Genetic Characterisation and QTL Mapping of Zinc Nutrition in Barley (*Hordeum vulgare*)

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Abstract

The genetics of micronutrient uptake and transport in plants is poorly understood. Only a few traits have been dissected genetically and these have largely been toxicity tolerance traits rather than desired traits for accumulation of essential nutrients. Advances in this area and consequently, any related plant breeding efforts, have been hampered by the practical difficulties of working with elements in trace amounts and the time consuming, expensive and technically difficult bioassays that are required. Molecular techniques offer the promise of identifying desirable genes which can be detected quickly and efficiently, thereby relieving the dependence on bioassays. The aim of this project was to map major genes or quantitative trait loci (QTLs) associated with zinc nutrition in the vegetative and reproductive tissues of barley (*Hordeum vulgare*).

A doubled haploid population derived from a cross between the Australian cultivar Clipper and an Algerian landrace Sahara 3771, together with a linkage map, was used to identify chromosomal regions associated with zinc accumulation. Sahara had previously been found to accumulate greater levels of zinc in vegetative tissues than Clipper. The Sahara allele at the locus X*Mne-1(B)* on the long arm of chromosome 4H was associated with increased zinc concentration and content in vegetative tissues at 4 weeks after sowing and with zinc concentration in vegetative tissues at anthesis.

The doubled haploid population was also screened for seed zinc concentration and content. The morphological marker for head row number (*hex-v*) located in the central region of chromosome 2H was associated with seed zinc content and seed weight. Two-row (Clipper) types had a greater seed weight and seed zinc content than 6-row (Sahara) types. Additional screening located a further three chromosomal regions associated with both seed zinc concentration (mg/kg) and seed zinc content (μg/seed). The presence of the Sahara allele at the locus X*bcd175* on the short arm of chromosome 2H conferred an average 20.2% increase in zinc concentration and a 26.3% increase in zinc content. The presence of Clipper alleles at the locus X*ksuD22* on the long arm of chromosome 2H and a locus of

unknown location (X*bcd265(B)*) increased zinc concentration by an average of 16.5% and 15.9% respectively. The average increase in zinc content per seed for the Clipper allele at these two loci was 24.6% and 22.2%. The presence of all three favourable alleles increased zinc concentration by 52.9% and zinc content by 75.1%. In addition, a region on the long arm of chromosome 5H (X*wg181*) was identified as being significantly associated with zinc transport into the seed, possibly from the apoplast.

This research represents a valuable contribution to the understanding of plant mineral nutritional genetics and the genetic relationships of zinc accumulation at different stages of the plant life cycle. Furthermore, this study has defined molecular markers for genetic loci that combined will enrich cereal seeds for zinc which is a major deficiency in human populations both in the developing and developed worlds. It also enables a map-based cloning approach to isolation of the genes that are responsible for the Zn loading trait as well as materials in which to confirm gene function by transformation, once they are cloned.

DECLARATION

This work contains no material which has been accepted for the award of any degree or diploma in any university or any 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 thesis being made available for photocopying and loan.

Paul Lonergan

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I would like to express my sincere thanks to the following people

Chapter 1

General Introduction

Zinc deficiency is the most widespread micronutrient deficiency in the world (Takkar and Walker, 1993), occurring on large areas of cropping land with resultant decline in yields and grain quality. Grain quality can be measured in terms of seed zinc content (μg/seed) or concentration (mg/kg). Low seed zinc content affects the health and vigour of plants grown from these seeds, particularly on zinc deficient soils (Rengel and Graham, 1995a,b), whereas low seed zinc concentration affects the health of people reliant on these grains for food (Gibson and Ferguson, 1998).

Some genotypes have the capacity to grow and yield well on zinc deficient soils (Graham *et al.*, 1992; Graham and Rengel, 1993; Rengel and Graham, 1995a,b; Cakmak *et al.*, 1996; Pearson and Rengel, 1997). Genotypes that are tolerant to zinc deficient soils are termed 'zinc efficient'. Efficient genotypes employ various physiological strategies such as increased phytosiderophore release and more efficient utilisation and compartmentalisation of zinc to ensure their survival (Rengel, 1999) but the genetic basis of these mechanisms is not well understood. This lack of understanding has hampered breeding efforts to increase zinc efficiency of various crops.

With respect to human nutrition, zinc is considered to be one of the 'big five' micronutrient deficiencies (together with iron, iodine, selenium and vitamin A) known to affect people worldwide (Welch and Graham, 1999). These deficiencies impair health and productivity, with impact primarily on women, infants and children from low-income families (Mason and Garcia, 1993), thus perpetuating a vicious cycle of poverty and poor

health. One of the proposed methods for alleviating zinc deficiency is through dietary modification/diversity (Gibson and Ferguson, 1998). One aspect of this approach involves growing cereal varieties of higher seed zinc concentration.

Although genetic diversity for zinc density in reproductive tissues has been observed for a number of crops (Graham *et al.*, 1999) there has been little effort to consciously breed for this trait. Again, progress has been hampered by a lack of understanding of the genetics involved in micronutrient transport and loading into the grain.

The advent of molecular techniques provides a new approach to selective breeding of micronutrient accumulation and uptake traits (Schachtman and Barker, 1999). In particular, the use of DNA markers as genetic tags for the desired trait (Helentjaris and Burr, 1989) could permit selection of improved cultivars following intraspecific hybridisation or alien introgression. The ever increasing number of genetic maps being produced by various groups provides a means by which to 'map' a trait to a particular chromosome, thereby allowing breeders to select for flanking DNA markers rather than using expensive, time consuming bioassays.

The aims of this project were to (i) map vegetative zinc accumulation genes or QTLs in barley using a doubled haploid population (ii) to map seed zinc accumulation using the same population and observe any correlation between vegetative and reproductive zinc accumulation, and (iii) to map zinc efficiency genes or QTLs and observe if they relate to zinc accumulation.

Chapter 2

Literature Review

Introduction

Zinc (Zn) is an essential trace element for plant growth but can also be phytotoxic at high shoot concentrations. The higher concentrations are generally associated with acidic soils which have been contaminated from a variety of sources. Agricultural soils, however, are generally not contaminated enough to cause phytotoxicity (Holmgren *et al.*, 1993) and it is Zn deficiency which more frequently limits production.

Zinc deficiency may be overcome by use of Zn fertilisers, either as a soil amendment or foliar spray or by growing Zn efficient cultivars. Zinc efficiency may be characterised as "a cultivar's ability to grow and yield well in soils too deficient in Zn for a standard cultivar" (Graham, 1984). Genetic diversity for Zn efficiency exists within plant species but for barley (*Hordeum vulgare* L.) the location and number of genes that code for this trait are unknown. Determination and location of these genes would assist genotype selection in breeding programmes where Zn efficiency is considered a desirable trait. There also exists genetic variation for seed Zn content and/or concentration. Location of the genes controlling these traits confers the potential to increase the nutritive value of seed, leading to better early plant vigour (particularly on Zn deficient soils) and improvement of human and animal nutrition.

2.1 Zinc Deficiency

2.1.1 History of zinc deficiency research

It was demonstrated by Javillier as early as 1912 that zinc sulphate applied to soil resulted in increased growth of wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), maize (*Zea mays* L.), lupins (*Lupinus angustifolius* L.) and peas (*Pisum sativum* L.) (Thorne 1957). Despite Javillier's work, it was not until the late 1920's that Zn started to become widely accepted as an essential element, after studies by Sommer and Lipman (1926) and Sommer (1928). They showed that several plant species, including barley, required Zn for normal growth. Following these studies, there were widespread reports of successful alleviation of Zn deficiency in the field by treatment with Zn fertilisers (Allison *et al.*, 1927; Barnette 1937; Camp and Reuther 1937).

Recognition of Zn deficient areas became more prevalent, especially where deciduous fruit crops were grown, but it was some time before the effect of the deficiency on a wide range of crops was fully understood. Viets *et al.* (1954) described barley, wheat and other grasses as insensitive to Zn deficiency but it is now accepted that lack of Zn causes poor growth and reduced yield in these crops.

2.1.2 Distribution of zinc deficiency in soil

Zinc deficiency has been reported on agricultural lands in 39 countries covering all populated continents (Takkar and Walker, 1993) and is considered the most widespread micronutrient problem. The affected areas represent a wide range of grain and horticultural crops and almost all soil types although Zn deficiency is usually associated with high-pH soils because of their low Zn availability or with coarse-textured, highly leached, acid soils because of their low total Zn content (Welch *et al.*, 1991). In India where the most extensive analysis of Zn deficiency has been performed, almost half of some 113,000 soil samples from across the country showed a deficiency of Zn (Takkar and Walker, 1993). This represented up to 70 % of agricultural areas in some states.

In Australia, Zn deficiency is widespread across the southern cereal belt (Hannam 1991) sometimes occurring over vast areas such as the Ninety Mile Desert on the border of Victoria and South Australia (Riceman 1948) where approximately 16 million ha of land is considered Zn deficient (Lindsay, 1972) or an area of 8 million ha in the south west of Western Australia (Donald and Prescott, 1975) which is considered the largest continuous Zn deficient area in the world. Such large-scale deficiencies have made Australia a world centre of research on micronutrient problems.

2.2 Chemistry of Zinc

Zinc is a transition metal of group II of the periodic table and as such has comparatively high electronegativity values and easily forms bonds with non-metals of significant covalent character (Kabata-Pendias and Pendias, 1992). It occurs most often as the covalent cation Zn^{2+} and shows a great affinity for combination with S anions and with several organic compounds.

2.2.1 Chemical forms of zinc

Zinc occurs in nature as many, widely varying minerals, including sulphides, sulphates, oxides, carbonates, phosphates and silicates. Zinc sulphides (sphalerite and wurtzite) are relatively common and it is their alteration by hydrolysis and sulphide oxidation that leads to production of the other minerals (Barak and Helmke, 1993). The major commercial Zn ores are sphalerite (ZnS), smithsonite ($ZnCO_3$) and hemimorphite ($Zn_4Si_2O_7(OH)_2.H_2O$).

Zn^{2+} equally accepts either tetrahedral coordination (coordination number = 4) or octahedral coordination (coordination number = 6). This reflects the full and spherical $3d$ subshell of its electronic structure which does not favour any one coordination over the other. Indeed, some minerals contain Zn in two different coordination numbers, such as hopeite [$Zn_3(PO_4)_2.4H_2O$] and hydrozincite [$Zn_5(OH)_6(CO_3)_2$] where both a tetrahedrally and an octahedrally coordinated Zn atom are present.

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Zinc can also substitute into a vast number of minerals as a minor constituent without major distortion of physical properties. It is this form that accounts for the bulk of its presence in rocks and soil making it the 24th most abundant element in the earth's crust.

In solution, divalent Zn ions tend to be surrounded by six water molecules arranged in an octahedron (Barrow, 1993). This aqueous Zn complex is considered a multiprotic acid because the water molecules may lose protons to give the hydrolysis series

$$Zn(H_2O)_6^{2+} \longrightarrow ZnOH(H_2O)_5^+ \longrightarrow Zn(OH)_2(H_2O)_4 \longrightarrow Zn(OH)_3(H_2O)_3^- \longrightarrow$$
$$Zn(OH)_4(H_2O)_2^{2-}$$
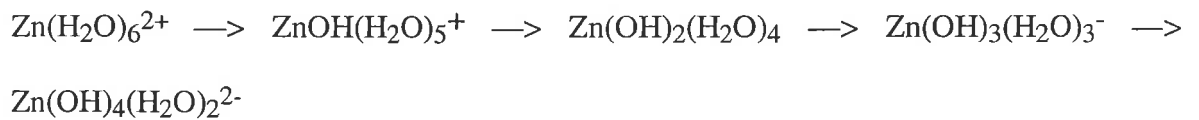
Zinc chloride complexes are also common ranging from $ZnCl(H_2O)_5^+$ to $ZnCl_4(H_2O)_2^{2-}$ (Barak and Helmke, 1993). Other complexes of Zn^{2+} occur with various organic and inorganic ligands including carbonate, sulphate, phosphate, organic acids, amino acids and synthetic chelates.

Within plants, Zn^{2+} forms stable, low molecular weight complexes which account for the majority of soluble Zn in the cell (Brown *et al.*, 1993). This water soluble Zn fraction is considered physiologically active and a better indicator of plant Zn status than total Zn content (Cakmak and Marschner, 1987).

2.2.2 Zinc chemistry in soils

Solubilization of Zn minerals during weathering releases mobile Zn^{2+} which becomes adsorbed by mineral and organic components in the solid phase of soils. Zinc concentration in a soil is related to the concentration in the soil parent material and variations are due primarily to different concentrations in these parent rocks. Kabata-Pendias and Pendias (1992) give values for Zn concentrations in sedimentary systems as being lower for sandstones or carboniferous rocks (10-30 mg kg^{-1}) than for shales (80-120 mg kg^{-1}) where it is held as adsorbed Zn and in the lattice structure of clay minerals (Krauskopf, 1972). The range in igneous formations varies from acid rocks (40-60 mg kg-1) to mafic rocks (80-120

mg kg^{-1}), a reflection of Zn's ability to substitute in silicates for Mg^{2+} and because Zn can occur as small grains of sphalerite in the mafic rock (Knezek and Ellis, 1980).

Sorption properties of the mineral part of soil material are associated principally with the clay and silt-size fractions which are a mixture of several aluminosilicate clay minerals. These are all 1:1 or 1:2 layer-type aluminosilicates in which Zn is negligible as a structural component but is highly adsorbed on to the surface of the mineral of high cation exchange capacity (CEC). In general, the higher the CEC the greater the amount of cation adsorbed.

2.2.3 Zinc chemistry in plants

In plants, Zn is neither oxidised nor reduced (Marschner 1986) and its chemistry differs from Mg^{2+}, Ca^{2+} or Mn^{2+} in that it forms more stable complexes with a particular affinity for tetrahedral complexes (Brown *et al.*, 1993). Zinc occurs in plants mainly as a complex with various low molecular weight compounds. It is present to a lesser degree as a free ion or component of proteins. Since various cell wall components such as lignin, cellulose and hemicellulose possess a high binding affinity for Zn, most of the free ion form is inactive, being bound in the apoplasm of roots. Reid *et al.* (1996) refer to the problems this binding has on Zn uptake measurements because most studies make no clear distinction between extracellular binding and actual membrane influx.

Once the divalent cation is absorbed by the plant it can form complexes with various ligand species. Zinc may also be absorbed in several other forms such as complex ions and Zn-organic chelates (Tiffin, 1972; Loneragan, 1975; Weinberg, 1977). Whether these complexes are the form transported through the xylem and phloem is unclear. There have been studies of Zn in the xylem exudate of several plants, which have reported Zn concentrations ranging from 4 to 22 mmol m^{-3} (Clark *et al.*, 1986; Hocking *et al.*, 1978; White *et al.*, 1981a) but none has made clear whether Zn exists as the free Zn^{2+} ion or if it is complexed with organic acids. Theoretical work of White *et al.* (1981b) using the CHELATE computer program suggests that in soybean (*Glycine max* L.) and tomato (*Lycopersicon esculentum* L.), Zn is complexed with citric and malic acids.

