efficiency. *Physiologia Plantarum* 95, 604-612.

Rengel, Z. and Graham, R. D. (1995a). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. I. Growth *Plant and Soil* 176, 307-316.

Rengel, Z. and Graham, R. D. (1995b). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake *Plant and Soil* 176, 317-324.

Reuter, D. J., Cartwright, B., Judson, G. J., McFarlane, J. D., Maschmedt, D. J. and Robinson, J. B. (1988). Trace elements in South Australian agriculture. *Department of Agriculture South Australia Technical Report* No. 139.

Robson, A. D. and Snowball, K. (1989). The effect of 2-(4-2',4'-dichlorophenoxy-phenoxy)-methyl propanoate on the uptake and utilization on zinc by wheat. *Australian Journal of Agricultural Research* 40, 981-990.

Robson, A. D. and Snowball, K. (1990). The effect of chlorsulfuron on the uptake and utilization of copper and zinc in wheat. *Australian Journal of Agricultural Research* 41, 19-28.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Sposito, G. and Mattigod, S. V. (1980). GEOCHEM: A computer program for the calculation of chemical equilbria in soil solutions and other natural water systems. Kearney Foundation of Soil Science, University of California, Riverside.

Webb, M. J. and Loneragan, J. F. (1988). Effect of zinc deficiency on growth, phosphorus concentration, and phosphorus toxicity of wheat plants. *Soil Science Society of America Journal* 52, 1676-1680.

Webb, M. J. and Loneragan, J. F. (1990). Zinc translocation to wheat roots and its implications for a phosphorus/zinc interaction in wheat plants. *Journal of Plant Nutrition* 13, 1499-1512.

Welch, R. M. and Norvell, W. A. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* 101, 627-631.

Welch, R. M., Webb, M. J. and Loneragan, J. F. (1982). Zinc in membrane function and its role in phosphorus toxicity. In Scaife, A. Ed. *Proceedings of the Ninth plant nutrition colloquium, Warwickshire, England.* Commonwealth Agricultural Bureaux. pp. 710-715.

Yim, K.-O. and Bayer, D.E. (1996). Root growth inhibition of rice by bensulfuron. *Weed Research* 36, 49-54.

CHAPTER 4

CHLORSULFURON REDUCES RATES OF ZINC UPTAKE BY WHEAT SEEDLINGS FROM SOLUTION CULTURE[1]

4.0 Abstract

Wheat plants differing in Zn efficiency (Excalibur; Zn-efficient, Gatcher and Durati; Zn-inefficient) were grown in HEDTA chelate-buffered nutrient solution in controlled conditions and supplied with 0 or 40 µg chlorsulfuron L^{-1}. Zinc uptake rates of 12-d-old plants were measured over 80 or 90 minutes using ^{65}Zn added to nutrient solutions. Increasing the Zn concentration of the solution (between 1 and 16 µ*M* ZnHEDTA) increased the rate of Zn uptake, while the percentage of Zn transported to shoots was decreased. Addition of chlorsulfuron to uptake solutions for 90 minutes did not influence rate of Zn uptake or transport of Zn to shoots. Pretreating plants with chlorsulfuron for 5 days decreased Zn uptake rates, but transport to shoots was proportionally increased. Three-day pretreatment with chlorsulfuron was the minimum required for significant differences in uptake and transport of Zn to occur. Plants exposed to chlorsulfuron for 3 days required a further 5 days of growth in chlorsulfuron-free solutions before uptake rates recovered to control plant rates. It is concluded that chlorsulfuron deleteriously but reversibly affects uptake of Zn across the plasma membrane after prolonged exposure.

4.1 Introduction

Sulfonylurea herbicides are widely used in Australia to control weeds in grain crops, especially in minimum tillage agriculture, due to their low application rates (10-25 g ha^{-1}; Parsons, 1992), high target specificity and low animal toxicity (Beyer *et al.*, 1988). The sulfonylurea herbicide chlorsulfuron was found to induce Zn deficiency in wheat crops growing on low-Zn soils in Western Australia (Bowran *et al.*, 1987). Zinc deficiency is a common problem in

---

[1]A manuscript based on this chapter has been accepted for publication in the journal *Plant and Soil*. The formatting of the text and figures has been modified to match the rest of this thesis.

many of the wheat growing regions in southern and western Australia (Reuter *et al.*, 1988). Zinc deficiency in early stages of wheat growth may lead to delayed maturity or significantly depressed yields (Nable and Webb, 1993).

Root and shoot growth were decreased by chlorsulfuron at low soil Zn concentrations (McLay and Robson, 1992; Robson and Snowball, 1989, 1990). Chlorsulfuron applied at recommended rates also decreased shoot Zn concentrations in wheat plants grown in Zn-deficient soil (McLay and Robson, 1992; Robson and Snowball, 1990). Decreased Zn concentrations only occurred when the chlorsulfuron was incorporated with the Zn fertiliser in soil (Robson and Snowball, 1990). Plants grown in nutrient solution did not show decreased growth or Zn concentrations, indicating that transport of soil Zn to roots by diffusion may be altered in the presence of chlorsulfuron (McLay and Robson, 1992).

The decrease in Zn concentration of wheat plants after chlorsulfuron application may be due to a simple reduction of root growth, limiting the soil volume explored by roots and decreasing the root surface area available to absorb Zn from the soil (McLay and Robson, 1992). In such a case, uptake of diffusion-limited nutrients such as Zn would be especially reduced (Osborne *et al.*, 1993). Root metabolism may also be influenced by the herbicide thus decreasing growth and indirectly reducing Zn uptake rate. Alternatively, chlorsulfuron may directly interfere with the transmembrane Zn uptake processes in roots.

The following experiments were conducted to determine whether Zn uptake by wheat plants growing in nutrient solution was influenced by chlorsulfuron treatment. Responses of cultivars differing in Zn efficiency (Graham *et al.*, 1992) were compared to determine if Zn uptake responses to chlorsulfuron were related to the level of Zn efficiency of cultivars. Times of exposure to chlorsulfuron and recovery from chlorsulfuron exposure were also examined.

4.2 Materials and Methods

4.2.1 Seed

Seed of Zn-efficient genotype Excalibur (*Triticum aestivum*) and Zn-inefficient genotypes Gatcher (*T. aestivum*) and Durati (*T. turgidum* L. conv. *durum* (Desf.) MacKey) were collected from trials conducted on Zn-deficient field sites; each cultivar had a low level of Zn in seed. Average Zn content (± SE) of Excalibur and Gatcher was 320 ± 30 ng Zn/grain and of Durati was 320 ± 40 ng Zn/grain.

4.2.2 Growing conditions

Seeds were surface-sterilised, germinated and grown in chelate-buffered nutrient solutions under controlled environmental conditions as described in Chapter 2. The final concentrations (in m*M*) of nutrients supplied in solution to plants were: 2.0, $Ca(NO_3)_2$; 0.5 $MgSO_4$; 1.5 KNO_3; 0.1 KCl; 0.01 H_3BO_3; 0.0001 Na_2MoO_4. Metal nutrients were supplied as chelates of HEDTA (Norvell and Welch, 1993) at the following concentrations (μ*M*): 0.2 Zn; 0.5 Cu; 1 Mn; 0.1 Ni; 100 Fe. In addition, 25 μ*M* K_3HEDTA was added to further decrease the activity of free micronutrient cations as an effective method of imposing deficiency stress on plants (Norvell and Welch, 1993). The free activity of Zn^{2+} was 4.2 p*M* in the growth solution (0.2 μ*M* ZnHEDTA; calculated using GEOCHEM, Rengel and Graham, 1995). The solution was buffered to pH 6.0 with 1 m*M* MES. To avoid Zn-deficiency-induced P-toxicity (Parker, 1993; Welch *et al.*, 1982), $NH_4H_2PO_4$ was added at 1 μ*M* day^{-1} after d 6. Plants were grown in half-strength nutrient solution for the first 6 days then changed to full strength solution on days 6, 10, 14, and 16.

4.2.3 Zinc uptake procedure

Experiments 1-3 were performed on 12-d-old wheat plants. Experiment 4 used 13-, 15- and 17-d-old plants. Eight plants per 1-L pot were exposed to 37 kBq L^{-1} of ^{65}Zn-labelled nutrient solution for 80 min. Uptake was stopped by transferring plants to 250 ml of ice-cold non-labelled solution otherwise identical

to the uptake solution for 25 minutes. Preliminary experiments indicated nutrient solution of the composition described above containing 25 μ*M* excess of K_3HEDTA used for uptake measurements decreased Zn uptake to levels below detection limits. To prevent this recurring, subsequent experiments did not use excess K_3HEDTA in uptake solutions.

Plants were rinsed in distilled water, gently blotted and separated into roots and shoots. Four replicates of two plants were used in each treatment. Each sample was placed in a polycarbonate tube and weighed before counting (fresh weight) and after drying at 50°C for 48 h (dry weight).

Samples were analysed using a Philips 4800W gamma-counter set to detect radiation between 0.939 and 1.3 MeV (disintegration energy of ^{65}Zn = 1.1 MeV). Background radiation was recorded using empty sample tubes. Samples (1 mL) of labelled solution from each pot were taken at the beginning of the uptake period as standards for each treatment.

Uptake rate of Zn by whole plants was calculated on a root FW basis. The percentage of total Zn taken up that was transported to shoots was calculated as (Shoot ^{65}Zn content × 100/Whole plant ^{65}Zn content).

4.2.3.1 Experiment 1; Effect of solution zinc concentration

The rate of Zn uptake by cv. Gatcher from solutions containing 1, 2, 4, 8 and 16 μ*M* ZnHEDTA (free Zn^{2+} activities calculated as 0.8, 1.6, 3.1, 6.2 and 12.3 n*M* respectively) was measured. Plants were pretreated with either 0 or 40 μg chlorsulfuron L^{-1} (0 or 112 n*M*) for 5 d before ^{65}Zn uptake measurements, then placed in fresh solution with no added chlorsulfuron during uptake. There were 10 treatments in all (5 Zn × 2 chlorsulfuron). Chlorsulfuron was applied as a solution of the commercial herbicide formulation Glean (75% chlorsulfuron), as McLay and Robson (1992) found there were no differences in the effects of the active ingredient and the herbicide. The concentration of 40 μg L^{-1} was chosen as it would not affect growth immediately (at least 7 h) but would significantly decrease root growth after a longer period (22 h; chapter 3) and approximates the concentration applied in other experiments (Dong *et al.*, 1995).

4.2.3.2 Experiment 2; Timing of chlorsulfuron addition

The effect of application time of chlorsulfuron on Zn uptake rate of Gatcher wheat was examined using a complete factorial arrangement of 0 or 40 μg chlorsulfuron L^{-1} supplied as a pretreatment for 5 days prior to uptake and 0 or 40 μg chlorsulfuron L^{-1} as a treatment during the uptake period. A 90-min ^{65}Zn uptake period was used, and uptake was measured from solutions containing 1 or 8 μ*M* ZnHEDTA. The final design was 2 5-d pretreatments × 2 uptake treatments × 2 Zn treatments = 8 treatments.

4.2.3.3 Experiment 3; Exposure time

To determine the minimum time of chlorsulfuron exposure required to obtain a significant change in Zn uptake rate, plants of the three cultivars were exposed to 40 μg chlorsulfuron for 1, 3 or 5 days (exposure from d 11, d 9 or d 7) prior to uptake measurements on d 12. A non-exposed control treatment (0 days) was included. The final design was 4 exposure times × 3 cultivars = 12 treatments.

4.2.3.4 Experiment 4; Recovery after minimum exposure time

Gatcher and Excalibur plants were supplied with 0 or 40 μg chlorsulfuron L^{-1} for the minimum exposure time required to produce an effect in Zn uptake (3 d, Exp. 3) and allowed to recover in chlorsulfuron-free nutrient solution for 1, 3, or 5 days. Uptake rates were therefore measured on d 13, 15 and 17 after planting. Non-treated controls were included for each recovery time to determine changes in uptake rate with plant age. The final design was therefore 2 cultivars × 3 recovery times = 6 treatments.

4.2.4 Statistics

Results were analysed by ANOVA and Fisher's LSD test. Differences between means were significant at $P \leq 0.05$. Percentage data are presented as non-transformed data since arcsin transformations for analysis (Zar 1984) did not change the significance of results.

4.3 Results

4.3.1 Experiment 1; Effect of solution zinc concentration

The Zn uptake rate increased with level of Zn in solution and was decreased by 5 days of pretreatment with chlorsulfuron (Fig. 4.1). Uptake rate increased with Zn concentration of solution at different rates depending on the pretreatment. Control plants reached a plateau after 8 μ*M* ZnHEDTA while chlorsulfuron-treated plants maintained lower rates of Zn uptake at each Zn concentration. Experiments 2, 3 and 4 used 1 (low Zn) and 8 (high Zn) μ*M* ZnHEDTA in the uptake solutions as these appeared to give a suitable range of uptake rates.

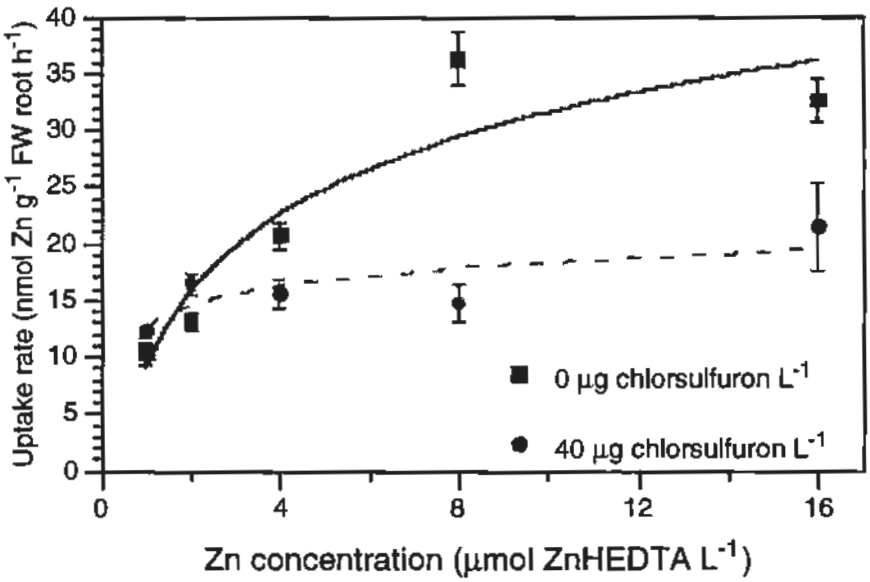

Figure 4.1. Zinc uptake rate of 12-d-old Gatcher wheat plants when transferred to solutions with a range of ZnHEDTA concentrations. Chlorsulfuron-pretreated plants were supplied with 40 μg chlorsulfuron L^{-1} for 5 days prior to uptake measurements. Control plants received no chlorsulfuron at any stage. Error bars represent ± SE (n = 4). Equations for the curves indicated are: Control uptake rate (solid line) = 9.78 × Ln(ZnHEDTA) + 9.05, r^2 = 0.86; Chlorsulfuron-pretreated uptake rate (dashed line) = 2.43 × Ln(ZnHEDTA) + 12.75, r^2 = 0.61.

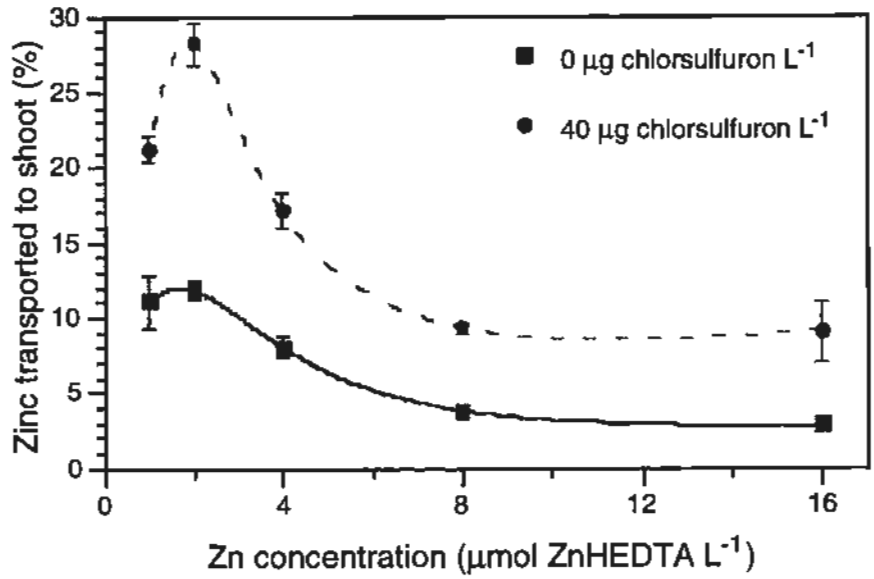

Figure 4.2. Effects of pretreatment with chlorsulfuron, supplied for 5 d prior to uptake measurements, and various concentrations of ZnHEDTA in the uptake solution on Zn transport to shoots as a percentage of total Zn taken up by 12-d-old Gatcher wheat. Roots and shoots were separated 30 min after the end of the 90 min uptake period.

The percentage of the total Zn taken up by the plant and transported to shoots (% transported to shoot) increased with an increase in Zn concentration up to 2 µ*M* ZnHEDTA then gradually declined to a constant amount as Zn concentration increased to 8 µ*M* ZnHEDTA and above. A similar pattern was observed for the chlorsulfuron-pretreated plants, except that the percentage of Zn transported to shoots was consistently greater than that for the control plants (Fig. 4.2).