There is strong evidence for complexation of Zn in the phloem. Van Goor and Wiersma (1976) found that all of the Zn in the phloem exudate of castorbean (*Ricinus communis*) was present as an anionic organic complex of M.W. between 1000 and 1500. More recently, Taylor *et al.* (1988) found a highly anionic complex of Zn (m.w. ~4000) in the phloem sap of orange trees (*Citrus sinensis* L.). Complexation in the phloem is not surprising considering the high pH of the phloem sap (usually greater than 8.0) and its high phosphate concentration. These conditions ensure very low free ion activities of all the micronutrient cationic metals (Fe, Mn, Zn, Cu and Ni) since they would precipitate as mixed oxides, hydroxides and phosphates. Complexation, however, allows for free movement in the phloem stream.

2.3 Diagnosis of Zinc Deficiency

Methods of assessing plant nutrient status include plant analysis, soil testing, foliar symptoms and physiological tests. The pooling of information from all such tests, together with an understanding of the biological and physiological processes involved, provides a more reliable assessment or diagnosis than any one method alone (Smith and Loneragan, 1997).

2.3.1 Plant mineral nutrient analysis

Plant analysis as a diagnostic tool dates back to early last century but it has been only in recent years that modern technology has enabled plant micronutrient analysis to develop at a rapid rate. The development of instruments such as inductively coupled plasma (ICP) emission spectrometers that are capable of quantitative multi-element analysis has vastly improved both the accuracy and ease of plant nutrient analysis.

The concept of critical nutrient concentration (CNC) forms the basis of most methods to assess plant nutrient status (Smith and Loneragan, 1997). Bouma (1983) described the CNC as that concentration in a particular plant part or tissue which is just sufficient or just deficient for maximum growth or yield. Such a precise definition is often impractical or impossible so it is often defined as that concentration where yield is reduced

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5% or 10% below the maximum (Ulrich and Hills, 1967). As critical concentrations vary with the age of a particular plant part, it is imperative that parts of the same physiological age be used (Smith and Loneragan, 1997). The CNC for Zn is especially sensitive to leaf age as seen in subterranean clover (*Trifolium subterranean* L.) where it declined from 25 mg/kg in the apical leaf to 18 mg/kg and then 10 mg/kg in successively older leaves (Reuter *et al.*, 1982). In practice the CNC is a narrow range of concentrations rather than one specific value. The CNC of Zn in leaves ranges from 10-15 mg Zn kg^{-1} dry weight for graminaceous species and from 20-30 mg Zn kg^{-1} dry weight for most dicotyledonous species (Marschner, 1993).

Problems associated with plant analysis relate primarily to sample collection. Much larger errors are likely to occur during sample collection and handling than during the actual analysis. Sampling should be done at a similar time and developmental stage for comparisons and be taken from appropriate plant parts (Bates, 1971; Reuter *et al.*, 1997). A technique commonly used to standardise physiological age is to sample petioles and/or blades of leaves at well defined stages of development, such as the most recently mature leaf blade, the youngest emerged leaf blade or the youngest open leaf blade (Smith and Loneragan, 1997).

2.3.2 Soil chemical analysis

Soil analysis is widely used to predict potential micronutrient deficiencies (or toxicities) prior to the growing season. In the case of Zn the top 0-10 or 0-15 cm of soil is sampled but this may be misleading for deep rooted perennial species since subsoils are generally deficient in extractable Zn (McLaren *et al.*, 1984). Calibration of soil tests is done by selecting a series of soils ranging from deficient in Zn to adequate and measuring growth responses to several rates of Zn (Brennan *et al.*, 1993). This response is then related to the soil-extractable Zn value, following which soils are broadly separated into Zn-deficient and Zn-adequate.

Many chemical solutions have been tried in the search for a universal extractant to predict Zn availability in soils (Lindsay and Cox, 1985; Sims and Johnson, 1991). Average

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levels

critical concentrations for major extractants are 0.5-1.0 mg Zn/kg for DTPA, 1.1 mg Zn/kg for Mehlich-1 and 1.0-5.0 mg Zn/kg for 0.1M HCl (Lindsay and Cox, 1985; Cox, 1987). DTPA (diethylene triamine penta-acetic acid) is the most frequently used extractant but, like others, it only characterises the pool size (capacity, quantity, potential availability) of a 'labile' fraction in soils comprising water soluble, exchangeable, adsorbed, chelated or occluded Zn (Marschner, 1986). Plants extract zinc from the soil solution, however, so that chemical extractions can only predict whether soils are capable of providing enough Zn for plant demand. The chemical extractants make no measure of the rate at which Zn becomes bioavailable.

2.3.3 Foliar symptoms of zinc deficiency

Zinc deficiency is known by various names depending on the plant under study. 'Little leaf', 'mottle leaf', 'rosette' and 'dieback' all refer to symptoms observed in deciduous fruit trees. The rosette symptom is caused by failure of terminal buds to elongate resulting in a tuft of small leaves at the end of twigs (Thorne, 1957). Severe deficiencies may result in dieback of branches.

Zinc deficient plants are characterised by a general retardation of normal growth and a lack of chlorophyll. Malformed leaves give rise to the 'rosette' symptom in young growth of dicots (Snowball and Robson, 1986) and 'fan shaped stem' in monocots (Grundon, 1987). The symptoms are normally seen first in the young leaves leading to the conclusion that Zn is immobile in the plant under conditions of deficiency.

In barley, symptoms develop on middle leaves but spread rapidly until the whole plant is affected (Grundon, 1987). Pale yellow, linear chlorotic areas appear in the mid section of the leaf, usually between the margin and mid-vein and progress outwards towards the tip and the base. Chlorosis is followed by grey or dark brown necrosis. In addition to these foliar symptoms, internode elongation is reduced resulting in very short stems that are often fan shaped with leaves crowded together at the top.

Diagnosis based on visible symptoms requires a systematic approach as summarised by Marschner (1986). Chlorosis or necrosis and the pattern of each are important criteria as is the age of the leaves affected. Symptoms appear preferentially on older leaves for mobile elements and on younger leaves for immobile elements.

Diagnosis of nutritional disorders based on foliar symptoms is largely a qualitative measure, providing information on the degree of the problem in question. Symptoms are clearly visible when a deficiency is acute and the growth rate and yield are severely depressed but as has been shown in some studies (Carroll and Loneragan, 1968; Reuter *et al.*, 1982) plants can experience Zn stress yet show no symptoms. At these mild levels of stress, deficiency is not clearly expressed as changes in leaf physiology but rather as changes in the growth rate or internal chemistry of the plant. Problems can also arise in diagnosing field grown plants due to combinations of deficiencies or deficiency of one nutrient occurring simultaneously with the toxicity of another. In addition, the appearance of symptoms in the field is typically patchy (Kubota and Allaway, 1972; Grundon, 1987).

2.3.4 Biochemical tests for zinc deficiency in plants

Enzyme activities are known to be responsive to micronutrient deficiency. With this in mind, carbonic anhydrase (CA) activity has been proposed as a diagnostic test for Zn deficiency (Bar-Akiva and Lavon, 1969; Dwevidi and Randhawa, 1974; Rengel, 1995a). These studies used CA activity as a measure of Zn status of several crops at progressive stages of Zn deficiency. Despite their simplicity, reliability and rapidity, biological tests are not widely used for diagnosis. This could be due to complications in enzyme patterns as a result of environmental growth factors or problems sampling and preserving tissue from field grown plants. In developing countries particularly, there is also likely to be a lack of suitable equipment for carrying out these tests.

2.4 Uptake of Zinc in Plants

2.4.1 Mobilisation of zinc to roots

Uptake is a dynamic process in which Zn must be continuously replenished in soil solution from the soil solid phase and transported to roots as uptake proceeds (Fageria *et al.*, 1991). Zinc transport to roots, absorption by roots and translocation within the plant occur simultaneously so that a rate change in one process will ultimately affect all others and may become the limiting factor in the uptake process. After solubilisation from the solid phase of soil, Zn moves to plant roots by means of mass flow and diffusion (Barber, 1962). Root interception, ie. root growth and root surface area are also important parameters determining the bioavailability of Zn (Marschner, 1993). Mycorrhizas associated with roots can also enhance uptake of Zn as well as other elements (Bowen *et al.*, 1974; Gerdemann, 1975).

2.4.1.1 Mass flow

Mass flow is the passive transport of nutrients to the root in soil solution, driven by transpiration. It is dependent on soil factors such as the Zn solution concentration which governs the amount of Zn available for mass flow and moisture content which determines the rate at which Zn moves along the water potential gradient.

Plants growing in soil extract most of their nutrients from the soil solution where Zn is present as free metal ion (15-30%) and as labile complex (Jeffery and Uren, 1983). This low concentration of Zn^{2+}, particularly in deficient soils, dictates that supply by mass flow is also very low. In this case diffusion becomes the major factor in supplying Zn to the plant.

2.4.1.2 Diffusion

Diffusion can be defined as the movement of nutrients from a region of high concentration to a region of low concentration (Fageria *et al.*, 1991). Studies by Wilkinson *et al.* (1968), using autoradiographic methods, show depletion of ^{65}Zn around roots due to diffusion. In the same study, increased transpiration ratios caused no difference in mean Zn

concentrations of tops and roots indicating mass flow was not responsible for much Zn movement to plants. In contrast to mass flow, diffusion operates only in the immediate vicinity of the root surface and is affected not only by soil conditions but also by plant factors such as root interception (Marschner, 1993).

2.4.1.3 Root interception

Since mass flow is of minor importance in Zn uptake the role of root growth in 'exploring' the soil volume becomes more important. The greater the volume of roots and hence surface area, the greater is the chance for diffusive movement of Zn into the roots. Although the percentage of soil volume occupied by root systems is small (0.55-1.1%), root growth is considered to provide a significant proportion of required Zn (Fageria *et al.*, 1991).

2.4.1.4 Arbuscular mycorrhiza (AM)

Arbuscular mycorrhizae (AM) are most commonly recognised for their role in phosphorus (P) nutrition of plants where plant P uptake is markedly improved by mycorrhizal colonization (Thompson, 1990, 1996). However, they can also enhance plant growth by improving Zn supply through absorption and translocation by external hyphae (Marschner, 1993). Studies by Thompson (1990) showed a threefold increase in plant Zn concentration of mycorrhizal wheat plants as compared to non-mycorrhizal wheat. Grain yield was 37% higher in the mycorrhizal wheat. Similar results were also observed for linseed (*Linum usitarissimum* L.) (Thompson, 1996). The effect is more pronounced on soils of low extractable Zn status or low Zn mobility (eg. high soil pH). Zinc uptake and dry shoot contents are increased in both pot studies (Swamvinathan and Verma, 1979; Faber *et al.*, 1990) and field studies (Jakobsen, 1983). Research by Kothari *et al.* (1991) showed that AM may account for 25% of total Zn uptake in maize.

2.4.2 Pathway from soil solution into roots

Uptake of free Zn^{2+} ions from soil solution by plant root cells is driven by electrical chemical potential gradients that exist across the root-cell plasma membrane (Graham and Welch, 1996). This gradient has two components: (1) the activity gradient of each

micronutrient ion and (2) the plasma membrane electrical potential gradient (Kochian, 1991). Plasma membrane potentials range from -120 to -180mV resulting in steep electrical gradients developed across the membrane (Welch, 1995). This, coupled with low intracellular activities of micronutrients, results in a very large driving force for micronutrient uptake. The main barriers of this movement into the root are the plasma membrane of the individual cortical cells and the endodermis, the innermost layer of cells of the cortex (Marschner, 1986).

2.4.3 Mechanisms of uptake

Most of what is known about micronutrient uptake is the result of research aimed at understanding iron (Fe) absorption by higher plants (Graham and Welch, 1996). Extrapolations have been drawn concerning Zn (Kochian, 1993), but active research directed specifically at Zn uptake is required before selection for more efficient mechanisms is possible.

Based on their response to Fe deficiency, higher plants have been categorised into two strategies for Fe uptake (Marschner, 1986). Strategy I relates to dicots and non-gramineous monocots and is characterised by root cell influx of free divalent cations, efflux of organic acids and H^+ ions, and inducible root cell plasma membrane reductases. Strategy II concerns gramineous plants only and is characterised by root cell influx of free divalent cations, efflux of free phytometallophores and influx of transition metal-phytometallophore complexes. These mechanisms are discussed below.

2.4.3.1 Influx of divalent zinc cations

Much of the research on ion absorption has focussed on plasma membrane and vacuolar membrane (tonoplast) transport systems. The lipid composition of both membranes is complex, consisting of phospholipids, glycolipids, sterols and sterol glycosides (Poole, 1988) arranged in a lipid bilayer which is relatively impermeable to ions. The carrier hypothesis of transport, developed by several early researchers (Jacobsen and Overstreet, 1947; Osterhout, 1950, 1952) proposes specific transport proteins inserted into the lipid bilayer which facilitate micronutrient and macronutrient uptake into the cell. The

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older hypotheses have now been rejected in favour of a divalent cation channel theory which allows for less specific transport of nutrients.

Ion channels have been identified in plasma membranes of higher plant cells for Ca^{2+}, K^+, Na^+ and Cl^- respectively (Tester, 1990; Spalding et $al.$, 1992). Tester (1990) found that Ca^{2+} channels could also transport other ions such as Mg^{2+}, Ba^{2+} and Rb^+ so it is possible that these Ca^{2+} channels may be plasma membrane passageways for other divalent micronutrient cations such as Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ni^{2+} (Welch, 1995).

It is now widely accepted that different classes of transport proteins occur in the cellular membranes of all living organisms (Kochian, 1991). These include ATPases that function as primary active transport systems (i.e. transport against an electrochemical gradient) and secondary active transport systems (i.e. systems that use energy stored in transmembrane H^+ gradients to drive active transport of another ion) and ion channels that mediate passive ion transport.

2.4.3.2 Rhizosphere acidification

Rhizosphere refers to the root-soil interface microenvironment which may differ in pH by up to 2 units from that of the bulk soil. Plant roots influence rhizosphere pH primarily through net excretion of HCO_3^- and H^+ ions (Graham and Welch 1996). Differences in net excretion are related to imbalances in the cation/anion uptake ratio. This ratio is most affected by the form of nitrogen supply (Marschner and Römheld 1983). Ammonia applied to neutral or alkaline soils reduces rhizosphere pH thereby enhancing mobilisation and uptake of Zn from the soil solution. Applied nitrate, on the other hand, increases rhizosphere pH resulting in decreased Zn concentration and mobility. A study by Thomson et $al.$ (1993) using a sandy loam (pH in water 6.8) found that ammonium-fed bean plants (*Phaseolus vulgaris* L.) absorbed twice as much Zn per unit root length as nitrate-fed plants. In this study, the pH of nitrate fertilised soil remained at 6.5-7.0 but ammonium-treated soil decreased pH from 6.8 to 5.5.

2.4.3.3 Reductases

Reductases are plasma-membrane-bound enzymes (Bienfait et al., 1982) that are known to reduce Fe(III) and Fe(II). Although there is some confusion as to the number and types of Fe(III) reductases in higher plants (Welch and Kochian, 1992), it is generally thought that they can be divided into two groups-: 1- standard reductases and 2- constitutive and inducible reductases. The first type reduces Fe(III) from electron acceptor molecules such as ferricyanide while the other two are capable of reducing Fe(III) in Fe(III)-chelates *in situ*. Welch (1995) proposed that reductases could also be capable of reducing Cu(II)- and Mn(III, IV)-chelates but confirmation of this awaits further research.

2.4.3.4 Phytometallophores

Zinc deficient gramineous species release phytosiderophores identical to those released under Fe deficient conditions (Zhang et al., 1989, 1991). The term phytometallophore or phytosiderophore ('plant iron bearer') identifies a class of low-molecular-weight metal chelate-forming molecules (non-proteinogenic amino acids containing α-hydroxyl carboxylate and α-amino carboxylate ligands) synthesised by higher plant cells (Römheld, 1987; Takagi et al., 1988). They form stable chelates with Zn and are effective in mobilising Zn from calcareous soils (Treeby et al., 1989).

Although phytometallophore release is speculated to be a general adaptive response to micronutrient metal deficiency (Marschner, 1993; Welch, 1995) a study by Gries et al. (1995) showed that the response can be genotype dependent. They measured phytometallophore release under Fe, Zn, Mn and Cu deficiencies in barley (cv. CM72) and found that release was a specific response to Fe deficiency only.

2.5 Movement of Zinc in Plants

2.5.1 Translocation and remobilisation

2.5.1.1 General

Long distance transport of solutes from roots to shoots occurs mainly in the nonliving xylem vessels. It is driven by the water potential gradient between roots and shoots and is unidirectional (Marschner 1986). Transport in the phloem, however, is bidirectional, being determined by the nutritional requirements of various plant organs and therefore occurs from source to sink. Transfer of mineral elements from the xylem to the phloem, referred to as loading, is mediated by specific cells called 'transfer cells' (Pate and Gunning, 1972). Although the forms of Zn in xylem and phloem are still uncertain, evidence from sap contents (Van Goor and Wiersma, 1976; White *et al.*, 1981a,b) theoretical predictions (White *et al.*, 1981b; Mullins *et al.*, 1986) and transport studies (McGrath and Robson, 1984) all support complexation in the transport streams.

2.5.1.2 Xylem transport

Nutrients unloaded into the xylem move to the top of the plant in the transpiration stream. The driving force for transpiration arises from the gradient in water potential that exists between the atmosphere and the water-filled pores in the mesophyll cell walls lining the stomatal cavities in the leaf (Kochian 1991). Water is 'pulled' from the root to the top and complexed ions as well as organic and inorganic solutes are transported along with the water.

Little is known about the mechanisms of micronutrient loading or unloading into and out of xylem vessels or the soluble forms of micronutrients in xylem sap (Welch 1995). The chemical composition of the xylem sap has been studied in the hope that this will add to the knowledge of how micronutrients move in the xylem but little progress has been made due to technical limitations in accessing xylem sap without significantly altering the processes and chemistry involved in long distance transport in the intact plant (Kochian, 1991). As it is difficult to detect such complexes *in situ*, simulation models and theoretical calculations have been the main sources of knowledge (Hanson, 1978; White *et al.*, 1981c). These

studies predict that the xylem sap contains several organic compounds (mainly organic and amino acids) in varying concentrations that could serve as ligands for transporting micronutrient metals in the transpiration stream.

2.5.1.3 Phloem transport

Zinc and the other micronutrient metals are thought to be transported predominantly in chelated form by organic molecules but knowledge of these ligands is very limited. Phloem sap contains a variety of chelate-forming components including organic acids, amino acids and peptides but to date none has been identified as a transport ligand for Zn or any of the other micronutrient metals.

Stephan and Scholz (1993) have proposed that the non-protein amino acid nicotianamine (NA) is the loading and possibly also the transport vehicle of transition metals in the sieve elements. The lack of NA in the tomato mutant *chloronerva* causes distinct disturbances in its Fe metabolism but external supply of NA remobilises Fe to growing leaflets and roots (Scholz *et al.*, 1992). Schmidke and Stephan (1995) found that NA and micronutrient metals were transported in a constant stoichiometrical ratio in the sieve tube sap and concluded that such cotransport in a molecular ratio suggests that NA complexes are the major loading and/or transport species of metal micronutrients.

2.5.2 Nutrient loading into seeds

Although much work has been reported on the pathway by which carbohydrates and macronutrients enter the developing fruit (Pate 1975, Jenner 1985 a,b,c, Wolswinkel 1987, 1991), little is known about the pathway of micronutrients. In the past, micronutrient transport has been assumed to be similar to that of the macronutrients i.e. via the phloem (Loneragan 1988, Longnecker and Robson 1993) even though the micronutrient metals are considered to be only partially mobile in the phloem (Kochian 1991). Studies by Pearson and Rengel (1995) however, suggested that while Zn may enter the grain of wheat via the phloem, manganese (Mn) may enter via the xylem. This opposes the accepted view of a discontinuity between mature xylem elements and the reproductive organs of plants (Zee and O'Brien, 1970; O'Brien *et al.*, 1985). Further studies, though, (Pearson *et al.*, 1995)

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suggested that Mn was transported across a membrane between the rachilla and the grain of wheat and possibly loaded into the phloem before transport to the grain.

There are conflicting reports concerning the site of this transfer. Herren and Feller (1994) proposed that in wheat, the transfer of Zn takes place mainly in the peduncle whereas Pearson and Rengel (1995) found that most exchange occurred in the rachis of the same species. Zinc may also be transported and remobilised from various plant organs via the phloem (Marschner, 1986). Retranslocation from leaves to seeds has been shown for subterranean clover (Riceman and Jones, 1958) pine trees (*Pinus radiata*) (McGrath and Robson, 1984) and wheat (Pearson and Rengel, 1994). Zinc is also known to accumulate in the glumes and palea/lemma of wheat in the early stages of development but is remobilised as the grain approaches maturity (Pearson and Rengel, 1994).

The capacity with which Zn is loaded into the phloem appears to be relatively low as shown by Pearson *et al.* (1995). A 10-fold increase in the Zn concentration in the culture solution resulted in only a 2.3-fold increase of loading into the grain. Other reproductive tissues such as the peduncle, rachis, glumes and palea, however, appeared to be concentration dependent with a 10-fold increase in Zn concentration in these tissues.

2.6 Function of Zinc in Plants

Zinc is known to play an important role in many enzymes, either as a structural component (Barak and Helmke, 1993) or as an enzyme activator (Kabata-Pendias and Pendias, 1992). Zinc enzymes exist in all six enzyme classes - oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases and are involved in essential metabolic processes such as carbohydrate and auxin metabolism, protein synthesis and reproductive processes.

2.6.1 Carbohydrate metabolism

Studies by Shkolnik (1984) indicate that Zn deficiency strongly affects the principal respiration pathway (glycolysis - the Krebs cycle - the respiratory electron transport chain).

This effect is thought to be caused by inactivation of crucial enzymes involved in starch and sucrose formation. The activity of aldolase, which catalyses the production of fructose-1,6-bisphosphate, was dramatically reduced in Zn deficient oats (Quinland-Watson, 1951). The activity of other enzymes including ribulose 1,5-bisphosphate carboxylase (RuBPC) have also been shown to decrease under Zn deficiency (Jyung *et al.*, 1972; Stiborova *et al.*, 1987).

Net photosynthesis can be reduced by nearly 70% in some species due to Zn deficiency (Brown *et al.*, 1993). The mechanisms that contribute to this are uncertain. Some workers have observed a sharp decline in carbonic anhydrase (CA) activity with increasing severity of Zn deficiency (Ohki 1976, Randall and Bouma, 1973). CA is localised in the chloroplast in C3 plants and in the cytosol of mesophyll in C4 plants. It was the first enzyme found to contain Zn and it catalyses the reversible conversion of carbonic acid to carbon dioxide and water.

$$H_2CO_3 <=> H_2O + CO_2$$

Randall and Bouma (1973) and Boardman (1975) argue that the effect of Zn deficiency is on the photosynthetic process itself rather than via CA whereas Seethambaran and Das (1985) showed increased photosynthesis in Zn deficient rice (*Oryza sativa* L.) with addition of CA. The fact that some species contain Zn-free CA (Fellner, 1963) confuses the issue further.

Zinc deficiency can also reduce photosynthesis by affecting chlorophyll content and chloroplast structure. Reed (1938) noticed arrested development and disintegration of chloroplasts in the leaves of Zn deficient plants together with modification of the palisade tissue and spongy parenchyma. Shrotri *et al.* (1978) observed that mesophyll chloroplasts in maize became swollen as were the bundle sheath chloroplasts. Similar effects have been reported for beans (Thomson and Weisner, 1962; Jyung *et al.*, 1975).

2.6.2 Auxin metabolism

Reduced levels of indole acetic acid (IAA) have been observed in Zn deficient plants (Skoog, 1940; Tsui, 1948; Cakmak *et al.*, 1989). The many theories proposed for this effect can be divided into two categories:

1- reduced levels of IAA are a result of increased oxidation of IAA to ICA (indole carboxylate acid) <u>or</u>

2- reduced levels of IAA are due to depressed production of IAA.

Domingo *et al.* (1990) showed increased levels of ICA in Zn stressed radish (*Raphanus sativus* L.). This concurred with Skoog's (1940) conclusion that Zn was necessary for maintaining auxin in an active state and that decreased levels of IAA were due to enhanced oxidation.

The second category of theories provide for an effect of Zn on production of IAA precursors or activity of enzymes. Tsui (1948) found reduced rates of synthesis of tryptophan, the precursor of IAA in Zn deficient tomato plants. Other researchers (Takaki and Kushizaki, 1970; Cakmak *et al.* 1989) however, have recorded increased levels of free tryptophan with increasing Zn deficiency. They conclude that conversion of tryptophan to IAA is specifically inhibited rather than the production of tryptophan.

None of the above experiments distinguished between the D- or L- forms of tryptophan. Work by Law (1987) showed D-tryptophan to be the precursor of IAA, and gibberellin (GA) increased the rate of transformation of L- into D-. Suge *et al.* (1986) showed a GA reduction in Zn deficient barley, maize and oats and suggested that perhaps GA is the primary candidate affected by Zn deficiency rather than IAA.

2.6.3 Protein synthesis

Zinc deficiency reduces the concentration of proteins in plants while increasing those of amino acids and amides (Römheld and Marschner, 1991). This is a result of a substantial decline in ribonucleic acid (RNA) caused either by lower activity of Zn-containing RNA polymerase (Soloiman and Wu, 1985), and/or by enhanced RNA degradation (Cakmak *et*

al., 1989) and/or by reduced structural integrity of ribosomes (Obata and Umebayashi, 1988).

Ribosomal RNA is attacked by ribonuclease (RNAase) which typically shows increased activity in Zn deficient plants (Dwivedi and Takkar, 1974). However, Seethambaram and Das (1984) noted reduction in RNA before increase of RNase activity suggesting that the effect of Zn deficiency is greater on RNA production than enhancement of RNase activity.

Zinc is also essential for the stability and function of genetic material. As well as stabilising the structure of RNA and DNA, it is believed to be essential for at least two chromatic proteins, chromatin TFIIA which is essential for transcription (Hanas *et al.*, 1983) and $g^{32}P$ protein which is involved in replication (Giedroc *et al.*, 1986). Zinc modifies the binding properties of proteins by substituting for magnesium (Mg) in the ternary enzyme-CO_2-metal complex or by reaction with enzyme SH groups (Stiborova *et al.*, 1987) forming domains referred to as 'zinc fingers'. Genes encoding these characteristic Zn-binding domains have been found in higher plants (Vodkin and Vodkin, 1989) and are thought to function as transcription factors as they do in other organisms (Brown *et al.*, 1993).

2.6.4 Membrane integrity

Loss of membrane integrity is the earliest biochemical change caused by Zn deficiency in animals (Bettger and O'Dell 1981). Several researchers (Welch *et al.*, 1982, Cakmak and Marschner 1988a) have observed the same effect in higher plants by measuring root exudates as an indicator of root plasma membrane permeability. These studies recorded greater leakage of root exudates from Zn deficient plants than from those supplied with sufficient Zn. Beyond these studies, it is generally accepted that Zn plays a role in maintaining the integrity of all biomembranes (Brown *et al.*, 1993) including chloroplast membranes and vacuolar membranes. This would explain chloroplast breakdown associated with Zn deficiency. It may also explain greater RNAase activity since RNAase is sequestered in the vacuoles of cells.

It is thought that Zn ions stabilise and protect cell membranes and enzyme proteins by binding sulphydryl groups and phospholipids (Chvapil, 1973). This prevents oxidative attack from toxic O_2 species such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), which are produced in high amounts in the chloroplasts of higher plants (Elstner, 1982). Cakmak and Marschner (1988b,c) recorded higher levels of O_2^- in Zn deficient plants and suggested that reduced activity of the Zn-containing enzyme superoxide dismutase (SOD) was responsible. The most important SOD in higher plants is a Cu-Zn metalloprotein that detoxifies O_2^-. Zinc plays a key structural role affecting the activity of this enzyme (Brown et $al.$, 1993).

2.7 Environmental Factors Affecting Zinc Nutrition

2.7.1 Temperature effects

Most research on Zn^{2+} uptake shows that it is temperature dependent. This has been demonstrated either directly in controlled temperatures (Martin et $al.$, 1965, Schmid et $al.$, 1965) or indirectly by using metabolic inhibitors (Giordano et $al.$, 1974, Reid et $al.$, 1996). The general findings of such research is that lower soil temperatures result in more severe Zn deficiency symptoms and decrease both Zn uptake and Zn concentration in plants. The work of Martin et $al.$ (1965) also suggests that, at low temperatures, plants are more likely to respond to Zn fertiliser and added P is more likely to induce Zn deficiency (see also section 2.6.4.1).

In the field, Zn deficiencies occur early in the growing season when soil temperatures are still relatively low. Symptoms of deficiency disappear as the season progresses and soils warm (Ferres, 1949; Millikan, 1953; Lindsay, 1972). The early season effect is most likely due to impaired root activity and growth (Lindsay, 1972; Marschner, 1993) along with a low root colonization with VA mycorrhiza. Such reduced colonisation can also result from a long fallow period (10 months or more) which occur as part of a planned rotation or are enforced by drought (Thompson, 1996). Inhibited translocation of Zn from roots to shoots may also be a factor (Schwartz et $al.$, 1987).