4.3.2 Experiment 2; Timing of chlorsulfuron addition

Uptake rate of Zn was significantly influenced by the factors of cultivar, Zn concentration in solution and chlorsulfuron pretreatment (Fig. 4.3, $P \leq 0.0001$ for each). Presence or absence of 40 µg chlorsulfuron L^{-1} in the uptake solution during the 90-min uptake period (chlorsulfuron treatment) did not significantly influence Zn uptake rates ($P \leq 0.71$), therefore data in Fig. 4.3 were pooled over the chlorsulfuron treatment. Pretreatment with chlorsulfuron for 5 days

decreased Zn uptake rate compared to control plants. Excalibur and Durati had similar rates of uptake while Gatcher took up Zn at a lower rate than the other two cultivars. The rate of Zn uptake was increased in 8 μ*M* ZnHEDTA solutions compared to low Zn solutions (Fig. 4.3).

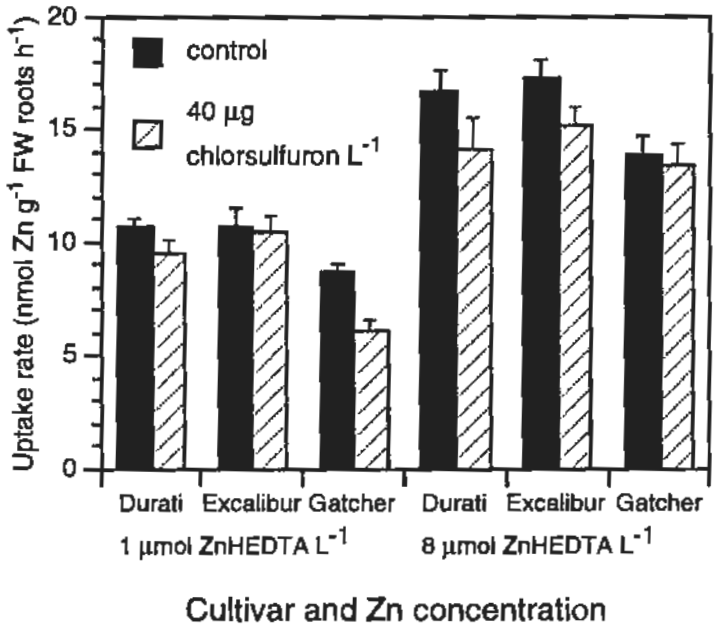

Figure 4.3. Zinc uptake rate of 12-d-old plants of three cultivars of wheat when transferred to solutions containing 1 or 8 μ*M* ZnHEDTA and labelled with ^{65}Zn. Chlorsulfuron-pretreated plants were supplied with 40 μg chlorsulfuron L^{-1} for 5 days prior to uptake measurements. Control plants received no chlorsulfuron. Data were averaged over chlorsulfuron treatment (presence or absence of 40 μg chlorsulfuron L^{-1} during the 90-min uptake period) because the main effect and all interactions involving chlorsulfuron treatment during uptake measurement were non-significant.

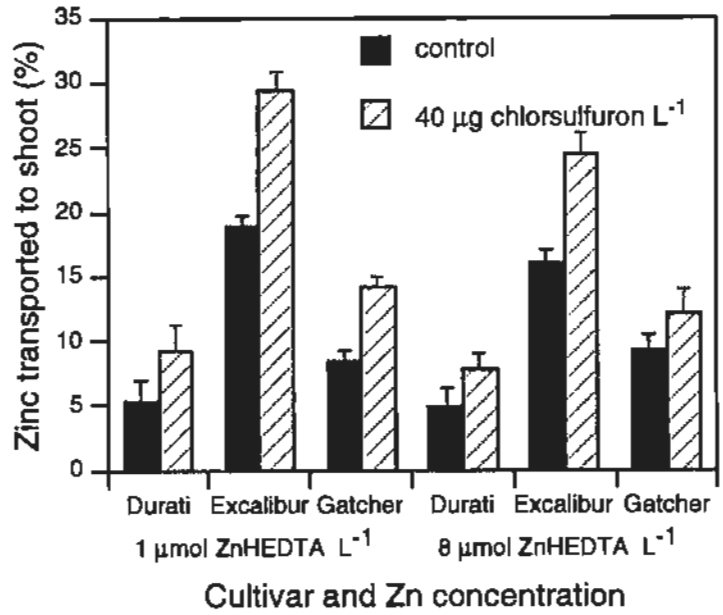

Figure 4.4. Zinc transport to shoots as a percentage of total Zn taken up by 12-d-old plants of three cultivars of wheat when transferred to solutions containing 1 or 8 µ*M* ZnHEDTA. Data averaged over chlorsulfuron treatment during uptake as for Fig. 4.3.

The percentage of Zn transported to the shoot was increased by chlorsulfuron pretreatment but not by chlorsulfuron treatment during the uptake period ($P \leq 0.97$, Fig. 4.4). Gatcher transported a larger percentage of the total Zn taken up from 1 µ*M* ZnHEDTA solutions than Durati (Fig. 4.4), although the rate at which Zn was taken up by Gatcher was lower than in Durati (Fig. 4.3). Excalibur transported the largest percentage of Zn taken up regardless of chlorsulfuron treatment.

4.3.3 Experiment 3; Exposure time

As exposure time to 40 µg chlorsulfuron L^{-1} increased, the rate of Zn uptake decreased significantly in all cultivars ($P \leq 0.0001$, Fig. 4.5). However, at least 3 days of exposure to 40 µg chlorsulfuron L^{-1} were required before a significant decrease in Zn uptake rate could be detected. Excalibur plants increased the rate of Zn uptake by a smaller amount than Durati and Gatcher plants when Zn

concentration of solution increased from 1 to 8 μ*M* ZnHEDTA (cultivar × Zn concentration interaction significant, $P \leq 0.007$, Fig. 4.5).

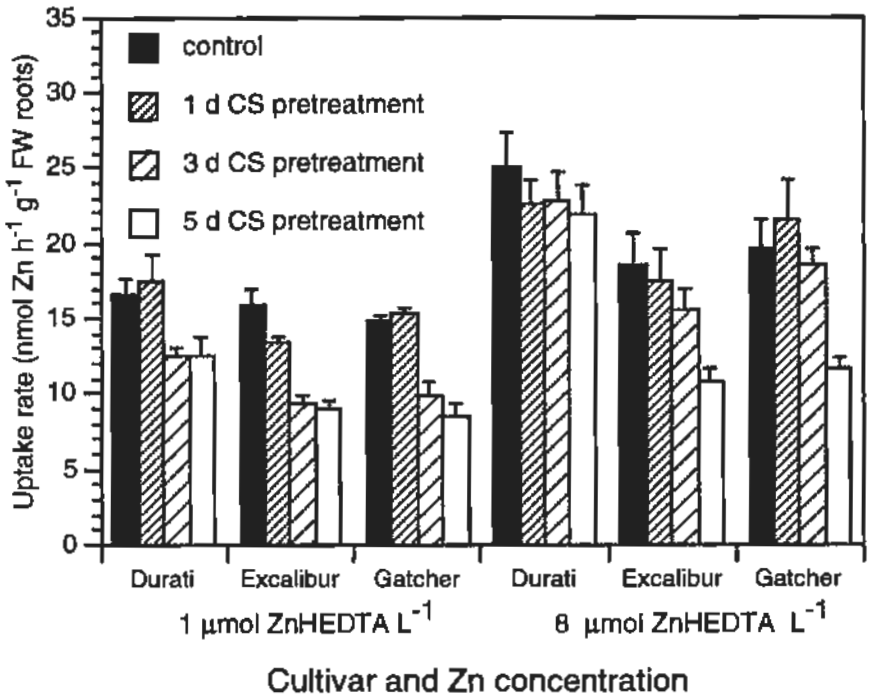

Figure 4.5. Zinc uptake rate for three cultivars of wheat pretreated with 40 μg chlorsulfuron L^{-1} for 0, 1, 3 or 5 d prior to transfer to chlorsulfuron-free solution containing 1 or 8 μ*M* ZnHEDTA. Uptake rates were determined using 12-d-old plants. CS = chlorsulfuron.

Results for percentage of Zn transported to shoots were pooled across cultivars (Fig. 4.6) even though the cultivar effect was significant ($P \leq 0.046$) as differences between the cultivars at each exposure time were small (< 3 %) and the rates of Zn transport to shoot (nmol Zn transported to shoot per g root FW per h) were not significantly different between cultivars (data not shown). Zinc transport to shoots increased as chlorsulfuron exposure time increased and was decreased by high Zn concentration of uptake solution. Excalibur and Gatcher transported similar amounts after each chlorsulfuron treatment while Durati transported less than Excalibur or Gatcher after 3 and 5 days exposure (data not shown). Gatcher wheat transported similar amounts of Zn to shoot in this experiment as in the first experiment (Fig. 4.2).

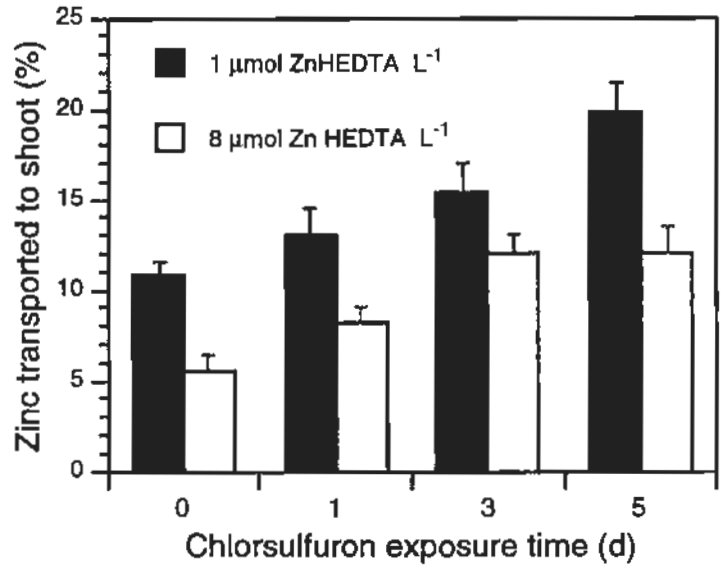

Figure 4.6. Zinc transport to shoots as a percentage of total Zn uptake of 12-d-old wheat plants pretreated with 40 µg chlorsulfuron L^{-1} for 0, 1, 3 or 5 d and transferred to chlorsulfuron-free solutions containing 1 or 8 µ*M* ZnHEDTA. Data were averaged over cultivars as differences in Zn transport between cultivars were small (< 3%).

Root FW of each cultivar was significantly reduced by pretreatment with chlorsulfuron ($P \leq 0.0001$, Table 4.1). Root DW remained similar over all exposure times ($P \leq 0.105$), although root DW of all genotypes had decreased slightly after 5 d of chlorsulfuron exposure in comparison to control (0 d exposure) plants.

Table 4.1. Root weight (mg plant^{-1}, mean ± SE, n = 4) of 12-d-old wheat plants of three cultivars exposed to 40 µg chlorsulfuron L^{-1} for 0, 1, 3, or 5 d before harvesting (Exp. 3). Cultivar × pretreatment interactions were non-significant ($P \leq 0.083$ and $P \leq 0.421$ for FW and DW respectively), therefore cultivar and pretreatment means are presented. Identical superscripted letters indicate treatments that do not differ significantly from each other in the same column or row (Fisher's LSD test).

	Chlorsulfuron Pretreatment (days)				
Cultivar	0	1	3	5	Cultivar means
	Fresh weight (mg plant^{-1})				
Durati	172 ± 14	143 ± 14	134 ± 6	148 ± 10	150 ± 6^{a}
Excalibur	195 ± 21	195 ± 6	165 ± 9	138 ± 5	173 ± 7^{b}
Gatcher	188 ± 13	167 ± 9	160 ± 10	120 ± 8	159 ± 7^{ab}
Pretreatment means	185 ± 9^{a}	169 ± 7^{ab}	153 ± 5^{bc}	135 ± 5^{c}	
$P_{cultivar} \leq 0.0109$					
$P_{pretreatment} \leq 0.0001$					
	Dry weight (mg plant^{-1})				
Durati	16 ± 2	11 ± 1	11 ± 2	13 ± 1	13 ± 1^{a}
Excalibur	13 ± 1	13 ± 1	13 ± 1	11 ± 7	12 ± 1^{a}
Gatcher	29 ± 6	25 ± 8	18 ± 3	15 ± 2	22 ± 3^{b}
Pretreatment means	19 ± 3^{a}	16 ± 3^{ab}	14 ± 1^{b}	13 ± 3^{b}	
$P_{cultivar} \leq 0.0001$					
$P_{pretreatment} \leq 0.0843$					

4.3.4 Experiment 4; Recovery after minimum exposure time

Plants were allowed to recover from the 3-d-exposure to 40 µg chlorsulfuron L^{-1} for 1, 3 or 5 d in chlorsulfuron-free solution, and uptake rate of plants aged 13, 15 and 17 d as well as effects of cultivar, chlorsulfuron pretreatment and Zn concentration in solution were measured. Uptake rate of control plants generally increased with increasing age in low-Zn solutions, but remained unchanged (Excalibur) or decreased (Gatcher) in high-Zn solutions (Fig. 4.7).

After one day of recovery in chlorsulfuron-free solution, chlorsulfuron-pretreated plants of each cultivar had lower Zn uptake rates compared to non-chlorsulfuron-treated control plants (Fig. 4.7). All chlorsulfuron-pretreated plants had recovered Zn uptake rates to reach or exceed control plant rates by 5 days of

recovery, and in some cases had recovered by 3 days. Supplying plants with more Zn increased the rate of Zn uptake, but did not always improve recovery time.

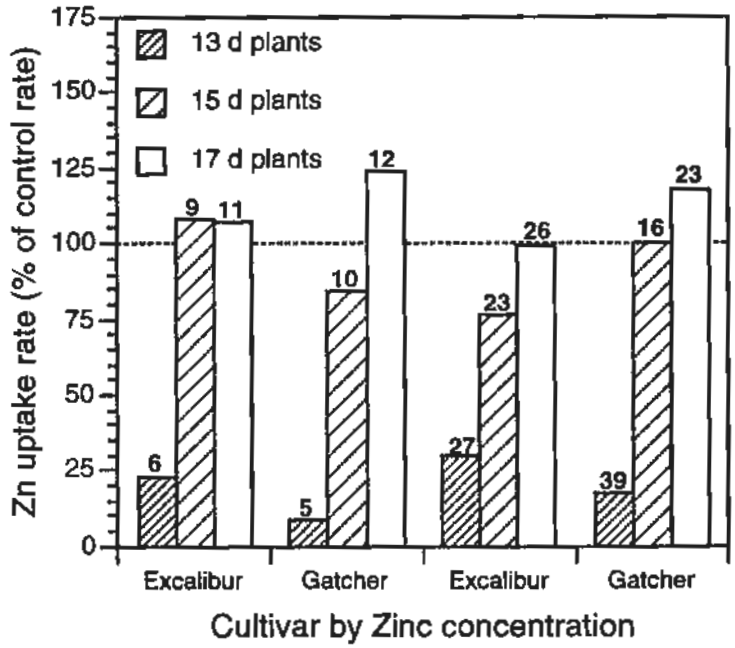

Figure 4.7. Zinc uptake rates of two cultivars of wheat (Excalibur and Gatcher) exposed to 40 µg chlorsulfuron L^{-1} from day 9 to day 12 and allowed to recover in chlorsulfuron-free solution for 1, 3 or 5 days (corresponding to 13-, 15- and 17-d-old plants). Zinc uptake was measured from nutrient solutions containing 1 or 8 µ*M* ZnHEDTA. Numbers above columns refer to Zn uptake rate of control (no chlorsulfuron) plants of the same age (nmol Zn g^{-1} FW roots h^{-1}). Percentages calculated as ([Pretreatment mean / Control mean] × 100).

The four-way analysis of variance for percentage of Zn transported to shoots indicated two significant two-factor interactions: Zn level × recovery time ($P \leq 0.0001$) and chlorsulfuron-pretreatment × recovery time ($P \leq 0.006$). There was no significant difference in Zn transport between cultivars. After 1 day of recovery, plants transported a greater percentage of Zn to shoots from solutions containing 8 µ*M* ZnHEDTA in comparison to those containing 1 µ*M* ZnHEDTA (Fig. 4.8). After 3 and especially after 5 days of recovery, plants transported a smaller percentage of Zn to shoots in high-Zn solutions than in low-Zn solutions. Percentages of Zn transported to shoots were similar after each recovery time

when supplied with 1 μ*M* ZnHEDTA, but decreased with recovery time at 8 μ*M* ZnHEDTA. Zinc transport to shoots of chlorsulfuron-pretreated plants decreased as recovery time increased, while Zn transport to shoots decreased to a constant level after 3 days in control plants (data not shown).

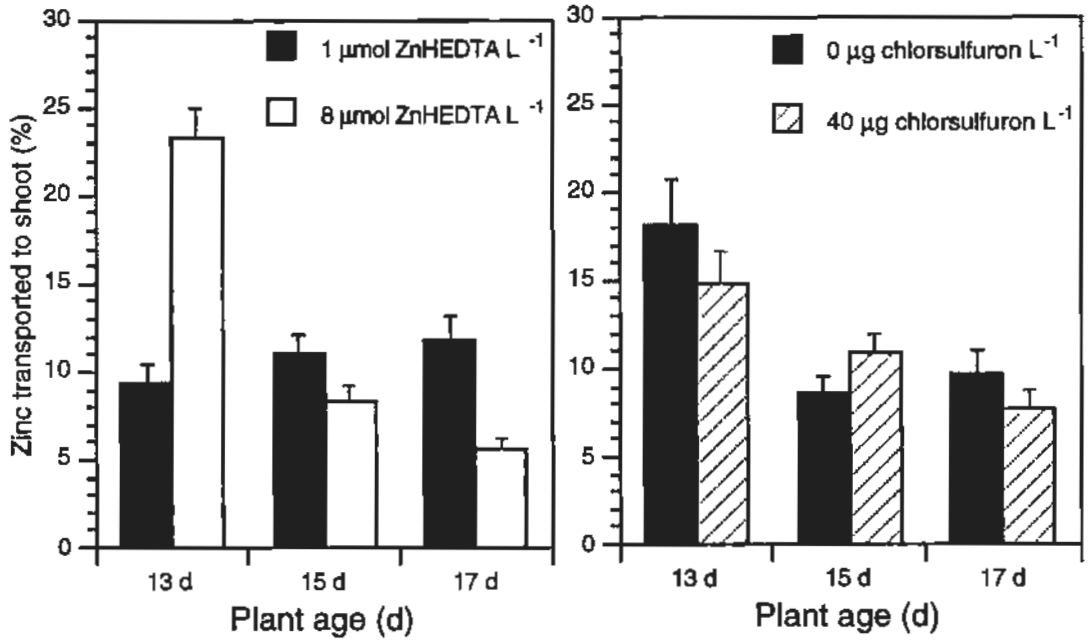

Figure 4.8. Effect of duration of recovery time in chlorsulfuron-free solution on Zn transport to shoots as a percentage of the total amount of Zn taken up. Data were averaged over the cultivar (Excalibur and Gatcher) and the chlorsulfuron pretreatment effects (presence or absence of 40 μg chlorsulfuron L^{-1} for 3 days, from day 9 to day 12). Vertical bars represent ± SE (n = 16).

4.4 Discussion

Plants were exposed to ^{65}Zn for 80-90 minutes as this was sufficient time to allow plants to take up ^{65}Zn and transport it to shoots. Zinc uptake is linear for the first 3-5 h (Reid *et al.*, 1996; Santa Maria and Cogliatti, 1988), and efflux of ^{65}Zn is unlikely to be a significant proportion of net flux after only 90 min (Santa Maria and Cogliatti, 1988). Measurements conducted in the present study therefore represent a net flux of Zn.

In all the experiments presented above, higher Zn concentration in the uptake solution increased the rate of Zn uptake by plants. Zinc uptake rates of tomato and rice roots increased with an increase in external Zn concentration in a

similar fashion (Bowen, 1987), but external free Zn concentrations were approximately 10^6-times those used in the experiments described in this study. Uptake rates of tomato and rice plants were saturated at 7.2 and 3.3 μmol Zn g^{-1} h^{-1}, respectively, when grown in solutions with 500 μ*M* $ZnCl_2$ (Bowen, 1987). Zinc uptake rates of 20-d-old wheat seedlings of approximately 170-190 pmol Zn g^{-1} FW root h^{-1} were obtained from solutions containing ^{65}Zn in 1-5 μ*M* $ZnCl_2$ (Chaudhry and Loneragan, 1972). The uptake rates obtained in the present study using wheat roots in chelated ^{65}Zn solutions were 100 times less than the rates obtained for tomato roots (Bowen, 1987) and 100 times greater than for wheat in non-chelated solution (Chaudhry and Loneragan, 1972). The results of Bowen (1987) are likely to overestimate uptake rates due to the extremely high external Zn concentrations used (Kochian, 1993). Older plants (20 d) may decrease their rates of Zn uptake (*cf.* K^+ transport by 7-and 21-d-old plants; Woodhouse *et al.*, 1978). Net Zn uptake rates of wheat from conventional solutions containing 2 μ*M* $ZnSO_4$ spiked with ^{65}Zn were of the same magnitude (13 nmol g^{-1} FW root h^{-1}; Santa Maria and Cogliatti, 1988) as the uptake rates from chelated solutions presented here.

Addition of chlorsulfuron to the nutrient solution during the 90-min uptake period did not significantly alter the rate of Zn uptake (Fig. 4.3), implying that chlorsulfuron did not have an immediate effect on metabolism of root cells. As wheat is relatively resistant to chlorsulfuron (Beyer *et al.*, 1988; Brown, 1990), which limits production of branched-chain amino acids (Brown, 1990; Ray, 1984), there should be some delay before detrimental effects appear. It should be borne in mind that root growth remained unchanged for at least 2 h after addition of 120 μg chlorsulfuron L^{-1} (Chapter 3).

A reduction in Zn uptake rate during the 80-90 min uptake period would have indicated either rapid metabolic inhibition of the cells, blocking of the transport of Zn into root cells or depolarisation of the membrane. Depolarisation of membranes has been suggested as a herbicidal mechanism for aryloxy-phenoxypropionate herbicides (Lucas *et al.*, 1984; Wright and Shimabukuro, 1987), but there is some doubt whether it would have been sufficient for the

observed effects (DiTomaso, 1994; DiTomaso *et al.*, 1991; Wright, 1994). Moreover, chlorsulfuron and herbicides from other chemical classes were found not to cause membrane depolarisation of oat roots even after 2-d exposure (Wright, 1994). In other experiments (Chapter 6), total respiration rates of wheat roots exposed to chlorsulfuron were not different from control plants, but the proportion contributed by the alternative oxidase (a stress-induced oxygen-consuming pathway, Purvis and Shewfelt, 1993) was increased in chlorsulfuron-treated plants (Chapter 6).

The Zn-inefficient cultivar Durati took up Zn at about the same rate as the Zn-efficient Excalibur (Fig. 4.5), suggesting the Zn efficiency mechanism may not be related to the capacity to take up Zn on a root FW basis. The Zn concentration that plants were grown in (0.2 μM ZnHEDTA corresponding to 4.2 pM free Zn^{2+}) may not have been sufficient for the Zn-efficient Excalibur to become Zn-deficient to a degree that would enable efficiency mechanisms to be fully expressed. No major differences in Zn uptake rates were detected in 12 cultivars differing in Zn efficiency (Rengel and Graham, 1995a, b). The percentage of Zn transported to shoots was greater in Excalibur than in Durati which may represent a Zn-efficiency mechanism if Zn availability is decreased later.

All uptake rates were measured on a root FW basis, thereby accounting for any differences in the root mass available to absorb Zn from solution. Pretreating plants with chlorsulfuron reduced both root FW and Zn uptake per g FW root per h (Table 4.1, Fig. 4.1). Zinc uptake rates were reduced after at least 3 days pretreatment with 40 μg chlorsulfuron L^{-1} but not after 1 day (Fig. 4.5). The percentage of Zn transported to shoots was increased as herbicide exposure time increased (Fig. 4.6), suggesting that plants were compensating for the decreased Zn uptake by roots during pretreatment. When supplied with higher concentrations of Zn, the amount transported to shoots represented a smaller proportion of the total amount taken up (*e.g.* Fig. 4.2).

Increasing Zn supply to plants may overcome the deleterious effects of chlorsulfuron pretreatment on root growth (Dong *et al.*, 1995; McLay and Robson, 1992). Chlorsulfuron-pretreated roots supplied with 32 μM ZnHEDTA

(corresponding to 22 n*M* free Zn) during the 80-min uptake period increased the rate of Zn uptake to approximately the same rate as that of control (untreated) root rates (data not shown). In addition, Zn supplementation (20 μ*M* ZnHEDTA) improved wheat root tip extension responses of plants treated with chlorsulfuron over 22 h (Chapter 3). Further testing of concentrations above 16 and 32 μ*M* ZnHEDTA is required to fully investigate the extent of Zn uptake responses to added Zn.

Nutrient uptake can be reduced by herbicides without reducing root growth parameters. Phosphorus concentration was reduced in wheat plants supplied with 7 μg chlorsulfuron kg^{-1} soil even though root length was not reduced (Osborne *et al.*, 1993). Low chlorsulfuron levels reduced copper concentrations at low soil copper levels while root weight was not reduced (Robson and Snowball, 1990). A hypothesis was put forward that chlorsulfuron reduces uptake of these elements by some means other than reduction in root mass or root length (Osborne and Robson, 1992). In the present study, the decrease of Zn uptake rate in chlorsulfuron-treated plants was not directly related to reduction of root mass (*e.g.* Fig. 4.5, Table 4.1), therefore is due to other indirect or additional mechanisms. Possible additional mechanisms include decreased metabolic requirements as a result of decreased growth rates, reduced protein synthesis resulting in fewer nutrient transport proteins in the cell membranes or reduced ability of membrane transporters to absorb nutrients from solution.

Chlorsulfuron concentrations similar to those used in this study reduced the number of root hairs in wheat plants grown in solution cultures (McLay and Robson, 1992; Robson and Snowball, 1990), and reduced the proportion of fine roots in soil (Dong *et al.*, 1995). Root hairs and fine roots contribute a large proportion of nutrients taken up (Nable and Webb, 1993), especially for diffusion-limited nutrients such as Zn and P (Osborne *et al.*, 1993). Chlorsulfuron may therefore decrease the specific root length or surface area of roots available to take up Zn. As a consequence, total Zn content of chlorsulfuron-treated wheat decreased, even though the long-term net Zn uptake rate on a root surface area basis was unchanged by chlorsulfuron (Dong *et al.*, 1995). In solution, Zn uptake

rates of chlorsulfuron-treated plants were decreased (*e.g.* Fig. 4.1), suggesting that even when diffusion was not a factor limiting Zn availability to roots, chlorsulfuron still had deleterious effects on Zn uptake.

Zinc is most probably transported across membranes via a transport protein (Kochian, 1993). As Zn is required for carbohydrate metabolism and protein synthesis (Marschner, 1995), and chlorsulfuron-treated plants have decreased valine and leucine production (Ray, 1984; Royuela *et al.*, 1991; Shaner and Singh, 1993), low Zn plants treated with chlorsulfuron could be expected to have even lower protein levels (Hatzios and Howe, 1982; Royuela *et al.*, 1991). Three days of chlorsulfuron exposure could reduce production of Zn transport proteins or intermediates and decrease uptake rates. Such proteins must be extremely sensitive to chlorsulfuron as 2-dimensional SDS-PAGE analysis of pea root tip proteins exposed to chlorsulfuron showed very little difference in general protein banding (Clayton and Reynolds, 1991).

In summary, rates of Zn uptake by wheat decreased as exposure time to chlorsulfuron increased. Decreases in root FW also occurred with exposure to chlorsulfuron, but uptake rates were not dependent on root weight. Effects of chlorsulfuron on Zn uptake rate were not immediate or permanent as at least 3 days exposure was required to significantly reduce uptake rate. Five days of recovery in chlorsulfuron-free solution allowed chlorsulfuron-treated plants to take up Zn at control plant rates. Cultivars differing in Zn efficiency were found to vary slightly in their ability to take up Zn from solutions. Chlorsulfuron-treated plants appeared to compensate for decreased uptake of Zn via roots by increasing the proportion of Zn transported to shoots.

4.5 References

Beyer, E. M., Duffy, M. J., Hay, J. V. and Schlueter, D. D. (1988). Sulfonylureas. In Herbicides: Chemistry, Degradation and Mode of Action. Kearney, P. C. and Kaufman, D. D. Eds. pp 117-189. Marcel Dekker, New York.

Bowen, J. E. (1987). Physiology of genotypic differences in zinc and copper uptake in rice and tomato. *Plant and Soil* 99, 115-125.

Bowran, D. G., Porritt, S. and Madin, R. (1987). Chlorsulfuron and diclofop-methyl effects on the copper and zinc status and yield of wheat. In Proceedings of the Eighth Australian Weeds Conference. Eds. Lemerle, D. and Leys, A. R. pp 404-407.

Brown, H. M. (1990). Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. *Pesticide Science* 29, 263-281.

Chaudhry, F. M. and Loneragan, J. F. (1972). Zinc absorption by wheat seedlings: I. Inhibition by macronutrient ions in short-term experiments and its relevance to long-term zinc nutrition. *Soil Science Society of America Proceedings* 36, 323-327.

Clayton, D. S. and Reynolds, T. L. (1991). Chlorsulfuron effects on protein synthesis and accumulation in cultured root tips of *Pisum sativum* L. *Journal of Plant Physiology* 137, 337-341.

DiTomaso, J. M. (1994). Evidence against a direct membrane effect in the mechanism of action of graminicides. *Weed Science* 42, 302-309.

DiTomaso, J. M., Brown, P. H., Stowe, A. E., Linscott, D. L. and Kochian, L. V. (1991). Effects of diclofop and diclofop-methyl on membrane potentials in roots of intact oat, maize, and pea seedlings. *Plant Physiology* 95, 1063-1069.

Dong, B., Rengel, Z. and Graham, R. D. (1995). Effects of herbicide chlorsulfuron on growth and nutrient uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant and Soil* 173, 275-282.

Graham, R. D., Ascher J. S. and Hynes, S. C. (1992). Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant and Soil* 146, 241-250.

Hatzios, K. K. and Howe, C. M. (1982). Influence of the herbicides hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pesticide Biochemistry and Physiology* 17, 207-214 .

Kochian, L. V. (1993). Zinc absorption from hydroponic solutions by plant roots. In Zinc in Soils and Plants. Ed. A D Robson. pp 45-58. Kluwer Academic Publishers, Dordrecht.

Lucas, W. J., Wilson, J. C. and Wright, J. P. (1984). Perturbations of *Chara* plasmalemma transport function by 2-[4-(2',4'-dichloro-phenoxy)-phenoxy]propionic acid. *Plant Physiology* 74, 61-66.

Marschner, H. (1995). Mineral Nutrition of Higher Plants, 2nd ed. Academic Press, London.

McLay, L. D. and Robson, A. D. (1992). The effect of chlorsulfuron and diclofop-methyl on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 43, 59-65.

Nable, R. O. and Webb, M. J. (1993). Further evidence that zinc is required throughout the root zone for optimal plant growth and development. *Plant and Soil* 150, 247-253.

Norvell, W. A. and Welch, R. M. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl) ethylenedinitrilotriacetic acid-buffered nutrient solution technique: I. Zinc ion requirements. *Plant Physiology* 101, 619-625.

Osborne, L. D. and Robson, A. D. (1992). Duration of zinc uptake inhibition by chlorsulfuron in wheat. *Australian Journal of Agricultural Research* 43, 1169-1174.

Osborne, L. D., Robson, A. D. and Bowran, D. G. (1993). The impact of chlorsulfuron and diclofop-methyl on nutrient uptake by wheat. *Australian Journal of Agricultural Research* 44, 1757-1766.

Parker, D. R. (1993). Novel nutrient solutions for zinc nutrition research: buffering free $zinc^{2+}$ with synthetic chelators and P with hydroxyapatite. *Plant and Soil* 155/156, 461-464.

Parsons, J. M. (1992). Australian weed control handbook, 9th ed. Inkata Press, Sydney.

Purvis, A. C. and Shewfelt, R. L. (1993). Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* 88, 712-718.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Rengel, Z. and Graham, R. D. (1995). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake. *Plant and Soil* 176, 317-324.

Reuter, D. J., Cartwright, B., Judson, G. J., McFarlane, J. D., Maschmedt, D. J. and Robinson, J. B. (1988). Trace Elements in South Australian Agriculture. *Department of Agriculture South Australia Technical Report*. No. 139.

Robson, A. D. and Snowball, K. (1989). The effect of 2-(4-2',4'-dichlorophenoxy-phenoxy)-methyl propanoate on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 40, 981-990.

Robson, A. D. and Snowball, K. (1990). The effect of chlorsulfuron on the uptake and utilization of copper and zinc in wheat. *Australian Journal of Agricultural Research* 41, 19-27.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Santa Maria, G. E. and Cogliatti, D. H. (1988). Bidirectional Zn-fluxes and compartmentation in wheat seedling roots. *Journal of Plant Physiology* 132, 312-315.

Shaner, D. L. and Singh, B. K. (1993). Phytotoxicity of acetohydroxyacid synthase inhibitors is not due to accumulation of 2-ketobutyrate and/or 2-aminobutyrate. *Plant Physiology* 103, 1221-1226.

Welch, R. M., Webb, M. J. and Loneragan, J. F. (1982). Zinc in membrane function and its role in phosphorus toxicity. In Proceedings of the Ninth Plant Nutrition Colloquium, Warwickshire, England. Scaife, A. Ed. pp 710-715. Commonwealth Agricultural Bureaux, Slough.

Woodhouse, P. D., Wild, A. and Clement, C. R. (1978). Rate of uptake of potassium by three crop species in relation to growth. *Journal of Experimental Botany* 29, 885-894.

Wright, J. P. (1994). Use of membrane potential measurements to study mode of action of diclofop methyl. *Weed Science* 42, 285-292.

Wright, J. P. and Shimabukuro, R. H. (1987). Effects of diclofop and diclofop-methyl on the membrane potentials of wheat and oat coleoptiles. *Plant Physiology* 85, 188-193.

Zar, J. H. (1984). Biostatistical analysis 2nd Edn. Prentice Hall, Englewood Cliffs, New Jersey.

CHAPTER 5

PROTEIN CONCENTRATIONS AND SUPEROXIDE RADICAL GENERATION

5.1 Introduction

Zinc is an important requirement for protein synthesis (Falchuk *et al.*, 1976, 1977; Marschner, 1995; see section 1.1.5.1). Plants grown in low-Zn conditions show distinct decreases in protein synthesis and content (Cakmak and Marschner, 1987, 1988; Cakmak *et al.*, 1989; Pinton *et al.*, 1994).

Zinc plays a protective role in cells (see section 1.1.5.1.3). Superoxide ($O_2^{\cdot -}$) and other radicals which are generated as a result of oxygen-consuming reactions in cells cause peroxidative damage to cell membranes and organelles (Fridovich, 1986). Zinc is a structural component of the copper/zinc superoxide dismutase protein (Cu/Zn SOD), which scavenges superoxide radicals and dismutates them to H_2O_2 (Fridovich, 1986; Thompson *et al.*, 1987). Zinc is also able to decrease $O_2^{\cdot -}$ generation by inhibiting the NADPH-oxidase that produces large quantities of $O_2^{\cdot -}$ inside the mitochondria (Jeffery, 1983). Damage caused by superoxide radicals in cells is therefore increased in Zn-deficient plants (Cakmak and Marschner, 1988).