2.7.2 Soil moisture content

Soil moisture affects nutrient supply via diffusion to the root surface (Marschner 1986). The coefficient of diffusion decreases with decreasing soil water potential causing a reduction in supply and uptake by the plant roots. As soil moisture falls the water potential at the root surface becomes much lower than that of the bulk soil (Zur *et al.*, 1982) and hydraulic contact between root surface and soil may be lost. Furthermore, increased mechanical impedance in drier soils inhibits root elongation thus reducing the root surface area available for uptake of nutrients (Marschner, 1986).

2.7.3 Effect of pH

Anderson and Christensen (1988) showed that pH is more important than any other single property in controlling Zn mobility. The availability of Zn decreases as soil pH increases due to increased adsorption by soil constituents (Bar-Yosef *et al.*, 1980) and hydrolysis of the Zn divalent cation (Harter 1983). Zinc equilibrium concentrations may decrease 30-45 times per unit increase in soil pH in the range 5.5-7.0 (Barber, 1984; Moraghan and Mascagni, 1991).

As pH increases above 5.5, Zn is adsorbed by hydrous oxides of aluminium (Al) and Fe (Kalbasi *et al.*, 1978; Kinniburgh and Jackson, 1982). As pH increases above 6.0, the unavailable hydrated form of the Zn cation ($ZnOH^+$) becomes more predominant (Harter 1983). This is further hydrolysed to $Zn(OH)_2$ above pH 8.0. In neutral and alkaline soils where pH is above 7.0, Zn deficiency is more pronounced due to lower availability of Zn (Rashid, 1993).

2.7.4 Nutrient-zinc interactions

The interaction between Zn and other nutrients occurs most frequently when both Zn and the nutrient in question are limiting. This most often occurs with P.

2.7.4.1 Phosphorus-zinc interactions

The literature is vast and contrary with much confusion arising from inadequate experimental design and/or critical evaluation (Loneragan and Webb, 1993). Even after

rejection of dubious data the subject remains complex and studies are divided into 2 distinct categories:-

1 increasing application of P decreases Zn concentration in plant shoots

2 increasing application of P does not decrease Zn concentrations in plant shoots

The most widespread interaction is where P decreases Zn concentration. Under conditions of marginal or limiting P and Zn the addition of P stimulates growth sufficiently to dilute Zn concentration resulting in Zn deficiency (Loneragan et al., 1979; Singh et al., 1988). Other studies, however, have found that increasing P has depressed Zn concentration in shoots more than can be explained by dilution alone (Loneragan, 1951; Sharma et al., 1968). In these cases it is supposed that P-induced Zn deficiency has depressed absorption of Zn by roots or inhibited translocation of Zn from roots to shoots.

According to Chaudhry and Loneragan (1970), there is no definitive evidence to support the hypothesis that P reduces translocation of Zn from roots to shoots but it is well established that P salts can depress absorption of Zn from soils. They can do this in two ways. Firstly, increasing P can suppress AM colonisation of roots which in turn reduces Zn absorption (Lambert et al., 1979; Singh et al., 1986). Secondly, applied P strongly enhances Zn adsorption on amorphous, hydrous oxides of Fe (Stanton and du Burger, 1967; Bolland et al., 1977; Saeed and Fox 1979). These oxides may play an important role in the sorption of Zn in soils and possibly affect its availability to crops (Ghanem and Mikkelsen, 1988).

2.7.4.2 Macronutrient-zinc interactions

The macronutrient cations calcium (Ca), Mg and potassium (K), as well as other alkali and alkaline earth cations, inhibit the absorption of Zn by plants grown in solution culture (Loneragan and Webb, 1993). Increasing Ca concentrations have been found to progressively decrease shoot Zn concentrations in legumes (Bell et al., 1989) and wheat seedlings (Chaudhry and Loneragan, 1972a,c). Similarly, K and Mg have been reported to

inhibit Zn absorption from solutions in which the Ca concentration was low (Chaudhry and Loneragan, 1972a,c).

In soils, however, the variable effect of Ca, Mg and K cations on Zn nutrition is due to the effects of their salts on soil pH. Studies by Wear (1956), for example, show an increase in plant Zn concentration when treated with $CaSO_4$ which decreased soil pH from 5.6 to 4.8, but a strong decrease in plant Zn concentration when treated with an equivalent amount of $CaCO_3$ which increased soil pH from 5.7 to 6.6.

2.7.4.3 Copper-zinc interactions

In terms of nutritional interactions, the principal interacting ion for Zn^{2+} is Cu^{2+} (Kochian 1991). In short term studies with excised leaf discs of sugar cane (*Saccharum officinarum* L.), Bowen (1969) showed that Cu^{2+} competitively inhibited Zn^{2+} uptake and suggested that both ions compete for the same transport site. This effect has also been demonstrated in studies with wheat and rice seedlings (Chaudhry and Loneragan 1972b; Giordano *et al.*, 1974).

Conversely, competitive inhibition of Zn on Cu uptake has been noted by several researchers (Chaudhry and Loneragan, 1970; Kauser *et al.*, 1976) leading to speculation that a single cation channel could mediate the uptake of both ions. In the experiments of Chaudhry and Loneragan (1970) the addition of Zn in the absence of Cu depressed Cu uptake leading to intensified Cu deficiency and a consequent decline in grain yield of wheat. Cu did not depress Zn uptake in the same experiment.

The apparent conflict between results in soil and solution culture may lie in the forms of Zn and Cu available (Loneragan and Webb, 1993). The divalent cations Zn^{2+} and Cu^{2+} are the dominant forms of Zn and Cu absorbed by the plant (Kochian 1991) and it was these ions that were present in the solution studies. In soils, however, Zn and Cu are present predominantly in complexed forms with a much higher percentage of Cu complexed than Zn (Barak and Helmke, 1993). The activity of Zn^{2+} ions would therefore be much higher at

absorbing sites making it an effective competitor on Cu^{2+} absorption but being less sensitive itself to competition from Cu^{2+} (Loneragan and Webb, 1993).

2.7.4.4 Boron-zinc interactions

Graham *et al.* (1987) have shown that low solution Zn concentrations enhanced plant boron (B) concentrations to toxic levels in barley in solution culture experiments. A similar effect was observed in wheat growing in soil (Singh *et al.*, 1990) and sour orange (*Citrus aurantium* L.) seedlings (Swietlik, 1995) where Zn deficiency enhanced B concentration while reducing growth. These findings have important implications for some alkaline soils of semiarid zones which are low in soil Zn availability coupled with high levels of soluble B. The observation that foliar applications of Zn are effective in alleviating B toxicity symptoms in Zn deficient plants (Swietlik, 1995) is of practical importance for crop production on such soils.

Conversely, increasing B concentrations had little effect on Zn concentration in shoots of barley and wheat (Nable, 1988) but greatly decreased total Zn content at very toxic levels. This is due to depressed growth with a consequent decrease in Zn uptake in most genotypes.

2.8 Genetics of Zinc Efficiency

Genotypic variation for tolerance to Zn deficiency has been widely reported for maize (Clark, 1978; El-Bendary *et al.*, 1993; Nair and Prabhat, 1977), soybean (Rao *et al.*, 1977; Hartwig *et al.*, 1991) wheat (Shukla and Raj 1974, Graham 1991, Graham *et al.* 1992; Cakmak *et al.*, 1996) and rice (Ponnamperuma, 1976; Majumder *et al.*, 1990), but rather less comprehensively for barley (Randhawa and Takkar, 1976; Graham *et al.*, 1992). For this reason, together with the difficulties in screening, progress in deciphering the genetics of Zn efficiency of barley and of the physiological and biochemical mechanisms associated with it has been relatively slow (Graham and Rengel, 1993). This in turn has hindered deliberate efforts at breeding superior Zn efficient genotypes.

2.8.1 Genetics of micronutrient efficiency

2.8.1.1 General

The genetics of micronutrient efficiency is characterised by single, major-gene inheritance (Epstein, 1972). The pioneering work on Fe efficiency in soybean (Weiss, 1943) showed that efficiency was due to a single, major dominant gene that controls the ability of the roots to reduce Fe^{3+} to Fe^{2+} (Brown *et al.*, 1961). Other studies showing single gene inheritance have included B and Mg efficiency in celery (*Apium graveolens* L.) (Pope and Munger, 1953a,b) and B efficiency in tomato (Wall and Andrus, 1962). Cu and Mn efficiency in rye (*Secale cereale* L.) and Mn efficiency in barley also appear to be controlled by single major genes (Graham, 1984; McCarthy *et al.*, 1988).

Several minor additive genes contributing to Fe efficiency were later identified by Fehr (1982). This system of both a major and several minor genes is likely to be true for the micronutrients in general (Graham and Welch, 1996).

2.8.1.2 Genetic variation for zinc efficiency

Differential Zn efficiency has been reported for many broadacre and horticultural species including navy bean (Ambler and Brown 1969; Jyung *et al.*, 1972; Jolley and Brown, 1991), tomato (Parker *et al.*, 1992), sorghum (*Sorghum vulgare*) (Shukla *et al.*, 1973; Ramani and Kannan, 1985), maize (Nair and Prabhat 1977; Clark, 1978; Ramani and Kannan, 1985; El-Bendary *et al.*, 1993), oats (Brown and McDaniel, 1978), wheat (Shukla and Raj, 1974; Graham, 1991; Graham *et al.*, 1992) and, soybeans (Rao *et al.*, 1976; Hartwig *et al.*, 1991). There have also been reports of differential Zn efficiency between species (Viets *et al.*, 1954; Tiwari and Pathak, 1982; Singh *et al.*, 1983; Tiwari and Dwivedi, 1990). Comparisons of this sort should be made with caution, however, as the small number of genotypes tested for any one crop can hardly be representative of that species. For this reason, also, it is difficult to determine the extent of Zn efficiency available within a particular germplasm (Graham and Rengel, 1993).

Field trials of cereals in Southern Australia (Graham, 1984, 1988, 1991; Graham *et al.*, 1992) show larger differences between species than within species. Rye and triticale (*X*

Triticosecale) cultivars are generally well adapted to Zn deficient soils and may be taken as a standard of efficiency to be achieved in all small grain cereals. Durum wheats (*Triticum durum* L.) are exceptionally poorly adapted as reflected in the fact that durum wheat production is limited to only the most fertile soils. Barleys and bread wheats are intermediate between these two extremes.

The genetics of Zn efficiency appear to be more complex than the other studied micronutrients, with studies by Graham (1984) suggesting several loci on as many different chromosomes are involved in Zn efficiency of rye. Large scale screening of rice lines has shown a range of responses and a suggestion that the genetic effects responsible for the Zn efficiency trait are mostly additive and to a lesser extent dominant (Majumder *et al.*, 1990). El-Bendary *et al.* (1993) found Zn accumulation in maize to be controlled by at least 4 additive genes. Differential responses to Zn in maize appeared to be caused by differences in translocation, requirements and utilisation of Zn and for accumulation of imbalanced amounts of mineral elements which interact with Zn. Varietal differences for Zn absorption by soybean in response to Zn fertilisers have been shown by a number of workers (Rao *et al.*, 1977; Rose *et al.*, 1981; Hartwig *et al.*, 1991). Hartwig *et al.* (1991) tested 330 F3 lines and from their phenotype distribution concluded that only a few genes control Zn absorption efficiency or inefficiency.

Mechanistic explanations for differential Zn efficiency have been difficult to determine due to the additive nature of inheritance and the likelihood that efficiency mechanisms are expressed at different levels (molecular, physiological, structural or developmental) of the plant organism (Rengel 1992). An understanding of the mechanisms behind these additive effects would be useful in breeding since each effect alone might not show high phenotypic difference but pyramiding all effects into one locally adapted cultivar would result in high overall Zn efficiency.

2.8.2 Mechanisms of micronutrient efficiency

Known mechanisms of micronutrient efficiency include the aforementioned ability of soybean roots to reduce Fe^{3+} to Fe^{2+} thereby increasing the bioavailability of Fe to the

plant. The major gene conferring Fe efficiency in tomato was shown to code for an Fe-transporting amine, NA (Brown and Wann 1982). Nicotianamine is the precursor for the synthesis of many phytometallophores which can chelate Fe^{3+} and mobilise it into the root cell. Studies on Fe efficiency in rice (Hoan *et al.*, 1992) showed that efficiency was linked to synthesis and release of the phytosiderophore, deoxymugineic acid.

Although definite evidence regarding mechanisms of differential Zn efficiency is lacking, it is known that phytometallophores can also chelate Zn^{2+}. Since NA is ubiquitous in higher plants (Prochazka and Scholz, 1984; Ripperger and Schreiber, 1982), it is possible that differences among alleles at a corresponding locus will permit enhancement of NA synthesis and thereby Zn uptake and transport (Graham and Welch 1996). Other possible mechanisms of differential Zn efficiency include differential compartmentalisation and/or utilisation of Zn within the plant cell, greater ability to support mycorrhizal colonisation and differential root geometry. These are proposed mechanisms only and their validity awaits further research.

It is important to remember that different mechanisms of Zn efficiency operate between soil culture and solution culture. In soil, plants rely on diffusion of Zn to the root surface for uptake whereas in a stirred solution culture Zn ions are uniformly distributed and not dependent on diffusion. This makes ranking of varieties difficult as experienced by Bei Dong *et al.* (1995) who found the wheat variety 'Excalibur' was the most Zn-efficient of 3 genotypes tested in Zn deficient soil but was the least efficient when tested in nutrient solution.

2.8.3 Screening methods

As plant breeding involves the testing of large numbers of genotypes, screening methods are required to be simple, fast and inexpensive. A better understanding of the physiological mechanisms controlling Zn efficiency is likely to lead to a more ideal screening technique (Graham and Rengel 1993).

The most common method of screening is in pots containing a Zn-deficient soil to which basal nutrients are added (Thongbai *et al.*, 1993; Rengel and Graham, 1995a,b; Rao *et al.*, 1977; Yang *et al.*, 1994; Nair and Prabhat, 1977). This is cheaper and quicker than field testing and allows for multi-level screening which is rare in field experiments. The importance of screening over a full range of stress was demonstrated by Paull (1990) in deciphering the genetics of tolerance to B toxicity. He found that several loci were involved, each operating at a different level of stress. As Zn efficiency is also likely to be controlled by several genes, full range response curves may be necessary to detect all loci.

Disadvantages of pot screening relate mainly to problems of space. Low temperatures are necessary to induce Zn deficiency which requires the use of controlled environment growth cabinets. Large pots, also, should be used to overcome problems of expression associated with root binding (Graham and Rengel, 1993).