Chlorsulfuron treatment of plants may also inhibit protein synthesis through the inhibition of ALS (see section 1.2.1.3). Rates of protein synthesis in maize were decreased by short term exposure (2 h) to relatively high concentrations of chlorsulfuron (100 μM; Hatzios and Howe, 1982). Ray (1984) found no change in protein synthesis of wheat in short term experiments with chlorsulfuron. Similarly, pea plants showed no differences in protein synthesis after 36 h exposure to chlorsulfuron (Clayton and Reynolds, 1991). Net protein content has not been measured after long-term exposure to chlorsulfuron; this may indicate whether prolonged inhibition of ALS decreases protein contents in plants.

Oxygen free radical generation can be induced by abiotic stresses, *e.g.* dessication (Smirnoff, 1993), high salt loads, (Hernandez *et al.*, 1993) and cold (Foyer *et al.*, 1994; Purvis *et al.*, 1995). Bipyridinyl herbicides (*e.g.* paraquat and

diquat) disrupt photosynthesis and generate oxygen free radicals which cause peroxidative damage to membranes (Bowler *et al.*, 1992). Sulfonylurea herbicides do not generate oxygen free radicals as their biological mode of action. However, sulfonylurea treatment may induce other stresses that increase superoxide radical generation; such a possibility does not appear to have been tested before. In particular, if chlorsulfuron is responsible for decreasing zinc concentrations in roots by decreasing Zn uptake (Chapter 2, Chapter 4), increased superoxide radical generation and decreased SOD levels may be observed. In such a case, superoxide radical levels could be used as an indicator of the degree of stress in wheat plants and as a method of screening for tolerance to Zn deficiency. The following experiments were conducted to determine if low Zn activity in nutrient solution decreased protein levels and increased superoxide radical production rates in wheat and whether chlorsulfuron had any influence on these parameters.

5.2 Methods

Plants were germinated and grown using the methods described earlier (section 2.2). As only a small amount of root material was required for these assays, plants were grown in 1-L pots with four cups per pot and four plants per cup. Four cultivars were tested: Durati, Gatcher and Excalibur, which have been described earlier (section 2.2), and Songlen, a Zn-inefficient breadwheat cultivar containing 344 ± 18 ng Zn/seed.

Experiment 5.1 was a fully factorial experiment with three main effects. Three cultivars (Durati, Gatcher and Songlen) were grown for 14 d in nutrient solutions (section 2.2.3) with 0.1 or 4 μM ZnHEDTA and treated with 0 or 40 μg chlorsulfuron L^{-1} from d 0. The design of the experiment was 3 cultivars × 2 chlorsulfuron levels × 2 Zn activities = 12 treatments. Four replicates were used for each treatment.

Experiment 5.2 examined the effect of different concentrations of chlorsulfuron (0, 4, 40 and 400 μg L^{-1}) on Durati and Excalibur wheats grown in 0.1 μM ZnHEDTA nutrient solution. Plants were exposed to chlorsulfuron from

d 0 and grown for 14 d. The design of this experiment was 2 cultivars × 4 chlorsulfuron levels = 8 treatments. Four replicates were used.

Experiment 5.3 examined the effect of time of exposure to chlorsulfuron on Gatcher wheat grown in nutrient solutions containing 0.1 or 4 μ*M* ZnHEDTA and treated with 0 or 40 μg chlorsulfuron L^{-1}. Chlorsulfuron was applied from d 0 and plants were grown for 14 and 21 d. The design of the experiment was 2 harvest times × 2 chlorsulfuron levels × 2 Zn activities = 8 treatments. Four replicates were used.

To assay superoxide radical production, a procedure based on that used by Cakmak and Marschner (1988) was employed. As superoxide production was calculated on a per mg protein basis, protein assays were performed using the Bradford (1976) method.

5.2.1 Protein extraction

Approximately 0.2 g (FW) of root material was sub-sampled from the entire root system and weighed. The sample was ground using an ice-cold (0 °C) acid-washed glass mortar and pestle in 0.75 mL of buffer solution (50 m*M* HEPES, 0.1 m*M* EDTA, 250 m*M* sucrose, titrated to pH 7.8 with KOH). The homogenate was transferred to a 2-mL Eppendorf tube with an additional 0.75 mL buffer before centrifuging at 15 000 *g* for 15 min in a bench centrifuge at 4 °C. The supernatant was retained and used for protein and superoxide radical assays without further treatment or purification.

5.2.2 Protein assay

To assay for protein concentration, a 0.05 mL aliquot of sample supernatant was mixed with 0.95 mL water and added to 1 mL of Coomassie Protein Assay Solution (Pierce Co.) and mixed. The absorbance of the mixture at 595 nm (Abs_{595}) was measured within 90 min of preparation, using a Hitachi model 100-20 spectrophotometer (Hitachi Ltd., Tokyo). The sample absorbance values were calibrated using protein standards (0, 5, 10, 20, 30, 40 and 50 μg mL^{-1}) of protein (bovine serum albumin fraction V; Sigma-Aldrich). The concentration of protein

in the diluted homogenate (μg mL^{-1}) was calculated using an exponential equation derived from the standard curve (Cricket Graph III, Computer Associates) and converted to μg protein g^{-1} root FW.

5.2.3 Superoxide radicals

The production of $O_2^{\cdot-}$ by NADPH-oxidase was measured using the reduction of cytochrome *c* (Markert *et al.*, 1984). Superoxide radical production was measured by recording the rate of cytochrome *c* reduction by wheat root homogenate using a procedure similar to that described by Cakmak and Marschner (1988). The following compounds were placed in a 2-mL cuvette and the rate of reaction observed at 550 nm: 0.8 mL 50 m*M* HEPES (adjusted to pH 7.8 with KOH); 0.01 mL H_2O; 0.01 mL 1 μ*M* KCN; 0.01 mL 0.1 m*M* EDTA; 0.01 mL 75 μ*M* cytochrome*c* ; 50-150 μg protein (0.15 mL supernatant); 0.01 mL 50 μ*M* NADPH. Total volume of the mixture was 1 mL. The rate of reaction was recorded for 3-5 minutes using the Hitachi 100-20 spectrophotometer. Control values were calculated by adding 25 μg horseradish leaf SOD (Sigma-Aldrich) to remove the superoxide radicals before they reduced the cytochrome *c*. Superoxide radical generation was calculated in mol $O_2^{\cdot-}$ generated per minute per mg protein using the following equation:

$$\frac{\Delta Abs_{550}}{21 \text{ mol m}^{-3} \times 10^6} \div \frac{\mu\text{g protein / ml} \times 0.15 \text{ ml}}{10^3} = mol\ O_2^{-} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$$

(Equation 5.1)

where ΔAbs_{550} = difference between rates of sample and control absorbance at 550 nm.

5.3 Results

5.3.1 Protein levels

Protein concentrations in roots differed between cultivars but were not significantly influenced by Zn activity of solution or chlorsulfuron treatment (Table 5.1). Root FW was significantly decreased by exposure to chlorsulfuron but was not influenced by solution Zn activity (Table 5.1). The slight increases in

protein concentration of chlorsulfuron-treated roots can be attributed to greater decreases in root FW than protein content (Table 5.1). There were no interactions between main treatment effects for any variable.

Table 5.1. Fresh weight, protein concentration, protein content and superoxide radical production (mean ± SE, n = 4) of roots of three wheat cultivars grown in 0.1 and 4 μM ZnHEDTA and exposed to chlorsulfuron (CS; 0 or 40 μg L^{-1}) for 14 d. Probability (P) values refer to significance of main effect treatments. There were no interactions between main effects for any variable.

Cultivar	Zn	CS	Root FW (mg $plant^{-1}$)	Protein (mg g^{-1} FW)	Protein (μg $plant^{-1}$)	$O_2^{\cdot-}$ (nmol g^{-1} min^{-1})
Durati	0.1	0	149 ± 15	6.3 ± 0.7	820 ± 125	0.83 ± 0.24
		40	56 ± 9	5.7 ± 0.4	310 ± 55	2.22 ± 0.59
	4	0	157 ± 10	5.3 ± 0.6	855 ± 159	2.47 ± 0.61
		40	48 ± 6	6.5 ± 0.2	301 ± 19	1.43 ± 0.07
Gatcher	0.1	0	89 ± 13	3.7 ± 0.4	344 ± 60	2.27 ± 0.42
		40	63 ± 6	4.0 ± 0.3	253 ± 36	3.44 ± 2.13
	4	0	89 ± 13	4.3 ± 0.3	381 ± 67	0.77 ± 0.40
		40	56 ± 7	4.6 ± 0.6	245 ± 56	6.28 ± 4.33
Songlen	0.1	0	165 ± 18	2.4 ± 0.2	406 ± 87	3.69 ± 1.12
		40	58 ± 3	3.6 ± 0.2	195 ± 5	2.92 ± 0.92
	4	0	123 ± 12	2.4 ± 0.2	304 ± 47	3.61 ± 0.97
		40	58 ± 8	3.0 ± 0.2	169 ± 29	3.69 ± 0.69
		ZnHEDTA	P ≤ 0.23	P ≤ 0.54	P ≤ 0.80	P ≤ 0.50
		Chlorsulfuron	P ≤ 0.01	P ≤ 0.11	P ≤ 0.01	P ≤ 0.15
		Cultivar	P ≤ 0.01	P ≤ 0.01	P ≤ 0.01	P ≤ 0.13

Root FW of Durati was decreased by 4 μg chlorsulfuron L^{-1} and of Excalibur by 40 μg chlorsulfuron L^{-1} (cultivar × chlorsulfuron interaction significant; Table 5.2). Protein concentration in each cultivar was increased by the same concentration of chlorsulfuron that decreased root FW in each cultivar. As root FW decreased with chlorsulfuron concentration, so too did total protein content, but to a lesser extent in each treatment.

Table 5.2 Fresh weight, protein concentration, protein content and superoxide radical production (mean ± SE, n = 4) of roots of Durati and Excalibur wheat grown in 0.1 μM ZnHEDTA and exposed to 0, 4, 40 or 400 μg chlorsulfuron L^{-1} for 14 d. Probability (P) values refer to significance of main effects and interaction treatment.

Cultivar	Chlorsulfuron	Root FW (mg plant^{-1})	Protein (mg g^{-1} FW)	Protein (μg plant^{-1})	$O_2^{\cdot-}$ (nmol mg^{-1} min^{-1})
Durati	0	216 ± 19	1.3 ± 0.2	294 ± 60	6.63 ± 2.04
	4	102 ± 18	4.0 ± 0.2	406 ± 70	6.16 ± 3.44
	40	47 ± 4	3.2 ± 0.1	150 ± 16	4.45 ± 0.42
	400	37 ± 5	4.1 ± 0.2	149 ± 18	3.40 ± 1.06
Excalibur	0	187 ± 16	2.5 ± 0.2	454 ± 45	3.60 ± 1.94
	4	219 ± 11	2.1 ± 0.1	459 ± 42	5.62 ± 1.78
	40	86 ± 8	3.5 ± 0.2	299 ± 20	6.78 ± 2.44
	400	40 ± 6	3.5 ± 0.1	139 ± 22	2.33 ± 0.33
	Cultivar	P ≤ 0.01	P ≤ 0.05	P ≤ 0.01	P ≤ 0.69
	Chlorsulfuron	P ≤ 0.01	P ≤ 0.01	P ≤ 0.01	P ≤ 0.48
	interaction	P ≤ 0.01	P ≤ 0.01	P ≤ 0.19	P ≤ 0.61

Root FW of Gatcher wheat increased between d 14 and d 21, although less so when treated with 40 μg chlorsulfuron L^{-1} (Table 5.3). Zinc activity of solution had no significant influence on root FW at either plant age. While root FW was significantly reduced by exposure to chlorsulfuron at both harvests, protein concentration was not influenced by any treatment (Table 5.3). Changes in protein content reflected changes in root FW (decreased by chlorsulfuron, unaffected by Zn and increased with plant age; Table 5.3).

Table 5.3. Protein concentration, FW, protein content and superoxide radical production (mean ± SE, n = 4) of Gatcher wheat roots grown in 0.1 or 4 μ*M* ZnHEDTA and exposed to chlorsulfuron (CS; 0 or 40 μg L^{-1}) for 14 and 21 d. Probability (P) values refer to significance of main effect treatments. There were no interactions between main effects for any variable.

Plant age	Zinc	CS	Root FW (mg plant^{-1})	Protein (mg g^{-1} FW)	Protein (μg plant^{-1})	$O_2^{\cdot-}$ (nmol mg^{-1} min^{-1})
14	0.1	0	210 ± 19	1.4 ± 0.1	296 ± 25	2.59 ± 0.60
		40	118 ± 9	1.7 ± 0.2	190 ± 12	3.37 ± 1.08
	4	0	196 ± 21	2.4 ± 0.3	469 ± 94	2.65 ± 0.55
		40	113 ± 4	1.8 ± 0.2	205 ± 30	3.38 ± 0.23
21	0.1	0	343 ± 56	1.8 ± 0.2	626 ± 150	4.01 ± 0.45
		40	149 ± 12	2.3 ± 0.3	343 ± 64	5.13 ± 1.01
	4	0	433 ± 35	1.8 ± 0.4	809 ± 225	5.17 ± 1.01
		40	128 ± 12	2.2 ± 0.2	292 ± 42	4.53 ± 0.83
	ZnHEDTA		P ≤ 0.57	P ≤ 0.17	P ≤ 0.32	P ≤ 0.80
	Chlorsulfuron		P ≤ 0.01	P ≤ 0.43	P ≤ 0.01	P ≤ 0.42
	Plant age		P ≤ 0.01	P ≤ 0.30	P ≤ 0.01	P ≤ 0.01

5.3.2 Superoxide radical production

Neither solution Zn activity nor chlorsulfuron treatment had any influence on superoxide radical production in roots (Tables 5.1, 5.2, 5.3). The overall response of superoxide radical production in a single cultivar was highly variable but could not be attributed to any of the experimental variables. There were slight but non-significant differences between the Zn-inefficient cultivars tested in Exp. 1 (Table 5.1). Zinc efficiency of cultivars had no significant influence on superoxide radical production (Table 5.2). Increasing plant age was the only experimental factor that increased superoxide production significantly (Table 5.3).

5.4 Discussion

5.4.1 Chlorsulfuron effects on protein levels

Plants treated with chlorsulfuron showed increases in root protein concentration of up to 1.5-fold over control plants (Tables 5.1, 5.2, 5.3). The increase in protein concentration appears to be due to chlorsulfuron reducing the growth of roots, rather than increasing protein synthesis.

Studies on the mode of action of chlorsulfuron concluded that short-term protein synthesis was not altered by chlorsulfuron treatment (Hatzios and Howe, 1982; Ray, 1982). After exposure to 1 mg L^{-1} chlorsulfuron (2.8 n*M*) for 6 h, 2-d-old maize roots increased short-term protein synthesis (measured by [^{14}C]-leucine incorporation) to 112% of control plant rates (Ray, 1982). After the same period, other measures of growth (root extension, DNA synthesis and mitotic index) were reduced by up to 80% of control plant rates (Ray, 1982). Protein synthesis of isolated soybean cells measured with the same technique ([^{14}C]-leucine incorporation) was inhibited 10-38% compared to control (untreated plants) after 2 h exposure to chlorsulfuron concentrations of 1 μ*M* (360 μg L^{-1}) and greater, while shorter exposure times had a lesser effect (Hatzios and Howe, 1982).

Because the biochemical site of action of chlorsulfuron was unknown when Ray (1982) and Hatzios and Howe (1982) performed their experiments, use of leucine to measure protein synthesis would not have been considered inappropriate. Using exogenously applied leucine may overestimate the rate of protein synthesis when acetolactate synthase is inhibited by chlorsulfuron. Inhibition of acetolactate synthase (ALS) prevents synthesis of valine, leucine and isoleucine (LaRossa and Schloss, 1984; see section 1.2.1.3), however, protein catabolism enables cells to recycle amino acids (Rhodes *et al.*, 1987; Royuela *et al.*, 1991). Total amino acid pools were increased after exposure to chlorsulfuron while the levels of valine, leucine and isoleucine were significantly decreased compared to untreated control plants of wheat (Royuela *et al.*, 1991) and *Lemna minor* (Rhodes *et al.*, 1987). Addition of leucine to cells with depleted branched-chain amino acid levels would result in rapid incorporation of leucine into

proteins, and use up the available valine and isoleucine, resulting in anomalously high rates of protein synthesis.

If ALS was not inhibited by chlorsulfuron immediately, branched-chain amino acid pools would not become depleted within 2-6 h (the times used by Ray, 1982, and Hatzios and Howe, 1982) and rates of protein synthesis would not be slowed. In this case, adding leucine would not increase protein synthesis rates. Using a non ALS-synthesised amino acid, Clayton and Reynolds (1991) found pea root protein synthesis measured by [^{3}H]-methionine incorporation after treatment with 28 n*M* (10 μg L^{-1}) chlorsulfuron for 36 h was identical to the rate measured incontrol roots.

Measurements of total protein concentration may be more appropriate as they measure the amount of protein present, rather than the rate of synthesis, which may be dependent upon various factors, such as the methods used or exposure times involved. When protein levels were measured using the (Bradford, 1976) assay, total protein accumulation in *Chlorella emersonii* was inhibited within 2-3 h by sulfometuron-methyl (Landstein *et al.*, 1995).

Inhibition of protein synthesis by chlorsulfuron was considered to be minor and unlikely to be the initial mode of action of chlorsulfuron (Hatzios and Howe 1982; Ray 1982). Because the concentrations of chlorsulfuron that were applied were well above expected field concentrations, inhibition of protein synthesis was considered unlikely to occur at field concentrations of chlorsulfuron (Hatzios and Howe, 1982). The effects of prolonged exposure to chlorsulfuron on protein synthesis have not been measured often, which, considering the mode of action, is surprising. Landstein *et al.*, (1995) found protein accumulation in algae was inhibited within 2-3 h of application.

5.4.2 Zinc and protein synthesis

Zn activity in solution had no significant effect on root FW or protein concentration of 14-d- and 21-d-old wheat plants (Tables 5.1-5.3). This contrasts with other studies that examined protein synthesis in relation to Zn availability. Bean (*Phaseolus vulgaris*) plants grown in Zn-deficient nutrient solution had

lower protein concentrations in leaves than those plants grown in Zn-adequate solutions (Cakmak *et al.*, 1989). When 17-d-old bean plants grown without Zn were resupplied with 4 μ*M* Zn for up to 4 days preceding measurements, protein concentrations increased with increasing time of Zn supply and eventually exceeded Zn-adequate control plant values (Cakmak *et al.*, 1989). Amino acid concentrations were decreased by Zn deficiency and recovered as Zn was supplied to Zn-deficient plants (Cakmak *et al.*, 1989).

The lack of response of protein levels in wheat plants in the experiments presented here may be due to the greater tolerance of wheat to Zn deficiency than beans. Root Zn concentrations of beans grown without Zn were 17 μg g^{-1}, while adequately-supplied roots had concentrations of 65 μg g^{-1} (Cakmak *et al.*, 1989). Wheat appears able to produce protein at a lower tissue Zn concentration (10-20 μg Zn kg^{-1} DW) than beans. Therefore, the low Zn activity of the solutions used in these experiments may have been sufficient to maintain protein synthesis in wheat roots. Overall, protein concentrations were more sensitive to chlorsulfuron than to Zn activity of solution.

5.4.3 Superoxide radical production

Superoxide radical production in wheat roots was of the same magnitude as in roots of bean and cotton found by Cakmak and Marschner, (1988) and Pinton *et al.*, (1994). However, the lack of response of $O_2^{\cdot -}$ production in roots to different Zn activities (Tables 5.1, 5.3) does not coincide with the results of Cakmak and Marschner, (1988) or Pinton *et al.*, (1994). The generation of superoxide radicals in cotton and bean roots was much greater when plants were grown in solutions without Zn than with adequate Zn (Cakmak and Marschner, 1988). Resupply of Zn (3-4 μ*M* Zn^{2+}) to plants for up to four days decreased $O_2^{\cdot -}$ production compared to plants maintained in zero-Zn solutions for the entire period (Cakmak and Marschner, 1988; Pinton *et al.*, 1994). Increases in superoxide radical levels in Zn-deficient roots were attributed to decreased SOD scavenging of $O_2^{\cdot -}$ molecules and increased generation of $O_2^{\cdot -}$ by NADPH-oxidases (Cakmak and Marschner, 1988a, b). Studies conducted on animal cells have shown NADPH

oxidation also decreased when supplied with Zn (Jeffery, 1983). The low Zn activity of solution (approximately 2 p*M* Zn^{2+}) used in the experiments presented here, as opposed to zero-Zn treatments used by Cakmak and Marschner (1988) possibly allowed sufficient Zn to be used in superoxide dismutase to remove the $O_2^{\cdot -}$ or to decrease NADPH-oxidation.

Superoxide radicals may be generated as a response to abiotic and biotic stresses at a number of cellular sites involved in electron transfer (Purvis and Shewfelt, 1993; see section 1.1.5.1.3). Wheat plants may be more tolerant of superoxide radical-generating stresses, or produce fewer superoxides in response to stress than other species. Superoxide radical generation may only be a transitory response to stresses, as control of reactive oxygen species is rapid (Hippeli and Elstner, 1996). If the superoxides were dismutated rapidly to other oxygen species (*e.g.* H_2O_2), which also cause peroxidative damage, but are not detected by the cytochrome *c* assay, then chlorsulfuron stress would not have appeared to have altered $O_2^{\cdot -}$ levels.

The sulfonylurea herbicides do not cause direct peroxidative damage as their primary mode of action, but could provide a general stress that increases superoxide radical generation. Increased superoxide radical generation from such stress may be short-lived in wheat, as chlorsulfuron is rapidly metabolised by wheat (Beyer *et al.*, 1988) and superoxide radicals are dismutated rapidly by SODs (Thompson *et al.*, 1997). As the exposure time to chlorsulfuron was comparatively long (14 and 21 d), even with replacement of chlorsulfuron at changes of nutrient solution the residual concentrations of chlorsulfuron in the plant tissues may not have been sufficient to increase superoxide radical generation.

In contrast to the possibility that sulfonylurea herbicides increase superoxide radical generation through a stress response, a number of sulfonylureas including chlorsulfuron have been found to decrease NADH-oxidase activity in cell membrance vesicles (Morre *et al.*, 1995a, b). The activity of the oxidase was inhibited by chlorsulfuron, which may in turn reduce the rate of $O_2^{\cdot -}$ production. As superoxide generation was measured in the roots rather than the leaves,

where bipyridinyl herbicides inhibit photosynthesis and increase superoxide generation (Chambers 1995), chlorsulfuron applied to roots may inhibit the NADH oxidase, decreasing superoxide levels.

In conclusion, 40 μg chlorsulfuron L^{-1} treatment has a much greater influence than increasing Zn concentration in nutrient solution from 0.1 to 4 μ*M* ZnHEDTA has on root protein concentration and root FW. Superoxide radical generation does not change significantly in roots grown in low-Zn activity and is not influenced by chlorsulfuron. Superoxide radical generation does not appear to be a suitable measure of either Zn deficiency or herbicide stress in wheat.

5.5 References

Beyer, E. M., Duffy, M. J., Hay, J. V., and Schlueter, D. D. (1988). Sulfonylureas. In Kearney, P. C. and Kaufman, D. D. Eds. *Herbicides: Chemistry, degradation and mode of action.* Marcel Dekker. pp. 117-189.

Bowler, C., van Montagu, M. and Inzé, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* 43, 83-116.

Bradford, M. M. (1976). A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Cakmak, I., Marschner, H. and Bangerth, F. (1989). Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolaris vulgaris* L.). *Journal of Experimental Botany* 40, 405-412.

Cakmak, I. and Marschner, H. (1987). Mechanism of phosphorus-induced zinc deficiency in cotton. III. Changes in physiological availability of zinc in plants. *Physiologia Plantarum* 70, 13-20.

Cakmak, I. and Marschner, H. (1988a). Enhanced superoxide radical production in roots of zinc-deficient plants. *Journal of Experimental Botany* 39, 1449-1460.

Cakmak, I. and Marschner, H. (1988b). Increase in membrane permeability and exudation in roots of Zn deficient plants. *Journal of Plant Physiology* 132, 356-361.

Clayton, D. S. and Reynolds, T. L. (1991). Chlorsulfuron effects on protein synthesis and accumulation in cultured root tips of *Pisum sativum* L. *Journal of Plant Physiology* 137, 337-341.

Falchuk, K. H., Ulpino, L., Mazus, B. and Vallee, B. L. (1976). *Euglena gracilis* DNA dependent RNA polymerase II: A zinc metalloenzyme. *Biochemistry* 15, 4468-4475.

Foyer, C. H., Descourvieres, P. and Kunert, K. J. (1994). Protection against oxygen radicals - an important defence mechanism studied in transgenic plants. *Plant Cell and Environment* 17, 507-523.

Fridovich, I. (1986). Biological effects of the superoxide radical. *Archives of Biochemistry and Biophysics* 247, 1-11.

Hatzios, K. K. and Howe, C. M. (1982). Influence of the herbicides hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pesticide Biochemistry and Physiology* 17, 207-214.

Hernandez, J. A., Almansa, M. S., Delrio, L. and Sevilla, F. (1993). Effect of salinity on metalloenzymes of oxygen metabolism in two leguminous plants. *Journal of Plant Nutrition* 16, 2539-2554.

Hippeli, S. and Elstner, E. F. (1996). Mechanisms of oxygen activation during plant stress - biochemical effects of air pollutants. *Journal of Plant Physiology* 148, 249-257.

Jeffery, E. H. (1983). The effect of zinc on NADPH oxidation and mono-oxygenase activity in rat hepatic microsomes. *Molecular Pharmacology* 23, 467-471.

Landstein, D., Epelbaum, S., Arad, S., Barak, Z. and Chipman, D. M. (1995). Metabolic response of *Chlorella emersonii* to the herbicide sulfometuron methyl. *Planta* 197, 219-224.

LaRossa, R. A. and Schloss, J. V. (1984). The herbicide sulfometuron methyl is bacteriostatic due to inhibition of acetolactate synthase. *Journal of Biological Chemistry* 259, 8753-8757.

Markert, M., Andrews, P. C. and Babior, B. M. (1984). Measurement of $O_2^{\cdot -}$ production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. *Methods in Enzymology* 105, 358-365.

Marschner, H. (1995). Mineral Nutrition of Higher Plants, 2nd Edn. Academic Press, London.

Morre, D. J., Fleurimont, J. and Sweeting, M. (1995a). Chlorsulfuron blocks 2,4-D-induced cell enlargement and NADH oxidase in excised sections of soybean hypocotyls. *Biochimica et Biophysica Acta - Biomembranes* 1240, 5-9.

Morre, D. J., Wu, L. Y. and Morre, D. M. (1995b). The antitumor sulfonylurea n-(4-methylphenylsulfonyl)-n'-(4-chlorophenyl) urea (LY181984) inhibits NADH

oxidase activity of HeLa plasma membranes. *Biochimica et Biophysica Acta - Biomembranes* 1240, 11-17.

Pinton, R., Cakmak, I. and Marschner, H. (1994). Zinc deficiency enhanced NAD(P)H dependent superoxide radical production in plasma membrane vesicles isolated from roots of bean plants. *Journal of Experimental Botany* 45, 45-50.

Purvis, A. C. and Shewfelt, R. L. (1993). Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* 88, 712-718.

Purvis, A. C., Shewfelt, R. L. and Gegogeine, J. W. (1995). Superoxide production by mitochondria isolated from green bell pepper fruit. *Physiologia Plantarum* 94, 743-749.

Ray, T. B. (1982). The mode of action for chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 10-17.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Rhodes, D., Hogan, A. L., Deal, L., Jamieson, G. C. and Haworth, P. (1987). Amino acid meatbolism of *Lemna minor* L: Responses to chlorsulfuron. *Plant Physiology* 84, 775-780.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Smirnoff, N. (1993). Tansley Review No. 52: The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* 125, 27-58.

Thompson, J. E., Legge, R. L. and Barber, R. F. (1987). The role of free radicals in senescence and wounding. *New Phytologist* 105, 317-344.

CHAPTER 6

THE INFLUENCE OF ZINC AND CHLORSULFURON ON ROOT RESPIRATION RATES

6.1 Introduction

6.1.1 Role of Zn in respiration

Zinc deficiency does not appear to be a major factor that determines respiration rate in plants, although there is a number of reports that suggest that Zn deficiency decreases oxygen uptake. Zn-deficient barley respired less than control plants (Satsukevich, 1975), as did Zn-deficient soybean (Wu and Xiao, 1992). Cotton yield and a number of physiological functions including respiration were increased by Zn fertilization in the field (Rakhmatdzhanov *et al.*, 1980). Grape vines treated with Zn maintained respiration rates compared to a decline in untreated controls as plants aged (Tagizade and Gasanov, 1975). Zn-deficient plants have decreased mitochondrial function which may impair their ability to oxidise respiratory substrates (Chvapil 1973; Shkolnik, 1984).

6.1.2 Respiration

Most energy in plants is derived from carbohydrates produced in photosynthesis and can be summarized by the following reaction which uses glucose as the primary carbohydrate:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O + \text{energy} \qquad \text{(Equation 6.1).}$$

The energy contained in glucose, is too large (670 kJ mol^{-1}) to be released in a single reaction without damaging cell components (Keeton and Gould, 1986). A series of reactions is used to oxidise the carbohydrates and generate adenosine triphosphate (ATP) which stores smaller amounts of energy (7.3 kJ mol^{-1}) that can be used readily in cellular reactions (Keeton and Gould, 1986). Pyruvate from the TCA cycle and the adenosine compounds NADH and FADH are oxidised by the electron transport chain in the mitochondrial inner membrane. The electron transport chain consumes oxygen and creates a proton gradient across the mitochondrial inner membrane that is used to drive ATP phosphorylation.

Thirty-six moles of ATP are produced from 1 mole of glucose (Keeton and Gould, 1986).

6.1.3 Electron transport chain and terminal oxidases

The electron transport chain consists of a series of multiprotein complexes that transfer electrons via ubiquinone molecules (Figure 6.1). Ubiquinone forms a mobile electron-distributing pool, accepting electrons from NADH (via complex I) and succinate (via complex II) (Moore and Siedow, 1991). Electrons from reduced ubiquinone (ubiquinol or $Q_{reduced}$) are then transferred to complex III. Electrons are transferred from complex III to IV by cytochrome c. Protons are exported by complexes I, III and IV, generating a proton gradient across the membrane. This gradient is used to phosphorylate ATP by allowing protons to move back into the mitochondrion via an ATPase (Moore and Siedow, 1991).

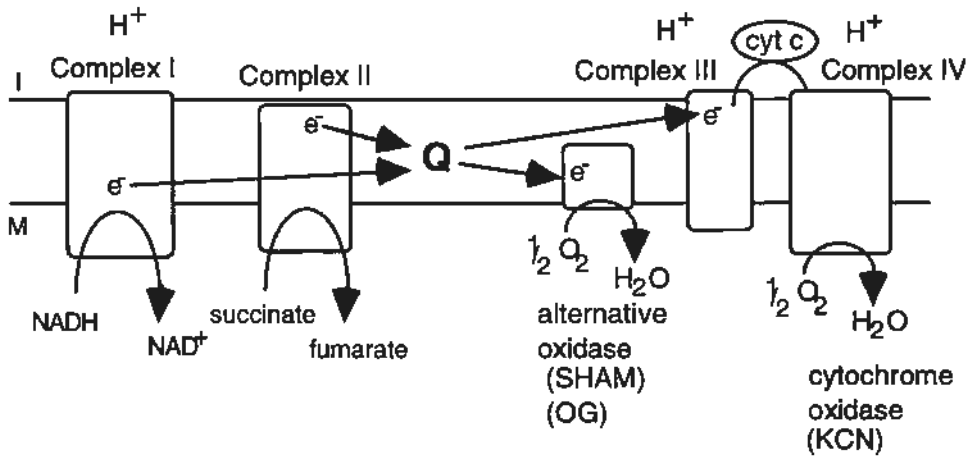

Figure 6.1. Schematic representation of the electron transport chain located on the inner mitochondrial membrane. The multiprotein units are indicated as complexes I to IV. Names in parentheses indicate oxidase inhibitors. Arrows indicate reduction/oxidation reaction sites involving transfer of electrons (e^-) or oxygen reduction. Q refers to the ubiquinone pool of oxidised and reduced quinones. I and M refer to the intermembrane space and matrix of the mitochondria respectively. (Figure adapted from Moore and Siedow, 1991, Purvis and Shewfelt, 1993 and Wagner and Krab, 1995).

The main site of oxygen consumption in the mitochondrion is at complex IV (cytochrome oxidase), in which oxygen is reduced (Moore and Siedow, 1991):

$$2e^- + \tfrac{1}{2}O_2 + 2H^+ = H_2O \qquad \text{(Equation 6.2).}$$

In plants and fungi, a second pathway is present, where electrons can be diverted at complex III through a protein known as the "alternative oxidase" (Day *et al.*, 1995b; Moore and Siedow, 1991). Oxygen is consumed by this oxidase, but the degree to which it operates and contributes to total oxygen consumption is generally much less than that of the cytochrome oxidase (Lambers *et al.*, 1996). Because electron flow through the alternative oxidase prevents the export of two protons for each electron entering the chain (see Fig. 6.1), energy is lost from the system as heat. The alternative oxidase is therefore much less effective in providing energy for cellular processes as less ATP is produced compared to the cytochrome pathway (Millar *et al.*, 1995).

6.1.4 Study of alternative oxidase

The best-known examples of the presence of the alternative oxidase are the Arum lillies which increase the respiration rates of the flower to 8-10 times pre-flowering rates. The alternative oxidase respiration is increased to almost 100 % of the total rates in order to volatilise oils which attract pollinating insects (Salisbury and Ross, 1992).

Interest in the alternative oxidase was stimulated with the discovery by Bahr and Bonner (1973a, b) that although electron flow could be diverted from the cytochrome oxidase to the alternative oxidase by inhibitors that block the action of the complexes, inhibition of the alternative oxidase did not divert electrons via the cytochrome pathway (Day *et al.*, 1996). This suggested that the alternative oxidase acted as an "overflow" mechanism when the cytochrome oxidase was saturated with electrons (Lambers *et al.*, 1996). Recent studies have indicated that electron flow may not always operate in this manner. Electrons may flow through the alternative pathway without the cytochrome pathway being fully engaged (Day *et al.*, 1996; Millar *et al.*, 1995); therefore, inhibitor studies may not

accurately represent the contribution of the alternative oxidase (Hoefnagel *et al.*, 1995; Millar *et al.*, 1995; Wagner and Krab, 1995).

Roles for the alternative oxidase other than in energy overflow are not fully known. When subjected to various abiotic stresses (*e.g.* cold, heat, darkness), which may interfere with the normal respiratory path, plants have the capacity to respire via the alternative pathway (Purvis and Shewfelt, 1993). The alternative oxidase may therefore be involved in responses to temporary changes in environmental conditions (Lambers *et al.*, 1996).