Ultimately, Zn efficiency must be tested in the field but should be done closer to the final selection phase as fewer numbers will minimise the cost of expensive field trials. Graham (1984) described a paired plot system of field screening consisting of a +Zn treatment (Zn fertiliser added) and a -Zn treatment (untreated). Zn efficiency is calculated by the equation-

$$Zn \text{ efficiency} = \frac{Yield\ (-Zn)}{Yield\ (+Zn)} \times 100$$

In this case, differences in general adaptation and yield potential are assumed to be small in comparison to the differences in Zn efficiency. The design also allows for a nearest neighbour (Wilkinson *et al.* 1983) or spatial (Gilmour *et al.*, 1997) analysis of variance to correct for inherent soil variability. Environmental effects (see Section 2.7) that differ between locations and seasons can complicate interpretations of field data (Brennan *et al.*, 1993) leading to multi-site testing over a number of years. Although Graham's method (1984) does not exclude such genotype x environment interactions, his paired plot approach

has found wide acceptance in assessing genotypic variation in tolerance to Zn deficiency in cereals (Graham *et al.*, 1992; Yilmaz *et al.*, 1996; Cakmak *et al.*, 1996).

More recent screening techniques have focussed on the production of phytosiderophores in Zn deficient solution culture (Zhang *et al.*, 1989; Cakmak *et al.*, 1994) but their correlation to field assessed Zn efficiency is poor. This could be due to the different efficiency mechanisms operating between soil and solution culture. It is anticipated that in the future molecular methods will play an ever increasing role in the screening for Zn efficiency.

2.8.4 Genetic variation for seed micronutrient density

As for agronomic efficiency, there also exists variation for seed micronutrient density. Data by van Beusichem and Graham (unpublished) showed a nearly 100% increase in grain Fe concentration of wheat cultivars from the lowest to the highest when grown together on the same site in the same season. Similarly, increases of 50% in Zn concentration have been shown in beans (van Beem *et al.*, 1992).

The heritable components of seed nutrition comprise micronutrient content and micronutrient concentration related by the formula:

content (μg/seed) = [concentration (mg/kg) x total seed weight (g)] ÷ no. of seeds

Both components depend on the micronutrient density of vegetative tissues and on the efficiency of the transport process. Thus, the micronutrient density of seeds could be increased by exploiting genetic variability that not only improves absorption from the soil but which improves internal transport to the grain (Graham and Welch, 1996). Welch *et al.* (1993) demonstrated increased accumulation of nutrients (including Zn) in the kernels of maize by incorporating the *o2* (opaque-2) and *Mal* (multiple aleurone) genes. As the mechanisms governing seed Zn accumulation become more clearly defined, it should be possible to breed for increased seed micronutrient density.

In terms of human nutrition, concentration is the more important component since food grains are sold almost exclusively by weight, especially in developing countries where there is no middle processing. It is these countries too, where human micronutrient deficiency problems are more pronounced. With this in mind, breeding programmes for such areas should be aimed at increasing seed micronutrient concentration while maintaining or improving yield. From an agronomic point of view, however, content is probably more important since the establishment of healthy, young seedlings is dependent, in part, on the nutritional status of the mother seed, especially when resown on micronutrient deficient soils (Asher 1987).

2.9 Genetic Analysis Using DNA Markers

In the late 1960s the discovery of restriction endonucleases (bacterial enzymes that can cleave DNA at defined sequences) led to the development of techniques for isolating and handling defined pieces of DNA. One major application of this technology, developed in the field of human genetics was the construction of comprehensive genetic maps (Botstein *et al.*, 1980; Donis-Keller *et al.*, 1987). The techniques developed are equally useful for plant application in the areas of varietal identification, genetic analysis of economic traits and breeding methodologies (Beckmann and Soller 1983; Burr *et al.*, 1983).

2.9.1 Restriction fragment length polymorphisms (RFLPs)

The study of segregational patterns of Mendelian genetic markers remains the most powerful route to the understanding of hereditary transmission. The availability of markers was initially a problem in analysing agricultural genomes but advances in molecular biology in the last 15 years have provided methodologies to increase the number of useful markers. These methodologies are based upon the availability of cloned DNA fragments that can be used to probe specific genomes for the presence of variation at the DNA level. Such variations appear as changes in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases. They have therefore been termed 'Restriction Fragment Length Polymorphisms' (RFLPs) (Grodzicker *et al.*, 1974; Botstein *et al.*, 1980).

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2.9.2 Genetic map construction

In order to construct a genetic map an appropriate mapping population must be selected. Selection will depend on the extent to which the progenies are also to be evaluated for agronomic characters. If only a genetic map is required then the parents for the cross can be selected simply on the basis of a high frequency of polymorphism (Laurie *et al.*, 1992). For basic mapping purposes, F2 or backcross populations are suitable. Both are easily produced but F2 populations have two advantages. Firstly, they require less crossing and generation time. Secondly, and more importantly, they provide twice as much information since two recombinant gametes are assessed per plant rather than one. This means that fewer individuals are required to estimate recombination frequencies to any given level of accuracy. In practice it has been common to use mapping populations derived from varieties previously used to characterise morphological or agronomic characters.

The application of RFLPs to the study of agronomically important traits requires additional types of mapping populations. Genes controlling quantitative traits (most commonly referred to as quantitative trait loci or QTLs) or single genes which are difficult to detect in individual plants, can be mapped more efficiently using populations of homozygous or near-homozygous lines. Barley is well suited to the analysis of quantitative traits because of the ease with which homozygous material can be produced. Doubled haploid lines are particularly useful and can be readily produced by the *H. bulbosum* method; a technique that involves making interspecific hybrids between cultivated barley *(Hordeum vulgare* L.) and a wild species *(H. bulbosum* L.) before selectively eliminating *bulbosum* chromosomes. Since Kasher and Kao (1970) first reported on the *bulbosum* method, others have reported obtaining barley doubled haploid plants through anther culture (androgenesis) (Clapham, 1973; Huang and Sunderland, 1982). A further advantage of doubled haploid populations is that they can be retested indefinitely since individual lines maintain their genetic integrity over selfed generations and new markers can therefore be fitted into the existing genetic map (Laurie *et al.*, 1992).

Furthermore, doubled haploid lines are very useful for genetic studies because they provide a population that is completely homogeneous and homozygous. This is useful for

the evaluation of quantitative characters since all genes have been fixed and desirable genes will not be lost due to segregation in later generations. A further advantage is that each individual genotype can be replicated in field trials to reduce the effects of environment on quantitative characters, thus increasing the power of discrimination between genotypes (Choo *et al.*, 1984).

2.9.3 Integration of quantitative trait loci (QTLs) into genetic maps

QTLs, although not qualitatively different from major genes, are more difficult to detect, requiring a more statistical approach. The traditional method of mapping QTLs has been to consider markers individually (Sax, 1923; Soller and Brody, 1976) by associating marker allelic variation with phenotypic variations for the character under study. Differences in marker genotype means are tested statistically and the likelihood for the presence of a putative QTL can be plotted at the marker positions along the chromosomes. This method has been further refined (Lander and Botstein, 1989) to an approach that assesses QTL likelihood at all locations, the 'interval mapping' method. This method is now widely used and the computer program MAPMAKER/QTL (Lincoln and Lander, 1989) is treated as an industry standard.

Further progress, mainly at the statistical level, is to be expected (Jansen, 1996) so that a better understanding of multilocus systems is possible. Recently developed methods for dissecting the effects of two or three linked QTLs (Jansen, 1992; Knapp, 1991; Haley and Knott, 1992; Martinez and Curnow, 1992) eliminate the effects of other QTLs experienced in single-QTL methods. This should improve the power and precision of applied mapping in breeding programmes. This would be especially useful in the case of Zn efficiency which is thought to be a polygenic trait.

Chapter 3

Screening of Doubled Haploid Parents Sahara and Clipper for Zinc Efficiency

3.1 Introduction

Zinc deficiency is considered to be the most widespread micronutrient deficiency in the world occurring in a wide range of soil types (e.g. acid and alkaline, with high and low organic matter, calcareous, sodic, sandy; Takkar and Walker, 1993). Such widespread deficiency depresses crop production and quality in cereals (Graham and Welch, 1996) impacting on the people reliant on these crops for their livelihood. Characterisation of efficient genotypes is important for agricultural production and sustainability in such areas.

Zinc efficiency has been characterised as "a cultivar's ability to grow and yield well in soils too deficient in Zn for a standard cultivar" (Graham, 1984). Genotypic variation for Zn efficiency has been reported in several crops but mapping of the genes responsible for this trait has been largely unexplored. In the past field screening has formed the basis for identifying efficient genotypes (Takkar *et al.*, 1983; MacNaeidhe and Fleming, 1990; Graham et al., 1992; Yilmaz et al., 1996); however, this is expensive, time consuming, labour intensive and prone to seasonal effects. The problem of non-homogeneity of soils is also of concern, particularly with experiments of low replication. A quicker, cheaper and more reliable method of identification, such as a controlled growth environment bioassay, is desired. The bioassay could then be used for genetic studies and mapping the genes controlling Zn efficiency.

Research by M.A.Pallotta (unpublished) identified a significant difference in vegetative Zn concentration and content between the Australian barley cultivar Clipper

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and an Algerian landrace, Sahara 3771 when grown in a manganese efficiency bioassay (Huang *et al.*, 1994). An RFLP map of barley chromosomes had been constructed using a population of 150 doubled haploid individuals derived from a cross between these two parents (Langridge *et al.*, 1995). This population, together with the RFLP marker set, were considered as promising tools which could be used to map Zn efficiency.

The initial aim of experiments in this chapter was to determine if there is a significant difference in Zn efficiency between the varieties Clipper and Sahara and, if so, at what soil Zn level this difference is expressed. Initial research, however, identified problems with the bioassay employed and further experiments were conducted in order to address these problems.

3.2 Experiment 3A - Establishing zinc levels to measure zinc efficiency

3.2.1 Materials and methods

The soil used, commonly referred to as Laffer Sand, was a siliceous sand (Uc 2.21, Northcote 1979) which had been collected from the A_2 horizon of a virgin site near Tintinara, South Australia (Nable and Webb, 1993; Thongbai et al. 1993). It had a pH of 6.8 (in water) and DTPA-extractable Zn = 0.07 mg/kg. Soil preparation was adapted from that of Rengel and Graham (1995a). The sand was sieved through a 2mm stainless steel sieve, washed 3 times with deionised water and air dried in a glasshouse. Calcium carbonate powder (0.3% w/w) was mixed thoroughly into dry soil before the following nutrients were added: (mg salt/kg dry soil) NH_4NO_3, 350; KH_2PO_4, 150; K_2SO_4, 120; $MgSO_4.7H_2O$, 90; $CuSO_4.5H_2O$, 10; $MnSO_4.4H_2O$, 7; H_3BO_3, 1; $CoSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 0.7; $H_2MoO_4.H_2O$, 0.5; $NiSO_4.7H_2O$, 0.15.

The soil was then divided into 12 equal portions and $ZnSO_4.7H_2O$ added to correspond to the following treatments: (mg Zn/kg dry soil) 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.6, 0.8, 1.6 and 3.2 hereafter referred to as Zn_0 - $Zn_{3.2}$.

Clear plastic sample jars (6.5cm diameter x 15cm height) covered with aluminium foil and lined with a polyethylene bag were filled with soil (equivalent to 650g dry weight)

and allowed to equilibrate overnight. Barley seeds of the varieties Clipper and Sahara with similar Zn contents (Clipper 2.71 µg/seed and Sahara 2.75 µg/seed) were surface-sterilised by soaking in 70% ethanol for 1 minute, rinsed with deionised water and then soaked in sodium hypochlorite (1.4% v/v) for 6 minutes before being rinsed again with deionised water. The seed was germinated on moistened Whatman No. 42, ashless filter paper for 36 hours at $20^{O}C$ before sowing (3 seeds per pot).

Plants were grown in a controlled-environment growth chamber with a 12-hour light/12-hour dark photoperiod at $20^{O}C$ day/$10^{O}C$ night. Light was supplied by high-pressure mixed-metal halide lamps delivering a photon flux density of 500 µmol/m^{2}/s at the surface of the pot. The experiment was set up as a randomised complete block with two replicates. Two genotypes were used at 12 rates of Zn. This amounted to 2 genotypes x 12 treatments x 2 replicates = 48 pots. Pots were rotated within a block every few days to minimise the effect of microenvironments caused by uneven light distribution and/or air flow. Pots were watered by volume daily and watered to weight every 3 days to maintain the soil moisture at 12% w/w.

After 6 weeks the youngest expanded leaf blades (YEBs) were taken from all plants before harvesting whole shoots. All plant material was oven-dried at 70°C for 24 hours. Dried samples were digested with 70% nitric acid and analysed for concentrations of the elements Zn, Mn, Fe, Cu, B, Ca, Mg, Na, K, P and S by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) (Zarcinas et al., 1985). All analyses were certified by the Australasian Soil and Plant Analysis Council (ASPAC). Certified standards were included in all analyses.

Data were analysed by a two-way analysis of variance using the computer program SuperANOVA™. The factors for the ANOVA model were Zn level and genotype. To overcome problems associated with non-homogeneity of variances, values for Zn concentration and Zn content were log transformed prior to statistical analysis. Pairwise comparisons of means were made using Tukey's Honestly Significant Difference (HSD) at $\alpha = 0.05$ (Zar, 1996).

In addition, simple linear regression was performed in order to assess whether the ICPAES results for YEBs were indicative of the Zn status of whole plants. This was done by regression of Zn concentration of YEBs against Zn concentration of the whole shoot. Formulae for logistic curves were calculated using routines of the GENSTAT^{TM} computer program. Curves were constructed using Deltagraph^{TM} software.

3.2.2 Results

<u>Visual Symptoms</u>

Clipper first showed foliar symptoms of Zn deficiency approximately 18 days after sowing in the Zn_0 treatments, with other treatments up to and including $Zn_{0.25}$ developing symptoms between this time and harvest on day 42. Severity of symptoms increased with decreasing soil applied Zn. Symptoms included pale yellow, chlorotic areas followed by a dark brown necrosis developing initially on middle leaves, later spreading to younger leaves. Symptoms of reduced internode elongation were also evident, particularly for Sahara. The only Sahara treatments to show foliar symptoms were Zn_0 at approximately 25 days and $Zn_{0.05}$ and $Zn_{0.1}$ just before harvest. Symptoms of reduced growth were similar for both varieties.

In addition to Zn deficiency, the older leaves of Clipper exhibited foliar symptoms of chlorosis and necrosis extending backward from leaf tips, consistent with boron toxicity. Symptoms first became noticeable at about 16-20 days over all treatments and were obvious and extensive by harvest. Sahara exhibited no such symptoms.