6.1.5 Herbicides and respiration

Inhibition of respiration is not a recognised mode of action for any herbicide class (Chambers, 1995). However, aryloxyphenoxypropionate herbicides (*e.g.* diclofop) can dissipate the membrane potential and effectively uncouple ATP phosphorylation from respiration (DiTomaso *et al.*, 1991; Wright 1994). Diclofop does not appear to alter respiration (Cohen and Morrison, 1984). The few reports of herbicide effects on plant respiration indicate that inhibition of respiration is not a common phenomenon. The herbicide 2,4-D decreased root respiration of peas at relatively high concentrations (Zhang *et al.*, 1989), and decreased mitochondrial respiration in barley grown under Zn-deficiency conditions (Zakharchishina, 1973). Bromoxynil decreased malate- and succinate-based respiration in peas, possibly by membrane uncoupling (Zottini *et al.*, 1994).

Sulfonylurea herbicides do not depolarise membranes as either their primary mode of action or after prolonged exposure times (Wright, 1994). Chlorsulfuron did not decrease oxygen consumption (Ray, 1982). However, as herbicides may be considered a form of abiotic stress (Lambers *et al.*, 1996; Purvis and Shewfelt, 1993), alterations in the alternative oxidase contribution may indicate that the prolonged presence of herbicides is altering the plant metabolism in some way.

In the experiments presented below, the hypotheses that wheat root respiration was decreased by low Zn activity of solution and the presence of chlorsulfuron were tested. The alternative and cytochrome oxidases were

examined using inhibitors (octyl gallate and cyanide, respectively) to determine their respiratory capacities.

6.2 Materials and Methods

6.2.1 Growing conditions and harvest procedure

Gatcher wheat was grown in nutrient solutions as described previously (section 2.2) for 14 d before assaying roots for cytochrome oxidase activity. Nutrient solutions containing 0.1 or 4 μ*M* ZnHEDTA and 0 or 40 μg chlorsulfuron L^{-1} (2 Zn activities × 2 chlorsulfuron concentrations = 4 treatments). Four replicates were used. A second set of plants grown for 22 d in conditions identical to the above experiment was assayed for alternative oxidase activity of roots. Only two replicates were used in this experiment. The final experiments assayed both alternative and cytochrome oxidase activities of Gatcher and Excalibur wheats grown in 0.1 μ*M* ZnHEDTA nutrient solution for 14 d with 0, 4, 40 and 400 μg chlorsulfuron L^{-1}; 2 cultivars × 4 chlorsulfuron concentrations = 8 treatments. Four replicates of each assay were used for each treatment.

Plants were taken to the laboratory where they were kept in ambient fluorescent light (< 50 μmol m^{-2} s^{-1}) for the duration of the experiments. Treatments were assayed in randomised order to minimize effects of any changes in respiration with time elapsed after removal of plants from the growth room.

Plants were removed from pots, roots blotted and the distal 5-8 cm excised. The excised roots were chopped into 1-2 mm lengths (sections) in a 0.2 m*M* $CaCl_2$ solution. Roots were allowed to recover from chopping injury for 30-60 minutes in the same solution. After the recovery period, the roots were strained from the solution, blotted dry and weighed, then placed in the oxygen electrode chamber.

6.2.2 Oxygen consumption measurement

The rate of oxygen uptake by root slices was measured at 25°C in a solution containing 250 m**M** sucrose, 10 mM KH_2PO_4, 10 mM TES, 5 mM $MgCl_2$. A Clark-type O_2 electrode with a magnetic stirrer (Rank Bros., Cambridge, UK) housed in a

clear perspex chamber and connected to a chart recorder was used to record the oxygen content of the solution. Approximately 0.15-0.25 g FW of roots was used for each replicate assay. Root sections were added to aerated medium in the chamber which was sealed with the electrode plug and allowed to reach a steady rate of oxygen consumption (2-3 min).

To observe alternative pathway respiration, the cytochrome pathway was inhibited by adding KCN (final concentration 1 m*M*). Oxygen consumption was allowed to reach a new steady rate (2-3 min). Octyl gallate was then added (final concentration 20 μ*M*) to inhibit alternative oxidase respiration and determine the residual respiration rate of roots.

To observe cytochrome oxidase respiration, FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; final concentration 1 μ*M*)) was added to uncouple respiration from proton transport and indicate the maximum possible rate of cytochrome oxidase activity (Lambers *et al.*, 1996). Octyl gallate was then added (20 μ*M*) to inhibit the alternative oxidase and force all electron flow through the cytochrome oxidase. After respiration reached a steady rate, KCN (1m*M*) was added to determine the residual respiration rate.

6.2.3 Western blot gel procedure

To determine the amount of alternative oxidase protein present in the roots a western blot assay was run using wheat root mitochondria. The following steps were performed at 4°C and the samples stored on ice between steps. The procedure is based on that employed by Zhang *et al.*, (1996). Approximately 2 g (FW) of roots was ground on ice in 4 mL of grinding medium (300 m*M* sucrose, 50 m*M* TES, 1 m*M* EDTA, 1 m*M* MgCl2, 1 % (w/v) PVP, 1 m*M* glycine, 0.4 % (w/v) BSA, 10 m*M* isoascorbic acid). The homogenate was transferred to centrifuge tubes in 20 mL of isolation medium and centrifuged at 4 500 rpm for 5 min. The supernatant was centrifuged at 12 000 rpm for 15 min. The pellet was resuspended in 10 mL of resuspension medium (300 m*M* sucrose, 20 m*M* TES, 0.1 % (w/v) BSA), then centrifuged at 4 500 rpm for 5 min. The supernatant was

centrifuged at 12 000 rpm for 15 min. The pellet from this final centrifugation was resuspended in 0.2 ml of resuspension medium.

As the alternative oxidase can be present in an oxidised or a reduced state, and this influences the banding of the enzyme in the final gel, the samples were solubilised in 5 mL of 10 m*M* pyruvate, 0.4 mg EDTA/mL for 30 min, then incubated in 5 mL of 20 m*M* TES, 2 *M* NaCl, 10 m*M* pyruvate with 10 m*M* mercaptoethanol to oxidise the enzyme and resolve the alternative oxidase into a single band. The samples were ultracentrifuged at 100 000 *g* for 30 min and the pellet (mitochondrial fraction) was resuspended in 0.1 mL TES buffer (20 m*M* TES, 10 m*M* pyruvate). The resulting samples were assayed for protein by the method of Lowry *et al.* (1951).

Samples containing 50 µg protein were loaded onto a polyacrylamide gel poured 24 h previously (9.6 mL H_2O, 5 mL 40 % (w/v) acrylamide, 5 mL 1.5 *M* TrisHCl, pH 8.8, 0.2 mL 10 % (v/v) sodium dodecyl sulphate (SDS), 0.2 mL 10 % (w/v) ammonium persulphate, 0.008 mL TEMED) in a Bio-Rad Protean II cell. The gel was bathed in a filtered (22 µm pore size) buffer solution of 25 mM Tris, 192 mM glycine and 0.1 % (v/v) SDS (pH 8.3) and run at 150 V, 15 mA for 4 hr. A set of pre-stained molecular weight standard proteins (Bio-Rad, Sydney) was also loaded to determine the approximate size of any proteins detected.

The gel and membrane were then placed between filter papers soaked in a transfer buffer solution (TBS; 100 mM Tris 2.5 M NaCl, pH 7.5). The proteins in the gel were transferred to a 0.2 µm PVDF (polyvinylidine difluoride) membrane (BioRad Trans-Blot® transfer medium) using a 9 V, 100 mA current for 1 hr across graphite plate electrodes. The PVDF membrane was removed and washed in TBS for 15 min, washed in TBS supplemented with 3 % BSA for 15 min, then incubated in primary antibody for 1 h. The membrane was washed in TBS supplemented with 0.5 mL L^{-1} Tween 80 detergent for 15 min, incubated in secondary antibody for 1 h and washed again TBS plus Tween 80 for 15 min. The membrane was incubated in alkaline phosphatase substrate until bands of colour appeared (up to 10 min), washed in H_2O and dried.

The gel was stained with a solution of 0.15 % (v/v) Coomassie blue in 50 % (v/v) methanol, 10 % (v/v) acetic acid for 15 minutes, then destained in a similar soultion without Coomassie blue for 15 minutes. After a final rinse in a solution of 5 % (v/v) methanol and 7 % (v/v) acetic acid for 30 min and dried with a vacuum drier.

6.3 Results

Total respiration of root sections from 14-d-old plants was decreased by low Zn activity of solution (Fig. 6.2). Chlorsulfuron pretreatment increased the cytochrome oxidase capacity (after addition of FCCP and octyl gallate) of root sections in both Zn activities, however this rate remained lower in low-Zn plants (Figure 6.2). When expressed as a percentage of the control rate, low Zn plants respired much faster when FCCP was added. Residual respiration (after addition of KCN) was slightly higher in chlorsulfuron-pretreated plants than control plants and was unaltered by Zn activity pretreatment.

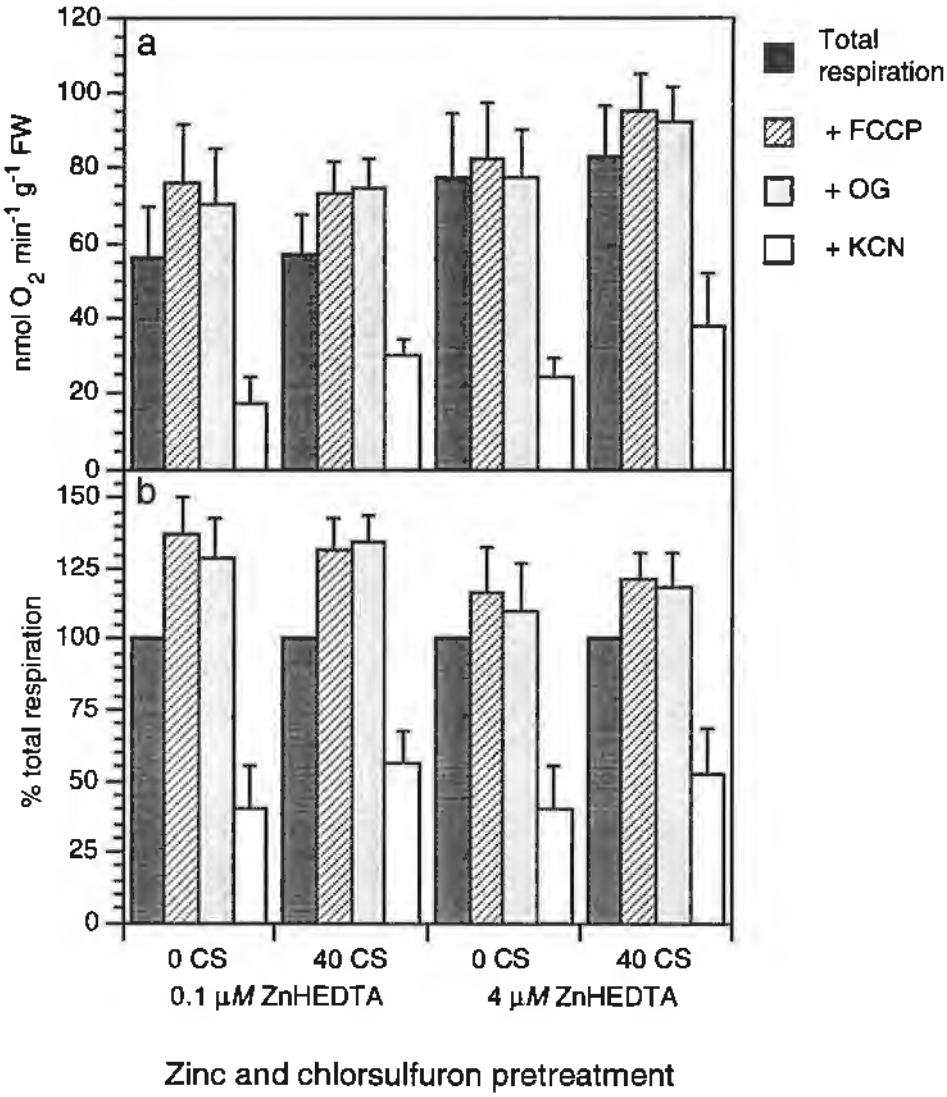

Figure 6.2. Respiration rates of root sections of 14-d-old wheat (mean ± SE, n = 4) indicating cytochrome oxidase activity after addition of 1 µM FCCP and 20 µM octyl gallate. Residual respiration was assessed by addition of 1 µM KCN. Fig. 6.2(a) indicates actual rates and 6.2(b) indicates percentages of total (uninhibited) respiration rates.

When 22-d-old Gatcher wheat was assayed for alternative oxidase activity, total respiration rates of low-Zn root sections were slightly higher than adequate-Zn roots (Fig. 6.3). Chlorsulfuron-pretreated roots had a considerably greater alternative oxidase capacity (shown by addition of 1 m*M* KCN) compared to control roots (60 % of total respiration vs 25 %). Low solution Zn activity slightly increased total respiration roots but had no effect on alternative or residual respiration. Residual respiration was slightly higher in chlorsulfuron-pretreated roots (Fig. 6.3).

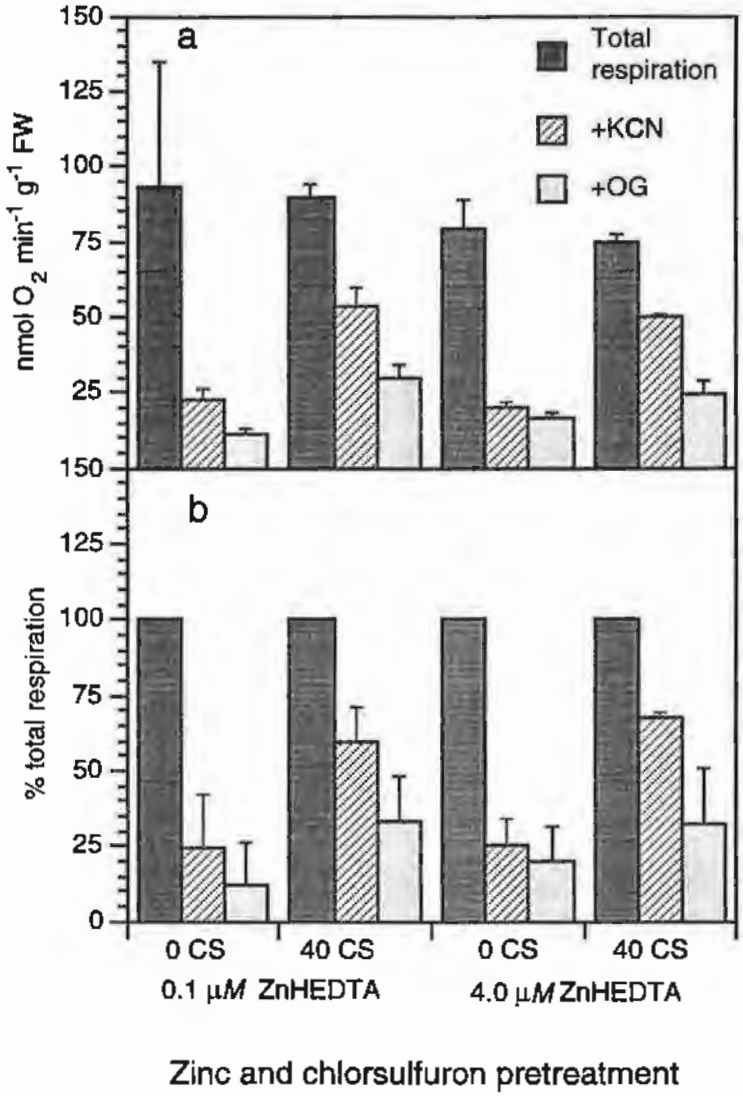

Figure 6.3. Respiration rates of root sections of 22-d-old Gatcher wheat (mean ± SE, n = 4) indicating alternative oxidase activity after addition of 1 m*M* KCN. Residual respiration was assessed by addition of 20 μ*M* octyl gallate (OG). Figure 6.3(a) indicates actual rates and 6.3(b) indicates percentages of total (uninhibited) respiration rates.

Total respiration rates of root sections of 14-d-old plants increased as the chlorsulfuron concentration increased, especially at 400 μg L^{-1} (Fig. 6.4a). Uncoupled respiration (after addition of FCCP) was higher than total respiration only in control plants, suggesting that respiration in chlorsulfuron-pretreated plants was already maximised and unable to be uncoupled (Fig. 6.4). When expressed as a percentage of total respiration, uncoupled respiration rates decreased as chlorsulfuron pretreatment concentration increased (Fig. 6.4b). The cytochrome oxidase capacity (after addition of octyl gallate) was decreased to a greater extent when chlorsulfuron pretreatment concentrations were high (40 and 400 μg chlorsulfuron L^{-1}). The residual respiration of roots (after addition of both inhibitors) was increased in roots pretreated with high concentrations of chlorsulfuron compared to control roots.

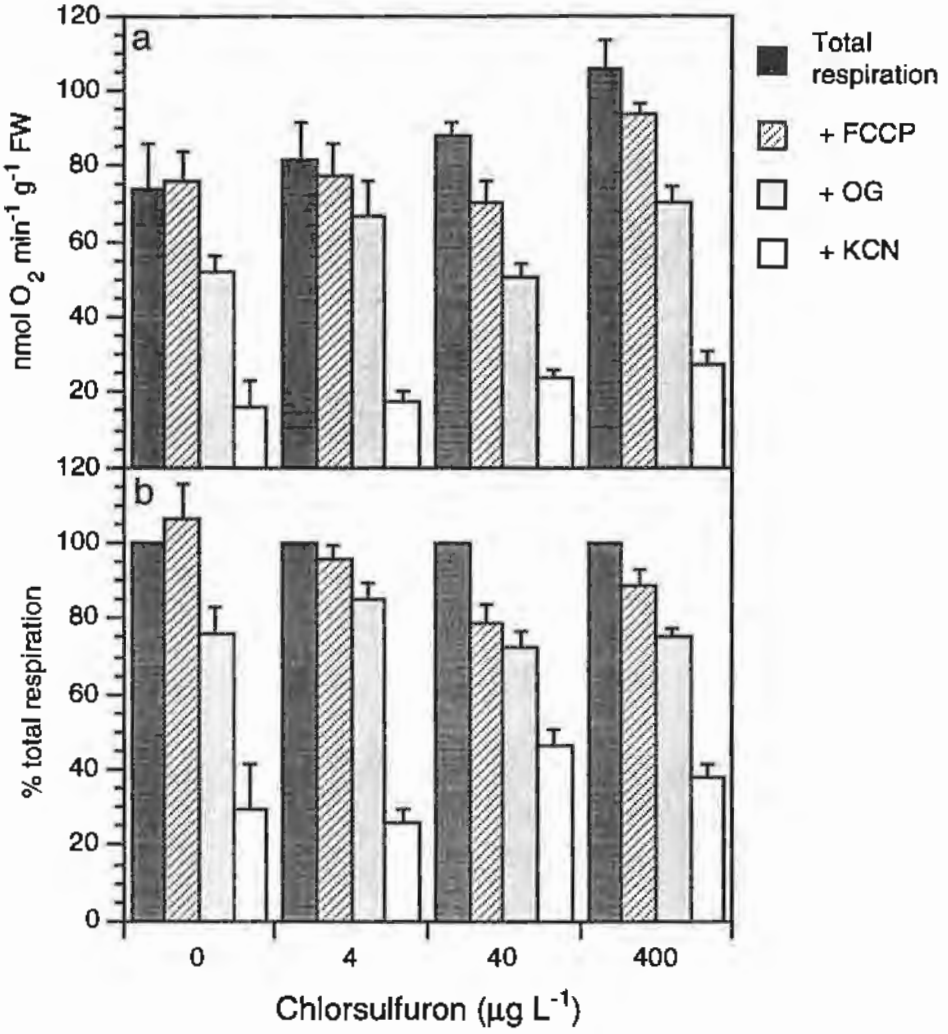

Figure 6.4. Respiration rates of root sections (mean ± SE, n = 4) of 14-d-old wheat grown in 0.1 µ*M* ZnHEDTA, indicating cytochrome oxidase activity after addition of 1 µ*M* FCCP and 20 µ*M* octyl gallate (OG). Residual respiration was assessed by addition of 1 µ*M* KCN. Plants were grown with 0 (control) 4, 40 or 400 µg chlorsulfuron L^{-1} for 14 d. Fig. 6.4(a) indicates actual rates and Fig. 6.4(b) indicates percentages of total (uninhibited) respiration rates.

High chlorsulfuron concentrations increased the total respiration and the alternative oxidase capacity of roots (Fig. 6.5)a. When expressed as a percentage of total respiration, there was no difference between the 40 and 400 μg chlorsulfuron L^{-1} pretreatments on alternative oxidase capacity, which was greater than the 0 and 4 μg pretreaments (Fig. 6.5)b. Residual respiration increased with chlorsulfuron treatment in the same manner as for Fig. 6.4b.

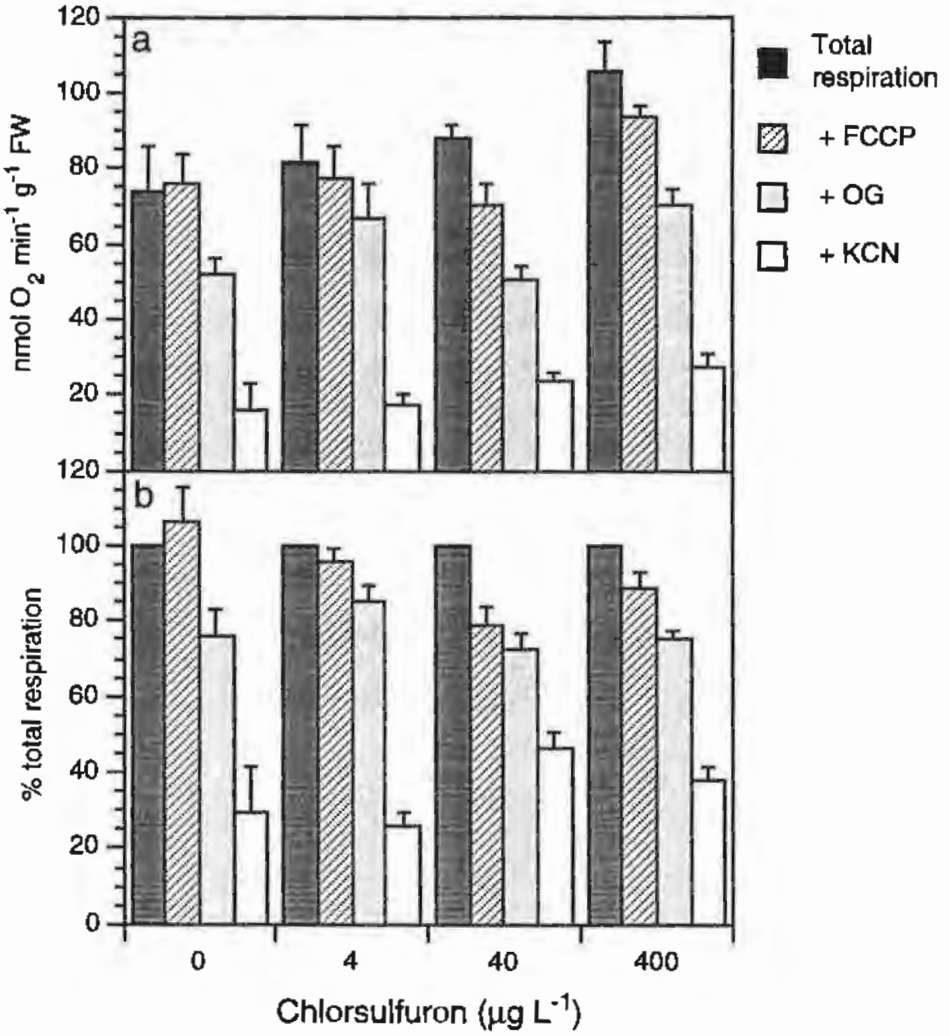

Figure 6.5 Respiration rates (mean ± SE, n = 4) of root sections of 14-d-old wheat grown in 0.1 μ*M* ZnHEDTA, indicating alternative oxidase activity after addition of 1 m*M* KCN. Residual respiration was assessed by addition of 20 μ*M* octyl gallate (OG). Plants were pre-grown with 0 (control), 4, 40 or 400 μg chlorsulfuron L^{-1} for 14 d. Fig. 6.5(a) indicates actual rates and 6.5(b) indicates percentages of total (uninhibited) respiration rates.

No evidence of alternative oxidase could be obtained from Western blot gels run using crude mitochondria of wheat roots. Several sets of wheat roots were sampled with no obvious improvments in detection of protein.

6.4 Discussion

Recent advances in respiration analysis have indicated that addition of inhibitors of mitochondrial respiration can interfere with the redox poise of the quinone pool (ratio of $Q_{reduced}$ to Q_{total}; Millar *et al.*, 1995; Hoefnagel *et al.*, 1995; Wagner and Krab, 1995). As the quinone redox poise influences the electron distribution between the cytochrome and alternative oxidases, addition of inhibitors may lead to inaccurate measurement of the degree of inhibition and hence the contribution of the particular pathway to total respiration (Day *et al.*, 1996; Millar *et al.*, 1995; Wagner and Krab, 1995). While this may have implications for the exact values of the *contribution* of the alternative oxidase in wheat roots in these experiments, the total *capacity* of each of the pathways can be measured easily using inhibitors (Hoefnagel *et al.*, 1995). The procedures used in the experiments presented here determine the potential of roots to respire using the alternative oxidase, rather than the actual proportion of respiration that is provided by the alternative oxidase (Hoefnagel *et al.*, 1995).

Chlorsulfuron-pretreated roots showed much greater alternative oxidase capacity than control roots when the cytochrome pathway was inhibited by KCN (Fig. 6.3, 6.5). Despite the warning that only qualitative data regarding the presence of alternative oxidase activity should be considered (Day *et al.*, 1996), it is obvious that there was a large influence of chlorsulfuron on alternative oxidase activity, while Zn activity of solution had much less influence (Fig. 6.2, 6.3). Total respiration was similar in control and chlorsulfuron-pretreated roots (Fig. 6.4, 6.5); however, a decrease in cytochrome oxidase activity with a corresponding increase in the proportion of alternative oxidase seems unlikely. It is more probable that the capacity of the alternative oxidase increased but its contribution to total respiration was unaltered. Some sulfonylureas, including chlorsulfuron, decrease NADH oxidation on the inner mitochondrial membrane (Morre *et al.*,

1995a, b), which may reduce total respiration rates. The effect of decreased NADH oxidation on the alternative oxidase was not reported by (Morre et al, 1995a, b) but should not have any direct influence on the assays as performed here.

There were no interactions between Zn and chlorsulfuron pretreatments that influenced total or inhibited respiration rates (Figs. 6.2-6.5), suggesting that the effects of Zn deficiency and chlorsulfuron act on independent functions within roots. Total respiration rate of 22-d-old wheat plants was increased by low Zn activity of solution, but was decreased by low Zn activity in 14 d-old-plants (Fig. 6.2). Barley plants treated with 2,4-D and supplied with Zn respired at a greater rate than Zn-deficient plants (Zakharchishina, 1973).

The observed increases in alternative oxidase capacity may not be a direct result of chlorsulfuron influence, but some other alterations in metabolism. Changes in alternative oxidase capacity due to altered Zn status are unlikely as plants were affected by chlorsulfuron more than by Zn activity of solution (Figure 6.2). Clayton and Reynolds (1991) found only four "stress-induced" proteins in chlorsulfuron-treated pea plants. Whether the stress proteins in peas represent the same stress imposed on wheat plants by chlorsulfuron is unknown, as peas are more susceptible to chlorsulfuron than wheat (Beyer *et al.*, 1988).

Root respiration appears to follow root growth, where decreased root growth limits oxygen consumption (Lambers *et al.*, 1996). Roots respire less when growing in low nutrient conditions (Lambers *et al.*, 1996), but this varies with plant age and the nutrients involved. Presumably, nutrients which decrease root growth when deficient will also decrease total respiration. Wheat grown without nitrogen increased respiration rates when resupplied with nitrate and ammonium (Barneix *et al.*, 1984 in Lambers *et al.*, 1996). The alternative oxidase component increased when plants were resupplied with ammonium, but decreased when nitrate was added (Barneix *et al.*, 1984). Phosphate-deprived plants did not alter total respiration rates, but the relative proportion of the alternative oxidase was increased compared to adequately-supplied plants (Rychter and Mikulska, 1990). Low solution Zn activity increased 18- to 22-d-old wheat root DW (Chapter 2) and 12-d-old root tip extension (Chapter 3) but

decreased total wheat root respiration of 14-d-old plants (Fig. 6.2). No influence of Zn activity on respiration of 22-d-old plants was observed (Fig. 6.3).

Respiration rates of a number of species varied with differential Zn supply. Zinc deficiency decreased respiration in tomato by inhibiting glycolysis and the TCA pathway (Paribok, 1973). Oxidative phosphorylation was unaffected by Zn deficiency as measured by phosphorus:oxygen ratios as was the "energy efficiency" (Paribok, 1968, in Shkolnik, 1984), but addition of Zn decreased sensitivity of respiration to cyanide (Chistyakov and Gundel, 1968, in Shkolnik, 1984). Respiration of Zn-adequate tomato plants was reduced to a greater extent than Zn-deficient plants when treated with respiration inhibitors (*e.g.* sodium azide; Paribok, 1972). Leaves of Zn-deficient tomato plants had a lower respiration rate than Zn-adequate plants (Paribok, 1972), however, there was no influence of Zn nutrition on tomato roots (Paribok, 1973).

Increased levels of trace elements (including Zn) in seeds increased respiration in 5-day-old barley plants and decreased it in 10-day-old plants (Satsukevich and Vol'-shchuk, 1976). Cobalt, Cu and Zn also decreased respiration in leaves of barley as plants approached maturity (Satsukevich, 1974). Wheat plants grown in low-Zn conditions may have altered their response to Zn availability between d 14 and d 21 after planting.

Environmental stresses had a number of influences on *Chlamydomonas* respiration that were similar to those in wheat (Weger and Dasgupta, 1993). Alternative oxidase capacity was increased by N, P and K deficiency without increasing total respiration, which is different to the effect seen in Fig. 6.3. Osmotic stress and high temeperatures on the other hand also increase the alternative oxidase capacity and decreased cytochrome oxidase capacity in *Chlamydomonas* (Weger and Dasgupta, 1993), similar to the stress observed in wheat plants treated with chlorsulfuron in Figs. 6.2 and 6.3.

Attempts to measure expression of alternative oxidase activity using western blot techniques were unsuccessful. No banding could be detected in any samples (50 μg protein) from several different extractions. It remains to be seen whether production of alternative oxidase is increased by chlorsulfuron pretreatment, or

whether specific activity of the enzyme is increased in chlorsulfuron-pretreated plants. Wheat treated with chloramphenicol (a compound that alters mitochondrial formation) for up to 14 d increased the total amount of alternative oxidase present in roots and leaves (Zhang *et al.*, 1996). Greater purification of wheat mitochondrial protein may improve the extraction of the enzyme, while methods of assaying alternative oxidase that avoid use of inhibitors may provide more precise estimates of the enzyme activity in chlorsulfuron-treated wheat.

Analysis of wheat root respiration indicated that chlorsulfuron treatment induces a stress that is detectable by an increased capacity for respiration via the alternative oxidase. Zinc deficiency does not alter the alternative oxidase capacity, but can alter the total respiration of wheat roots, depending on the age of the plants.

6.5 References

Beyer, E. M., Duffy, M. J., Hay, J. V., and Schlueter, D. D. (1988). Sulfonylureas. In Kearney, P. C., and Kaufman, D. D. Eds. *Herbicides: Chemistry, degradation and mode of action.* Marcel Dekker. pp. 117-189.

Chambers, A. (1995). Field crop herbicide information, 4th edn. Victorian Department of Agriculture, Rutherglen Research Institute, Victoria.

Chvapil, M. (1973). New aspects in the biological role of zinc: A stabilizer of macromolecules and biological membranes. *Life Sciences* 13, 1041-1049.

Day, D. A., Krab, K., Lambers, H., Moore, A. L., Siedow, J. N., Wagner, A. M. and Wiskich, J. T. (1996). The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. *Plant Physiology* 110, 1-2.

Day, D. A., Whelan, J., Millar, A. H., Siedow, J. N. and Wiskich, J. T. (1995b). Regulation of the alternative oxidase in plants and fungi. *Australian Journal of Plant Physiology* 22, 497-509.

DiTomaso, J. M., Brown, P. H., Stowe, A. E., Linscott, D. L. and Kochian, A. D. (1991). Effects of diclofop and diclofop-methyl on membrane potentials in roots of intact oat, maize, and pea seedlings. *Plant Physiology* 95, 1063-1069.

Hoefnagel, M. H. N., Millar, A. H., Wiskich, J. T. and Day, D. A. (1995). Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. *Archives of Biochemistry and Biophysics* 318, 394-400.

Keeton, W. T., and Gould, J. L. (1986). Biological science. Norton, New York.

Lambers, H., Atkin, O. K., and Scheurwater, I. (1996). Respiratory patterns in roots in relation to their functioning. In Waisel, Y., Eshel, A., and Kafkafi, U. Ed(s). *Plant roots: The hidden half. 2nd edn.* Marcel Dekker. pp. 323-362.

Millar, A. H., Atkin, O. K., Lambers, H., Wiskich, J. T. and Day, D. A. (1995). A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. *Physiologia Plantarum* 95, 523-532.

Moore, A. L. and Siedow, J. N. (1991). The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochimica et Biophysica Acta* 1059, 121-140.

Morre, D. J., Fleurimont, J. and Sweeting, M. (1995a). Chlorsulfuron blocks 2,4-D-induced cell enlargement and NADH oxidase in excised sections of soybean hypocotyls. *Biochimica et Biophysica Acta - Biomembranes* 1240, 5-9.

Morre, D. J., Wu, L. Y. and Morre, D. M. (1995b). The antitumor sulfonylurea n-(4-methylphenylsulfonyl)-n'-(4-chlorophenyl) urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membranes. *Biochimica et Biophysica Acta - Biomembranes* 1240, 11-17.

Paribok, T. A. (1972). Tomato respiration in relation to zinc availability. *Fiziologiya i Biokhimiya Kul'turnykh Rastenii* 4, 379-384.

Paribok, T. A. (1973). The effect of varying the zinc supply on tomato respiration. *Fiziologiya Rastenii* 20, 158-164.

Purvis, A. C. and Shewfelt, R. L. (1993). Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* 88, 712-718.

Rakhmatdzhanov, U. R., Ponomarev, V. G. and Ergashev, A. (1980). Effect of zinc fertilizers on physiological processes and productivity of cotton grown in Tajikistan. *Agrokhimiya* 1, 80-84.

Ray, T. B. (1982). The mode of action for chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 10-17.

Rychter, A. M. and Mikulska, M. (1990). The relationship between phosphate status and cyanide-resistant respiration in bean roots. *Physiologia Plantarum* 79, 663-667.

Salisbury, F. B., and Ross, C. W. (1992). Plant physiology, 4th edn. Wadsworth, Belmont, California.