<u>Shoot dry weights</u>

Total dry weight of shoots increased with increasing soil applied Zn (Figure 3.1a) reaching maximum growth at $Zn_{0.2}$-$Zn_{0.3}$ for Sahara and $Zn_{0.1}$-$Zn_{0.2}$ for Clipper. Determination of an exact level of maximum yield is difficult due to variation caused by a low number of replicates. This is particularly true for Clipper and is highlighted in constructing a logistic curve of shoot dry weight versus applied Zn (Figure 3.1b). Clipper had a lower squared coefficient of correlation (0.69) compared to Sahara (0.92). The shoot weight response of Clipper in the region $Zn_{0.15}$-$Zn_{0.8}$ was variable but clearly there was a large increase in shoot growth for both varieties between Zn_0 and $Zn_{0.1}$ with increases diminishing as treatments approached Zn sufficiency at about $Zn_{0.15}$. A similar result is observed for the relationship between shoot dry weight and shoot Zn concentration (Figure 3.1c).
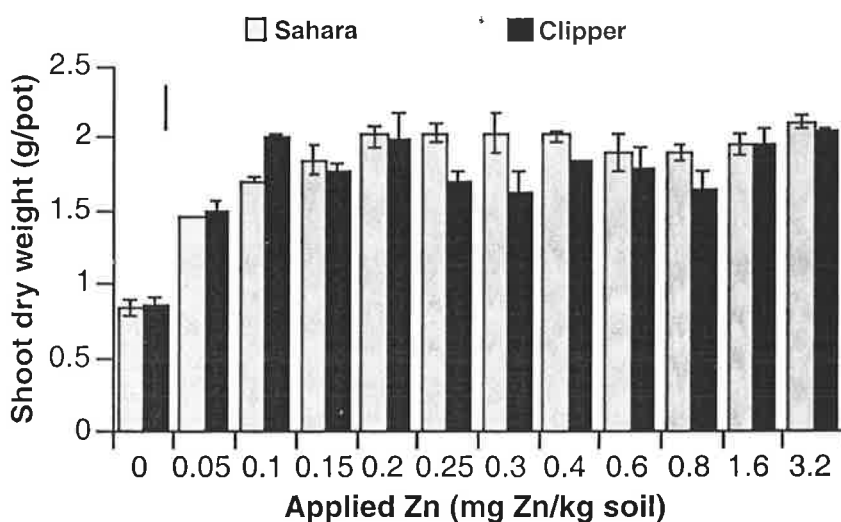
Figure 3.1a

Effect of soil applied zinc on shoot dry weight of barley varieties Sahara and Clipper grown in Laffer sand for 42 days. Values are an average of two replicates with standard errors shown. The vertical bar represents Tukey's $HSD_{0.05}$ for the main effect of zinc treatment.

X Sahara

$y = -14.62 + 16.61/(1 + 8.15\text{Exp}(-18.32(x - -0.128)))^{(1/8.15)}$

$R^2 = 0.92$

☐ Clipper

$y = -0.28 + 2.09/(1 - 1.37\text{Exp}(-26.07(x + 0.031)))^{(1/-13.65)}$

$R^2 = 0.69$
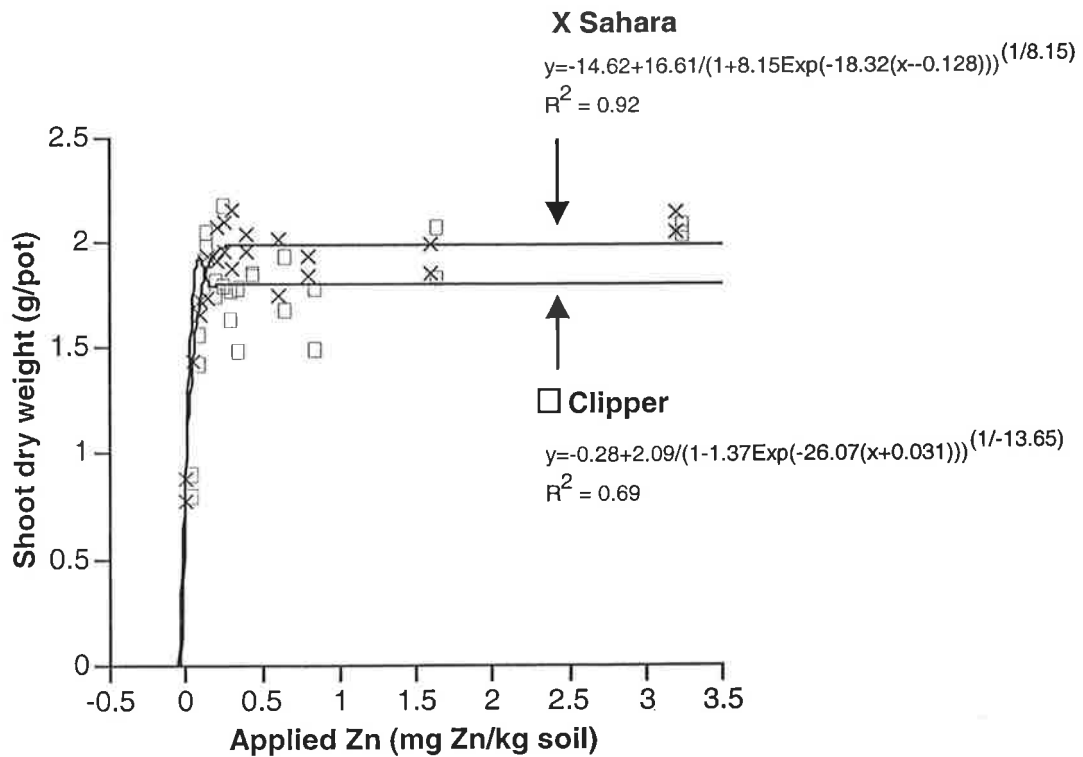
Figure 3.1b

Generalised logistic curve showing response of shoot dry weight to soil applied zinc for barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each symbol represents a single replicate.

☐ Clipper

$y = -0.199 + 2.08/(1 + 0.687*\text{Exp}(-0.4*(x - 4.252)))^{(1/0.687)}$

$R^2 = 0.42$

X Sahara

$y = -0.853 + 2.72/(1 + 20.53*\text{Exp}(-23.43*(x - 6.05)))^{(1/20.53)}$

$R^2 = 0.82$
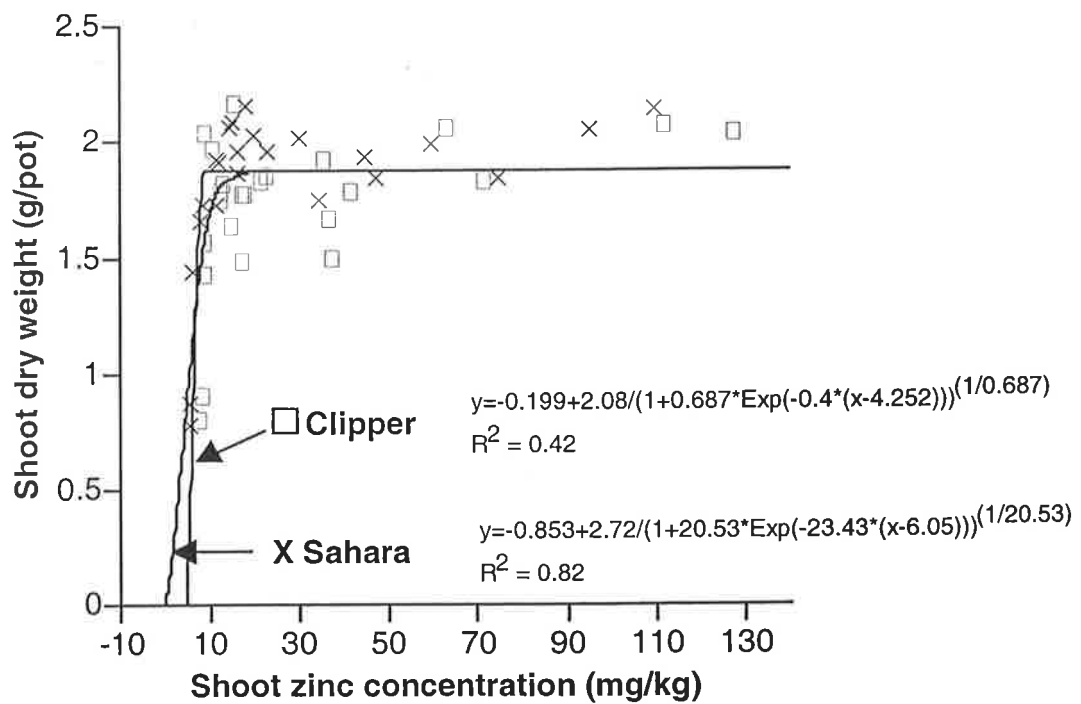
Figure 3.1c

Generalised logistic curve showing relationship between shoot zinc concentration and shoot dry weight for barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each symbol represents a single replicate.

Shoot zinc concentrations and zinc contents

Shoot Zn concentrations increased with increasing soil applied Zn (Figure 3.1d). There was a highly significant Zn treatment effect ($P \leq 0.0001$) but no difference between varieties and no significant interaction (see Appendix 1 (Section 3.1) for ANOVA table of log transformed data).

Shoot Zn content also increased with the level of soil applied Zn (Figure 3.1e) showing a strong ($P \leq 0.0001$) Zn treatment effect (see Appendix 1 (Section 3.1) for ANOVA of log transformed data). In contrast to Zn concentration, there was also a genotype by treatment effect ($P \leq 0.05$). There was no significant genotype effect.
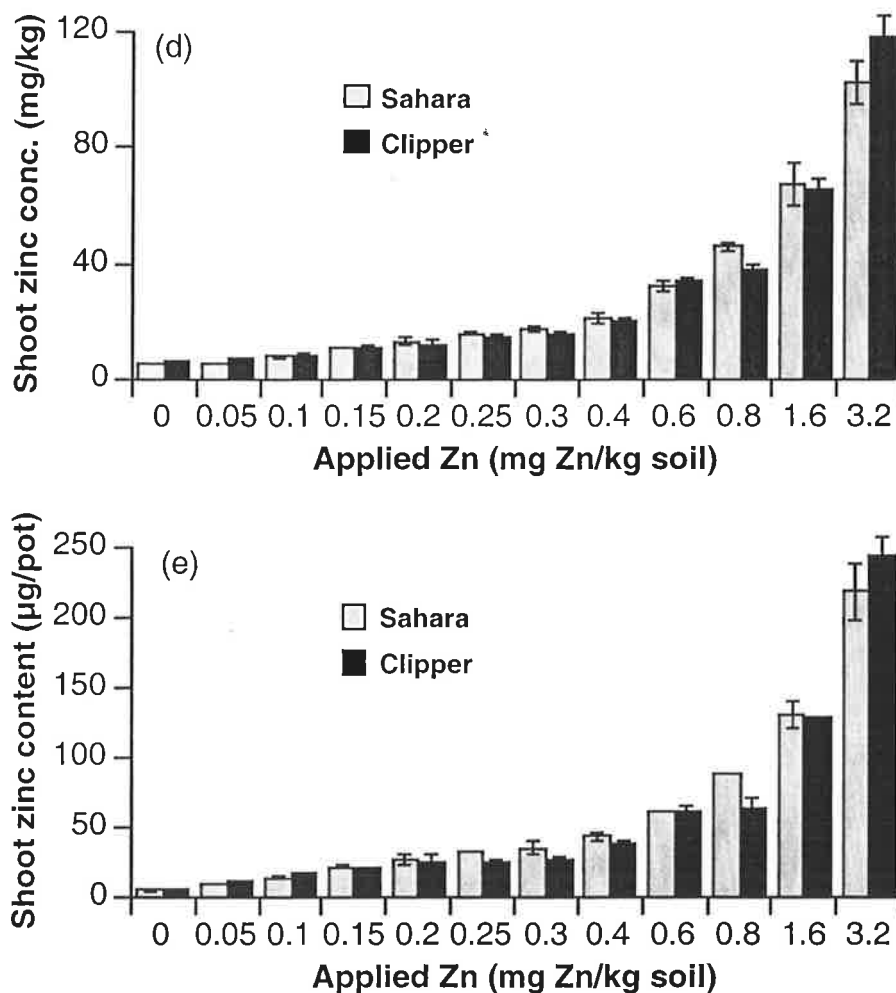
Figures 3.1d and e

Effect of soil applied zinc on shoot zinc concentration and zinc content of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Values are an average of two replicates with standard errors shown.

YEB Zinc Concentrations

A simple regression of YEB Zn concentration against total shoot Zn concentration showed quite different behaviour between the two varieties (Figure 3.1f). The correlation was reasonably linear for Clipper at low Zn concentrations, but at higher concentrations the increase in YEB Zn did not increase at the same rate as total shoot Zn, approaching an asymptotic maximum at approximately 45-50 mg Zn/kg in the YEB. While total shoot Zn concentrations continued to increase, YEB Zn concentrations reached a maximum average of 44.0 mg/kg at $Zn_{1.6}$, decreasing to 39.5 mg/kg at $Zn_{3.2}$. Conversely, the relationship between Zn concentrations in YEBs and whole shoots for Sahara was highly significant and linear, except for Zn_0 where relatively high Zn concentrations in YEBs (12-13 mg/kg) did not reflect that measured in whole shoots (5.7-5.9 mg/kg).
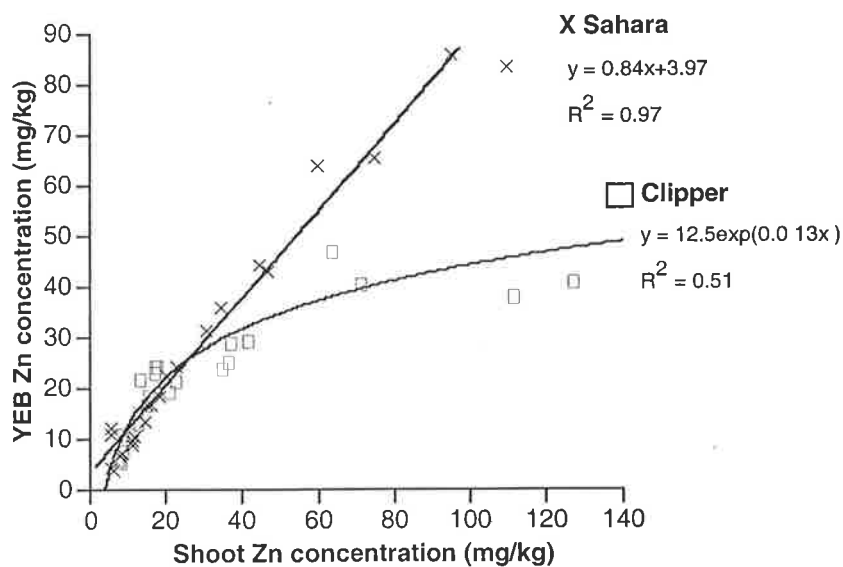
Figure 3.1f

Relationship between YEB zinc concentration and whole shoot zinc concentration of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Clipper shows an exponential relationship whereas Sahara shows a linear relationship. Each symbol represents a single replicate.