Satsukevich, V. B. (1975). Effect of copper, zinc and cobalt on activity of redox enzymes and respiration in barley seedlings of the first seed generation. *Vestsi Akademii Navuk BSSR, Biyalagichnykh Navuk* 3, 98-101.

Satsukevich, V. B. (1974). Manifestation of the effect of trace elements on respiration, activity of respiratory enzymes and yield of barley in the first

seed generation. *Vestsi Akademii Navuk BSSR, Biyalagichnykh Navuk* 5, 16-22.

Satsukevich, V. B. and Vol'-shchuk, O. V. (1976). Physiological and biochemical indices of barley given additional application of trace elements. *Vestsi Akademii Navuk BSSR, Biyalagichnykh Navuk* 1, 31-36.

Shkolnik, M. Ya (1984). Trace elements in plants. Elsevier, Amsterdam.

Tagizade, A. Kh and Gasanov, M. (1975). The effect of minor elements and gibberellin on the respiration rate of grapes. *Uchen. Zapiski-Azerb. Un-t. Ser. Biol. Nauk* 3-4, 64-67.

Wagner, A. M. and Krab, K. (1995). The alternative respiration pathway in plants - role and regulation. *Physiologia Plantarum* 95, 318-325.

Weger, H. G. and Dasgupta, R. (1993). Regulation of alternative pathway respiration in *Chlamydomonas reinhardtii* (Chlorophyceae). *Journal of Phycology* 29, 300-308.

Wright, J. P. (1994). Use of membrane potential measurements to study mode action of diclofop methyl. *Weed Science* 42, 285-292.

Wu, M. C. and Xiao, C. Z. (1992). The physiological effect of zinc on soyabeans. *Acta Agriculturae Boreali Sinica* 7, 73-77.

Zakharchishina, V. O. (1973). Effect of 2, 4-dinitrophenol on respiration of mitochondria in barley grown under zinc deficiency conditions. *Visnik Kharkivskogo Universitetu, Biologiya* 89, 77-80.

Zhang, F., Römheld, V. and Marschner, H. (1989). Effect of zinc deficiency in wheat on the release of zinc and iron mobilizing root exudates. *Zeitschrift für Pflanzenernährung und Bodenkunde* 152, 205-210.

Zhang, Q. S., Hoefnagel, M. H. N. and Wiskich, J. T. (1996). Alternative oxidase from *Arum* and soybean - its stabilization during purification. *Physiologia Plantarum* 96, 551-558.

Zottini, M., Scoccianti, V. and Zannoni, D. (1994). Effects of 3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil) on bioenergetics of higher plant mitochondria (*Pisum sativum*). *Plant Physiology* 106, 1483-1488.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

The observation that wheat plants growing in low-micronutrient soils became deficient in those nutrients when sprayed with chlorsulfuron (Bowran *et al.*, 1987) has prompted a number of studies that have quantified the response of wheat grown in Zn-deficient conditions and exposed to chlorsulfuron (Robson and Snowball, 1989, 1990; McLay and Robson, 1992; Osborne and Robson, 1992). The findings of these studies indicated that root growth and nutrient uptake were decreased simultaneously by chlorsulfuron treatment, hence separating the mechanism that decreased Zn uptake from the mechanism that decreased root growth was difficult (Osborne and Robson, 1992). The aim of the experiments presented in this thesis was to determine the mechanisms that cause the decline in shoot Zn concentration and the responses of the plant to Zn deficiency and chlorsulfuron stress.

Three mechanisms that could be responsible for reducing Zn concentrations in shoots of chlorsulfuron-treated plants were examined in Chapters 2-4:

i) The reduction of total root mass (root DW) by chlorsulfuron will reduce the volume of soil occupied by the roots, therefore the amount of Zn available to chlorsulfuron-treated plants may be reduced.

ii) The rate of Zn uptake in chlorsulfuron-treated plants may be decreased compared to control plants.

iii) Transport of Zn to the shoots of chlorsulfuron-treated plants may be diminished, therefore reducing the total Zn content in shoots.

Decreased root growth reduces the volume of soil occupied by roots and decreases uptake of diffusion-limited nutrients such as Zn (Dong *et al.*, 1995). Osborne and Robson (1992) were unable to separate the effects of chlorsulfuron on decreased Zn uptake from decreased root growth when measured at 14-d intervals. In the study presented here, root DW was decreased after 4 days exposure to chlorsulfuron, by which time shoot Zn concentrations were also decreased (Chapter 2). Significant differences in root growth (DW) between chlorsulfuron-treated and

control plants could not be determined with shorter exposure times, hence it was difficult using these methods to determine if root mass was decreased before Zn uptake was decreased. Short-term measurements of root growth showed that root tip extension was decreased as early as 2-3 h after addition of chlorsulfuron (Chapter 3), paralleling results of Ray (1982). Zinc uptake rates were unaffected by exposure to chlorsulfuron for 90 min (Chapter 4), indicating that short-term inhibition of Zn uptake does not occur, contrasting with the effects of metabolic inhibitors such as DNP (Giordano *et al.*, 1974; Reid *et al.*, 1996; Schmid *et al.*, 1965). This demonstrates that chlorsulfuron decreases the rate of Zn uptake after inhibition of root growth, and this decrease in Zn uptake rate is more likely to be due to a long-term decline in the ability of roots to take up Zn than to a rapid inhibitory effect of chlorsulfuron.

Significant decreases in Zn uptake rates were not detected until plants had been exposed to chlorsulfuron for at least 3 d prior to the uptake period (Chapter 4). These decreases in Zn uptake rates support the data from Chapter 2 and elsewhere (Osborne *et al.*, 1993; Robson and Snowball, 1990) that Zn, Cu, Mn and P contents were decreased by concentrations of chlorsulfuron that had no significant influence on root DW. After 3 d exposure to chlorsulfuron, both root FW and Zn uptake rates were decreased (Chapter 4), hence the total amount of Zn taken up by chlorsulfuron-treated plants was less than control plants. Therefore, in addition to chlorsulfuron reducing root growth (Chapter 2) and root tip extension (Chapter 3), the experiments in Chapter 4 showed that chlorsulfuron directly decreased Zn uptake rates.

Chlorsulfuron can inhibit transport of solutes within plants (Hall and Devine, 1993) and may also inhibit transport of Zn and other nutrients within chlorsulfuron-treated plants. However, results from experiments in Chapter 4 showed that the percentage of Zn transported from roots to shoots was increased rather than decreased in chlorsulfuron-pretreated plants. Despite this increase, the smaller root mass of chlorsulfuron-pretreated plants available to absorb Zn from solutions, along with the decreased rate of Zn uptake, results in chlorsulfuron-treated plants having lower shoot Zn contents than control plants.

The inhibition of Zn uptake after exposure to chlorsulfuron was reversed by transferring plants to chlorsulfuron-free nutrient solution and growing them for a further 5 days (Chapter 4). Chlorsulfuron applied to roots in solution culture can be removed easily by washing roots and changing solutions, enabling rapid recovery from herbicide damage, while chlorsulfuron activity in soils would remain high for several weeks as the half-life of chlorsulfuron is around 4-8 weeks depending on the pH of the soil (Fredrickson and Shea, 1985). Wheat grown for 4-6 weeks in soil treated with chlorsulfuron showed symptoms of Zn deficiency, while 8-week-old plants had begun to recover (Osborne and Robson, 1993). A similar recovery occurred in wheat plants between 5 and 6 weeks (Dong *et al.*, 1995). Solution-grown plants are therefore able to recover from chlorsulfuron treatment much more quickly than soil-grown plants. The rapid recovery of Zn uptake rate suggests that chlorsulfuron can only inhibit uptake of Zn while present and does not cause permanent damage to the plant. In split-root experiments, Zn uptake was decreased only when chlorsulfuron was mixed with Zn in the soil and not when Zn and chlorsulfuron were supplied in separate halves of split-root containers (Robson and Snowball, 1990).

Water relations of wheat plants were also suspected of being altered by chlorsulfuron treatments. Water uptake of chlorsulfuron-treated wheat was decreased in soil experiments (Robson and Snowball, 1990), but this may have been due to the smaller root systems of chlorsulfuron-treated plants taking up less water. In solution experiments, the water content of both roots and shoots was decreased by chlorsulfuron treatment (Chapter 2). Zn deficiency can decrease transpiration and water potential, indicating water stress (Sharma *et al.*, 1994, 1995). Low Zn activity of solution decreased the water content of both roots and shoots (Chapter 2).

Both Zn deficiency and herbicide exposure constitute considerable stresses on plant metabolism. The processes of protein synthesis, respiration and the activity of enzymes like superoxide dismutase rely on Zn for their activity. These processes may be used as indicators of Zn-deficiency-stress and may potentially be used to assess whether chlorsulfuron influences Zn nutrition of wheat. However, results obtained in the present study indicated there was no interaction between chlorsulfuron and

Zn activity on protein accumulation in roots (Chapter 5). Zn-deficient plants produce less protein than Zn-adequate plants and functioning of Zn-containing proteins such as superoxide dismutase is also reduced (Cakmak *et al.*, 1989). Measurements of protein concentration in the present study indicated that Zn activity of solution had little influence on wheat root growth (FW) or protein concentration (Chapter 5).

In previous studies, superoxide radical generation was increased in Zn-deficient plants (Cakmak and Marschner, 1988; Pinton *et al.*, 1994). In this study (Chapter 5) there was no influence of solution Zn activity on $O_2^{\cdot-}$ generation. Wheat superoxide dismutase may be less sensitive to Zn deficiency than the species tested by Cakmak and Marschner (1988), such that cells can remove $O_2^{\cdot-}$ even in low-Zn plants. Wheat also appeared more tolerant to Zn deficiency, so may produce fewer $O_2^{\cdot-}$ than bean plants. Plant age was the only factor that significantly influenced $O_2^{\cdot-}$ generation. Zinc-inefficient cultivars may experience increased superoxide radical generation as a result of prolonged exposure to chlorsulfuron treatment or Zn-deficient conditions, however, this may require an improved technique to accurately measure responses.

Root respiration is affected by a number of abiotic factors, including nutrition, but responses often depend on the nutrient involved. Respiration may also vary depending on plant age (Satsukevich and Vol'-shchuk, 1976). Respiration of wheat root slices measured in this study (Chapter 6) was influenced by both Zn activity and chlorsulfuron treatment. Low Zn concentration of the nutrient solution increased total respiration by up to 5%, while chlorsulfuron had no significant effect on total respiration rates of root sections but increased the capacity for alternative oxidase activity compared to control plants. The alternative oxidase can be induced by various stresses, including herbicides (*e.g.* paraquat; Bowler *et al.*, 1992), but the response to sulfonylurea exposure was previously unknown. Alternative oxidase capacity was presumably induced by chlorsulfuron stress, however it was not possible to determine if alternative oxidase enzymes were respiring more efficiently or if the amount being produced was greater than untreated plants as seen in chloramphenicol-treated plants (Zhang *et al.*, 1996), as expression of alternative oxidase was not observed with Western blot gels (Chapter 6). Further studies may

indicate whether chlorsulfuron induces increased alternative oxidase activity, or increase expression of the enzyme itself.

The results presented in this study have shown that the decreased rate of Zn uptake in chlorsulfuron-treated wheat plants is independent of the inhibition of root growth. As a result of these two responses, chlorsulfuron-treated plants are smaller and more Zn-deficient than untreated control plants.

As the experiments in this thesis were conducted in solution of pH 6.0, the effects on uptake and growth may be more severe or plants may take longer to recover from exposure to chlorsulfuron in more alkaline soils such as those found in the southern and western Australian wheatbelts. Further studies into responses at different pH and soil conditions may indicate whether use of sulfonylureas should be continued in soils with low plant-available Zn levels.

7.2 References

Bell, P. F., Chaney, R. L. and Angle, J. S. (1991). Free metal activity and total metal concentrations as indices of micronutrient availability to barley [*Hordeum vulgare* (L.) 'Klages']. *Plant and Soil.* 130, 51-62.

Bowler, C., van Montagu, M. and Inzé, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* 43, 83-116.

Bowran, D. G., Porritt, S., and Madin, R. (1987). Chlorsulfuron and diclofop-methyl effects on the copper and zinc status and yield of wheat. In Lemerle, D., and Leys, A. R. Eds. *Proceedings of the Eighth Australian Weeds Conference, 1987.* pp. 404-407.

Cakmak, I., Marschner, H. and Bangerth, F. (1989). Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolaris vulgaris* L.). *Journal of Experimental Botany* 40, 405-412.

Cakmak, I. and Marschner, H. (1988a). Enhanced superoxide radical production in roots of zinc-deficient plants. *Journal of Experimental Botany* 39, 1449-1460.

Cakmak, I. and Marschner, H. (1988b). Increase in membrane permeability and exudation in roots of Zn deficient plants. *Journal of Plant Physiology* 132, 356-361.

Dastgheib, F., Field, R. J. and Namjou, S. (1994). The mechanism of differential response of wheat cultivars to chlorsulfuron. *Weed Research* 34, 299-308.

Dong, B., Rengel, Z. and Graham, R. D. (1995). Effects of herbicide chlorsulfuron on growth and nutrient uptake parameters of wheat genotypes differing in Zn-efficiency. *Plant and Soil* 173, 275-282.

Flaburiari, A. and Kristen, U. (1996). The influence of chlorsulfuron and metsulfuron methyl on root growth and on the ultrastructure of root tips of germinating maize seeds. *Plant and Soil* 180, 19-28.

Fredrickson, D. R. and Shea, P. J. (1986). Effect of soil pH on degradation, movement, and plant uptake of chlorsulfuron. *Weed Science* 34, 328-332.

Giordano, P. M., Noggle, J. C. and Mortvedt, J. J. (1974). Zinc uptake by rice, as affected by metabolic inhibitors and competing cations. *Plant and Soil* 41, 637-646.

Hall, L. M. and Devine, M. D. (1993). Chlorsulfuron inhibition of phloem translocation in chlorsulfuron-resistant and -susceptible *Arabidopsis thaliana*. *Pesticide Biochemistry and Physiology* 45, 81-90.

Hatzios, K. K. and Howe, C. M. (1982). Influence of the herbicides hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pesticide Biochemistry and Physiology* 17, 207-214.

Lemerle, D. (1993). Influence of rainfall and temperature on sensitivity of barley (*Hordeum vulgare*) to chlorsulfuron. *Australian Journal of Agricultural Research* 44, 23-32.

McLay, L. D. and Robson, A. D. (1992). The effect of chlorsulfuron and diclofop-methyl on the uptake and utilization of zinc by wheat. *Australian Journal of Agricultural Research* 43, 59-65.

Osborne, L. D. and Robson, A. D. (1992). Duration of zinc uptake inhibition by chlorsulfuron in wheat. *Australian Journal of Agricultural Research* 43, 1169-1174.

Osborne, L. D. and Robson, A. D. (1993). What is the significance of herbicide nutrient interactions on wheat? *Plant and Soil* 156, 529-532.

Osborne, L. D., Robson, A. D. and Bowran, D. G. (1993). The impact of chlorsulfuron and diclofop-methyl on nutrient uptake by wheat. *Australian Journal of Agricultural Research* 44, 1757-1766.

Pederson, R. N., Black, I. D., Dyson, C. B. and Hannam, R. J. (1994). Effects of the herbicide metsulfuron-methyl on root length, nutrient levels, grain protein, and yield of barley. *Australian Journal of Experimental Agriculture* 34, 499-504.

Ray, T. B. (1982). The mode of action for chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* 17, 10-17.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Reid, R. J., Brookes, J. D., Tester, M. A. and Smith, F. A. (1996). The mechanism of zinc uptake in plants - characterisation of the low-affinity system. *Planta*, 198, 39-45.

Rengel, Z. and Graham, R. D. (1995). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. I. Growth *Plant and Soil* 176, 307-316.

Robson, A. D. and Snowball, K. (1989). The effect of 2-(4-2',4'-dichlorophenoxy-phenoxy)-methyl propanoate on the uptake and utilization on zinc by wheat. *Australian Journal of Agricultural Research* 40, 981-990.

Robson, A. D. and Snowball, K. (1990). The effect of chlorsulfuron on the uptake and utilization of copper and zinc in wheat. *Australian Journal of Agricultural Research* 41, 19-28.

Royuela, M., Arrese-Igor, C., Muñoz-Rueda, A. and Gonzalez-Murua, C. (1991). *In vitro* and *in vivo* effects of chlorsulfuron in sensitive and tolerant plants. *Journal of Plant Physiology* 139, 235-239.

Satsukevich, V. B. and Vol'-shchuk, O. V. (1976). Physiological and biochemical indices of barley given additional application of trace elements. *Vestsi Akademii Navuk BSSR, Biyalagichnykh Navuk* 1, 31-36.

Schmid, W. E., Haag, H. P. and Epstein, E. (1965). Absorption of zinc by excised barley roots. *Physiologia Plantarum* 18, 860-869.

Sharma, P. N., Tripathi, A. and Bisht, S. S. (1995). Zinc requirement for stomatal opening in cauliflower. *Plant Physiology* 107, 751-756.

Welch, R. M. and Norvell, W. A. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* 101, 627-631.

Yim, K.-O. and Bayer, D. E. (1996). Root growth inhibition of rice by bensulfuron. *Weed Research* 36, 49-54.

Zhang, Q. S., Hoefnagel, M. H. N. and Wiskich, J. T. (1996). Alternative oxidase from *Arum* and soybean - its stabilization during purification. *Physiologia Plantarum* 96, 551-558.

Zhang, Q., Mischis, L. and Wiskich, J. T. (1996). Respiratory responses of pea and wheat seedlings to chloramphenicol treatment. *Australian Journal of Plant Physiology* 23, 583-592.