Other Elements

Shoot boron concentrations ranged from 23-49 mg/kg for Clipper and 13-23 mg/kg for Sahara (Figure 3.1g). These concentrations are high, but not toxic, with the critical concentration (toxicity) for barley in the range 70-150 mg/kg (Reuter *et al.*, 1997).

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Concentration of Fe in shoots was greatly increased under Zn deficiency (Figure 3.1h). The shoot Fe concentration was greater in Sahara than in Clipper at treatments $\leq$.$Zn_{0.1}$, but concentrations were similar at the higher Zn treatments. Manganese concentrations behaved in a similar manner (Figure 3.1i) and increased rapidly below $Zn_{0.2}$ for Sahara and below $Zn_{0.1}$ for Clipper.
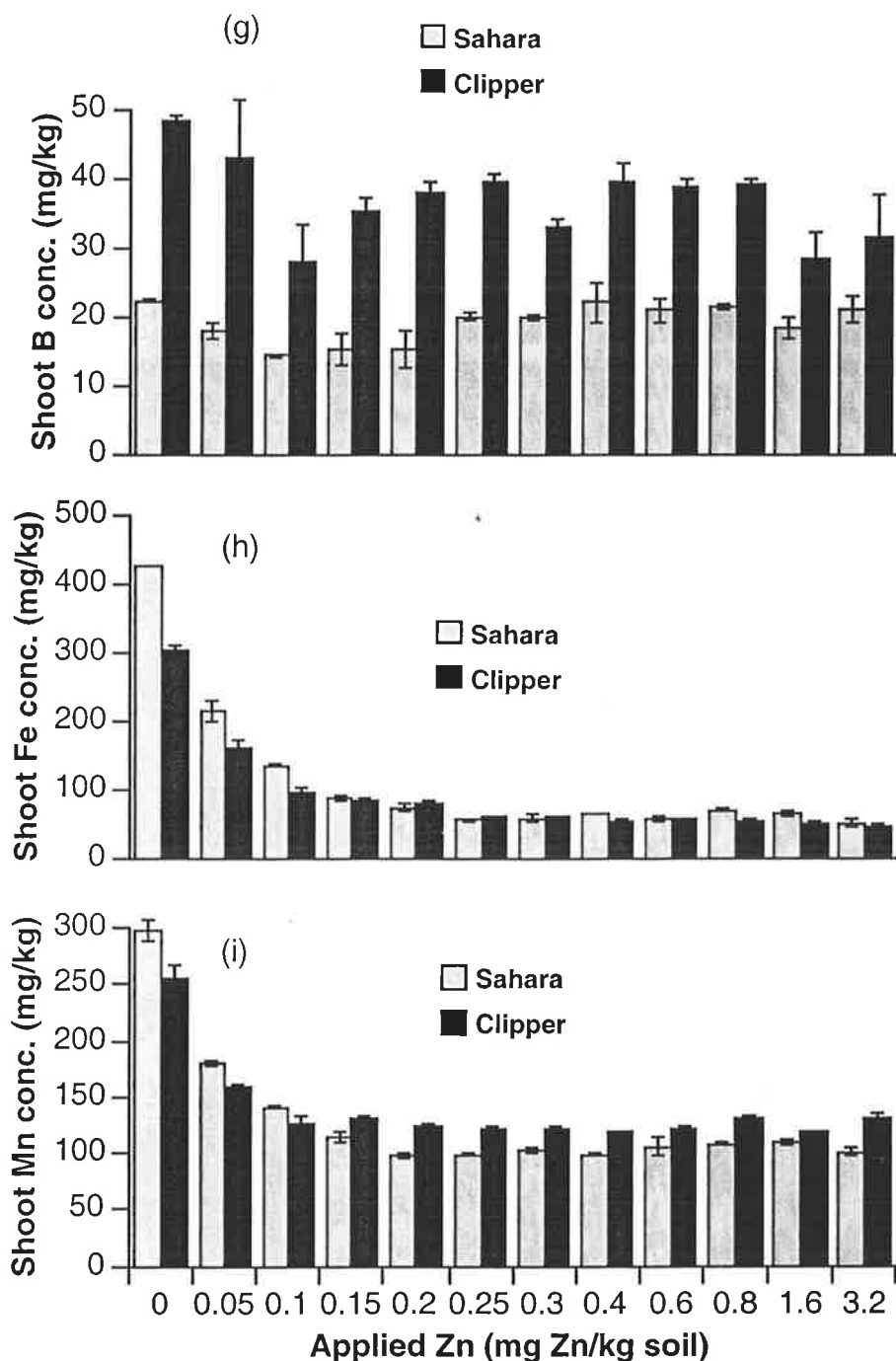
Figures 3.1g, h and i

Effect of soil applied zinc on shoot boron, iron and manganese concentrations of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Values are an average of two replicates with standard errors shown.

3.3 Experiment 3B - Measuring zinc efficiency of the barley varieties Clipper and Sahara

3.3.1 Materials and methods

The results of experiment 3A identified high shoot boron concentrations and a high degree of error between replicates for some variables, most notably shoot dry weight. In order to correct these problems it was decided firstly to wash the Laffer Sand more vigorously (10 times) in an attempt to reduce plant B accumulation by removing adsorbed B. It was also decided to increase the number of replicates from two to five for each genotype by treatment group in an effort to reduce standard errors. This necessitated a reduction in treatments so that the experiment didn't become unmanageably large.

The choice of Zn treatments was based on the results of experiment 3A, concentrating on the lower end of the scale where large increases in shoot weight between treatments were recorded. Zinc treatments corresponded to 0, 0.01, 0.05, 0.1, 0.3 and 3.2 mg Zn/kg dry soil hereafter denoted as Zn_0 - $Zn_{3.2}$.

Apart from these two modifications, methods of soil preparation, seed sterilisation, growing conditions and harvest were the same as for experiment 3A. Data were again analysed as a two-way analysis of variance with genotype and Zn treatment forming the relevant factors. Values for Zn concentration and Zn content were again log-transformed due to non-homogeneity of variances.

3.3.2 Results

<u>Visual Symptoms</u>

Clipper at Zn_0 was again the first variety by treatment combination to show foliar symptoms of Zn deficiency (at approximately 14 days). Symptoms of Zn deficiency were similar to those seen in experiment 3A and decreased with increasing soil applied Zn up to treatment $Zn_{0.3}$ in which plants exhibited no deficiency symptoms. As was the case with experiment 3A, foliar symptoms were more prevalent on Clipper while reduced internode elongation was more pronounced on Sahara. Necrosis, characteristic of boron toxicity, was again evident on Clipper only, but appeared at all soil Zn treatments with similar intensity.

Shoot dry weights

There was a strong (P ≤ 0.0001) Zn treatment effect (see Appendix 1 (Section 3.2) for ANOVA table). Shoot dry weights increased with increasing soil applied Zn up to $Zn_{0.3}$ before decreasing slightly at $Zn_{3.2}$ (Figure 3.2a), suggesting that this Zn level is too high to attain maximum yield. Further evidence is provided by considering the relationship between shoot weight and shoot Zn concentration of individual replicates (Figure 3.2b) which shows higher shoot Zn concentrations (>70 mg/kg) associated with declining shoot weights. Critical Zn concentrations for toxicity in whole shoots of barley are in the range 120-520 mg/kg (Davis and Beckett, 1978). Although the concentrations measured in Experiment 3B are slightly less than this, it would appear that in this system shoot Zn concentrations of 70-160 mg/kg are toxic. The decline in shoot weight is sharper for Sahara suggesting it is more sensitive to Zn toxicity. This result stands in contrast to Experiment 3A where high shoot Zn concentrations did not result in decreased shoot dry weight.
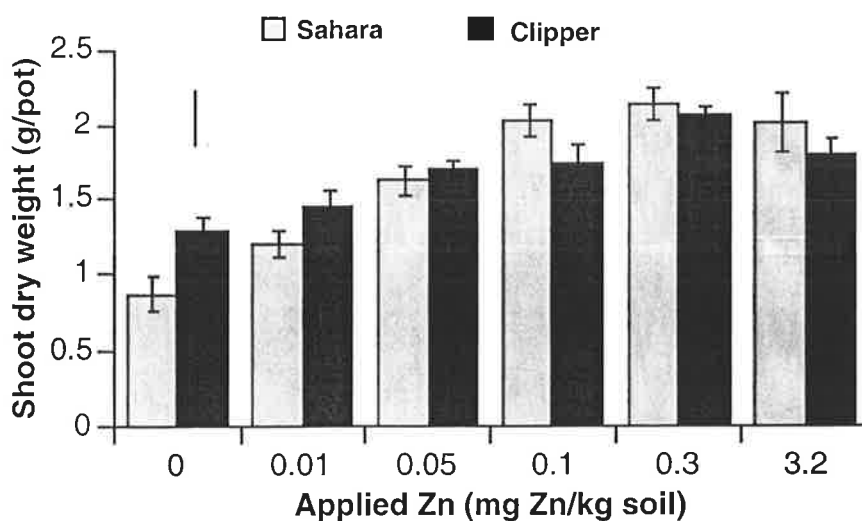
Figure 3.2a

Effect of soil applied zinc on shoot dry weight of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Values are an average of 5 replicates with standard errors shown. The vertical bar represents Tukey's $HSD_{0.05}$ for the main effect of zinc treatment.

X Sahara
$y=3.33+(-2.93(0.938^x))+(-0.012x)$
$R^2 = 0.52$

☐ **Clipper**
$y=2.48+(-1.80(0.904^x))+(-0.006x)$
$R^2 = 0.52$
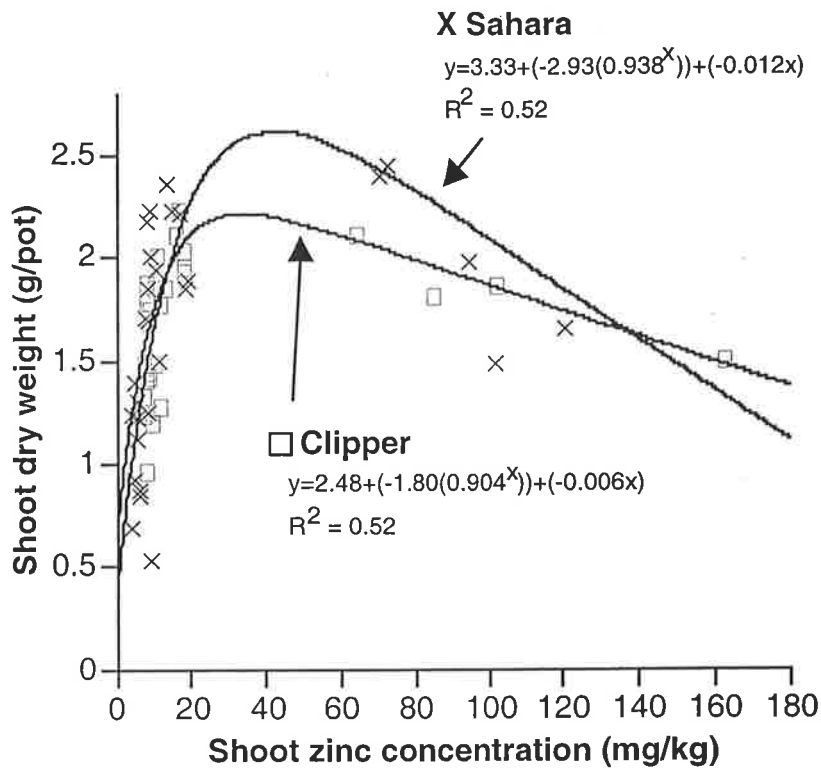
Figure 3.2b

Response curve showing relationship between shoot dry weight and shoot zinc concentration for barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each symbol represents a single replicate.

The construction of a logistic curve for shoot dry weight versus applied Zn (Figure 3.2c) was affected by the large variance among replicates at $Zn_{3.2}$. This treatment led to shoot growth decline in some replicates but not others. The curves for relative yield versus applied Zn are similarly affected (Figure 3.2d).

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X Sahara

$y = 1.94 + ((-1.04 + 13.16x)(0.0002^x))$

$R^2 = 0.79$

□ **Clipper**

$y = 1.77 + ((-0.483 + 4.73x)(0.012^x))$

$R^2 = 0.60$

Shoot dry weight (g/pot)

Applied Zn (mg Zn/kg soil)
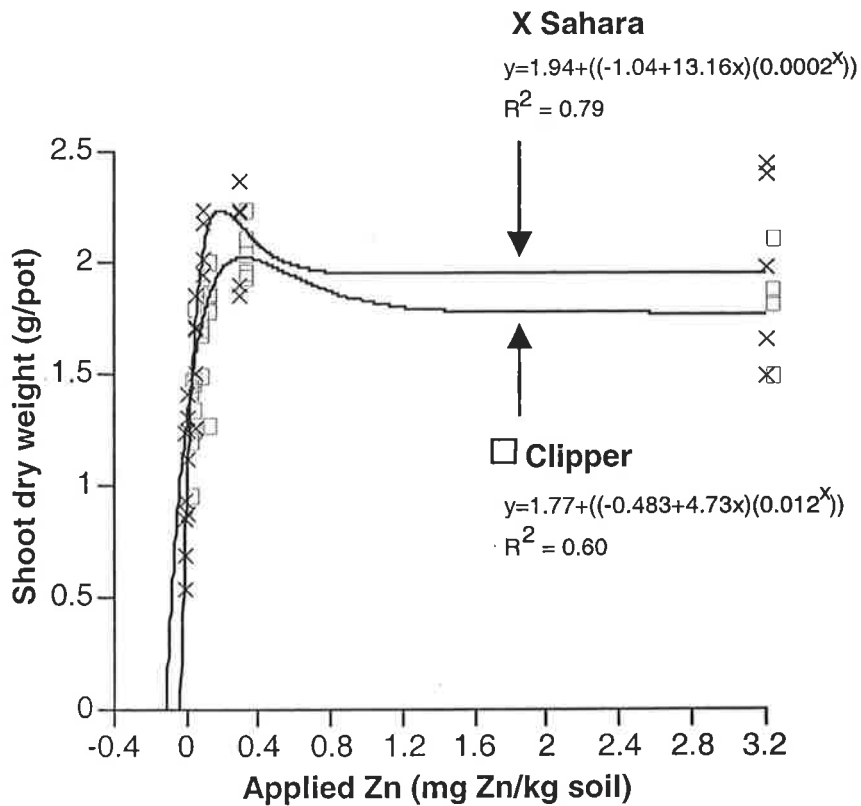
Figure 3.2c

Generalised logistic curve showing the response of shoot dry weight to applied zinc for barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each symbol represents a single replicate.

□ **Clipper**

$y = 79.08 + (-21.49 + 212.1x)0.012^x$

$R^2 = 0.60$

X Sahara

$y = 76.46 + (-44.39 + 557.6x)0.0001^x$

$R^2 = 0.75$

Relative yield (%)

Applied Zn (mg Zn/kg soil)
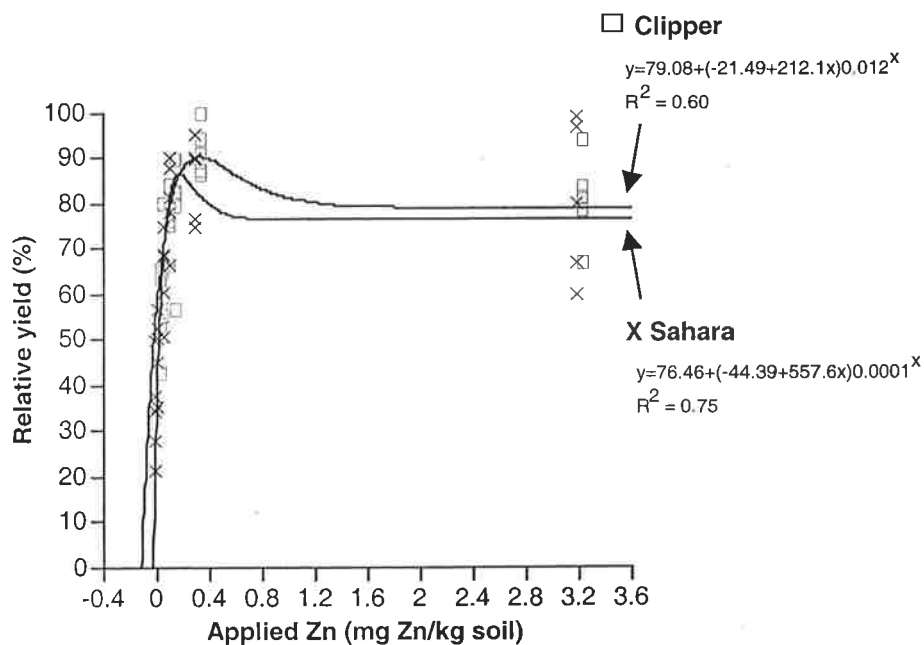
Figure 3.2d

Generalised logistic curve showing effect of applied zinc on relative yield (% of maximum yield) of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each symbol represents a single replicate.

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Shoot zinc concentrations and zinc contents

Shoot Zn concentrations increased with increasing soil applied Zn above $Zn_{0.01}$ (Figure 3.2e). There was a significant (P<0.0001) Zn treatment effect but no difference between genotypes and no interaction (see Appendix 1 (Section 3.2) for ANOVA table of log transformed data). There was no difference between Zn_0 and $Zn_{0.01}$, nor between $Zn_{0.05}$ and $Zn_{0.1}$.
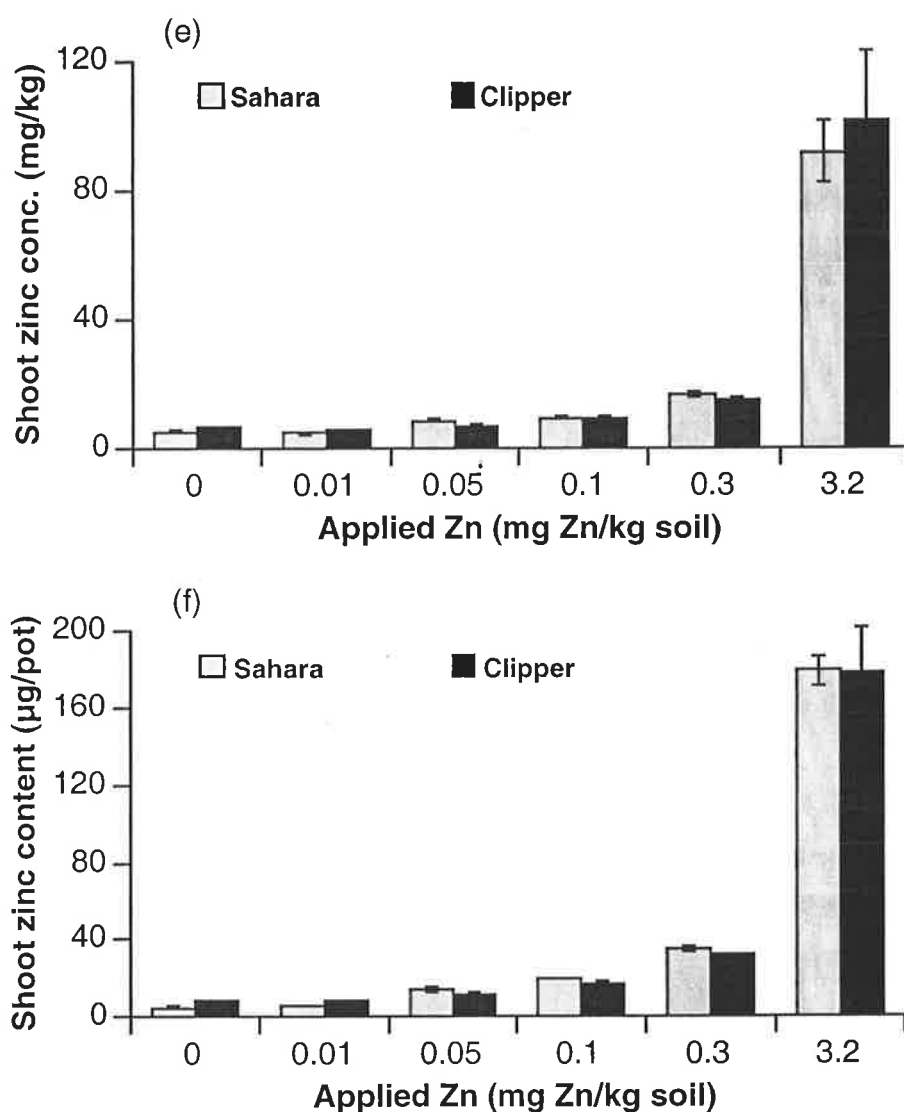
Figures 3.2e and f

Effect of applied zinc on shoot zinc concentration and content of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Values are an average of five replicates with standard errors shown.

Shoot Zn contents also increased with increasing soil applied Zn (Figure 3.2f) and showed a strong (P<0.0001) Zn treatment effect but no varietal difference (see Appendix 1 (Section 3.2) for ANOVA table of log transformed data). In addition there was a strong

(P<0.0001) genotype by treatment interaction, presumably due to Sahara's more rapid decline in shoot weight as treatments approach Zn_0.

YEB Zinc Concentrations

The behaviour of YEB Zn concentrations for the two varieties was consistent with that observed in Experiment 3A. Simple regression of YEB Zn concentration against total shoot Zn concentration (Figure 3.2g) shows a linear relationship between the two variables for Sahara. Clipper again showed an exponential relationship with YEB Zn concentrations approaching an asymptotic maximum at approximately 80 mg Zn/kg.
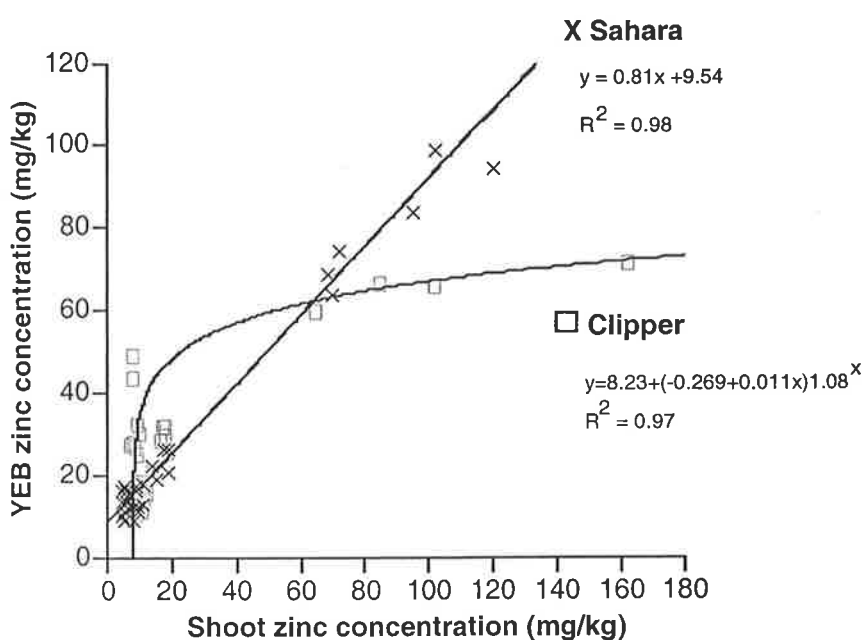
Figure 3.2g

Relationship between YEB zinc concentration and whole shoot zinc concentration of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Clipper shows an exponential relationship whereas Sahara is linear. Each symbol represents a single replicate.

Other Elements

Washing the sand 10 times (compared to 3 times in Experiment 3A) caused shoot boron concentrations to be reduced by approximately 50% for Sahara over all treatments in comparison to Experiment 3A (see Appendix 2 for shoot concentration data). Reduction for Clipper, however, occurred only at very low levels of added Zn (Zn_0 and $Zn_{0.01}$).

Despite this reduction, the boron toxicity-like symptoms experienced in Experiment 3A were still obvious on Clipper at all Zn treatments.

Shoot concentrations of iron, manganese, copper, phosphorus and sulphur were also reduced compared to Experiment 3A, particularly at low levels of soil applied Zn (see Appendix 2 for shoot concentration data). Shoot magnesium concentrations were reduced by approximately 30% in general and up to 50% for Sahara at Zn_0 (see Appendix 2). Shoot concentrations of potassium, however, increased in comparison to Experiment 3A at all treatments for Sahara and above Zn_0 for Clipper (see Appendix 2). Sodium levels were increased significantly for Sahara at all treatments but only slightly for Clipper (see Appendix 2).

3.4 Experiment 3C - Effect of boron on zinc efficiency bioassay

3.4.1 Materials and methods

Results of Experiment 3B showed that shoot boron concentrations in variety Clipper remained high. This led to investigation of the quality of water used for washing soil and watering plants. ICPAES analysis determined it to be less than adequate for this type of bioassay (see Section 3.4.2). A relatively large concentration of boron in the water (0.025 mg/L) was considered to be a possible factor in the cause of B toxicity together with the additional H_3BO_3 added in the nutrient mixture. In order to resolve these problems a small factorial experiment was carried out. The same two genotypes, Clipper and Sahara, were grown in 3 separate soil treatments at two levels of Zn and two levels of boron. The design was therefore 2 genotypes x 3 soil treatments x 2 Zn levels x 2 boron levels x 2 replicates = 48 pots.

Soil was sieved through a 2mm stainless steel sieve before the following treatments were carried out -

1) washed - as per Experiment 3A (i.e. 3 times) using RO water (B concentration =0.025 mg/L).

2) pure washed - 3 times using millipore water (B concentration = 0.001 mg/L).

3) unwashed

After drying in a glasshouse, calcium carbonate powder (0.3% w/w) was thoroughly mixed into each soil before the following nutrients were added: (mg salt/kg dry soil) NH_4NO_3, 350; KH_2PO_4, 150; K_2SO_4, 120; $MgSO_4.7H_2O$, 90; $CuSO_4.5H_2O$, 5 (note: this is half the amount added in Experiments 3A and 3B); $MnSO_4.4H_2O$, 7; $CoSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 0.7; $H_2MoO_4.H_2O$, 0.5; $NiSO_4.7H_2O$, 0.15.

Each soil was then divided into 2 equal portions and the following Zn treatments applied -

1) -low Zn = 0 mg Zn/kg dry soil as $ZnSO_4.7H_2O$ hereafter denoted as Zn_0.

2) -high Zn = 1.6 mg Zn/kg "   "   " hereafter denoted as $Zn_{1.6}$.

Each of these treatments was further divided into two equal portions and the following boron treatments applied -

1) -low B = 0.018 mg B/kg dry soil as H_3BO_3 hereafter denoted as B_{low}.

2) -high B = 0.175 mg B/kg " " "   "   hereafter denoted as B_{high} (this is the same level as that used in Experiments 3A and 3B).

Soil was added to pots as previously but a purer source of millipore water (B=0.001 mg/L) was used for watering than was used in Experiments 3A and 3B. Seed sterilisation and growing conditions were the same as for previous experiments. At harvest time, whole tops only were sampled and prepared for ICPAES analysis as before. The sampling of YEBs was abandoned due to their poor linear correlation with whole shoots for variety Clipper as experienced in Experiments 3A and 3B. Data were analysed as a 4-way analysis of variance. The factors for the ANOVA model were genotype, soil treatment, Zn treatment and boron treatment. Values for Zn concentration, Zn content, boron concentration and boron content were log transformed prior to analysis in order to overcome problems associated with non-homogeneity of variances.

3.4.2 Results

<u>Water Quality</u>

ICPAES analysis of water used for washing soil and watering plants in Experiments 3A and 3B showed relatively high concentrations of boron and sodium (Table 3a).

<u>Table 3a</u>

Concentrations (mg/L) of selected elements in water used for washing soil and watering plants in Experiments 3A, B and C. The first column refers to the experiment, type of water and water use. RO refers to water prepared by reverse osmosis while DD1 and DD2 refer to double-deionised water prepared by two different Milli-Q® water purification systems.

Water ID	Fe	B	Cu	Na
Exp 3A,B-RO washing	0.003	0.025	0.007	1.3
Exp 3A,B-DD1 watering	0.002	0.025	0.005	1.1
Exp 3C-DD2 pure wash	n.d.	0.01	0.005	0.4
Exp 3C-DD2 watering	n.d.	0.001	0.004	0.1

n.d. - not detectable

It was estimated that each pot received approximately 2.0-2.5 L of water during these experiments which equates to 0.050-0.063 mg of boron added through watering alone in Experiments 3A and 3B. The amount of H_3BO_3 added in the nutrient mixture was equivalent to 0.114 mg of boron per pot. Watering the plants grown in Experiments 3A and 3B therefore increased the amount of added boron by approximately 50%. This was considered unacceptable for this bioassay so a more pure source of water which would contribute only 0.002-0.003 mg of extra boron was located for Experiment 3C (Table 3a).

<u>Visual Symptoms</u>

Foliar symptoms of Zn deficiency were apparent on Clipper $Zn_{0,pure}$ and $Zn_{0,washed}$ at approximately 14-18 days after sowing and Clipper $Zn_{0,unwashed}$ at approximately 25-27 days after sowing. Symptoms on Sahara $Zn_{0,pure}$ and $Zn_{0,washed}$ appeared about 10-12 days after Clipper for the same treatments but Sahara showed no foliar Zn deficiency symptoms

for the unwashed treatment.

Boron toxicity-like symptoms on Clipper, similar to those observed in Experiments 3A and 3B, were greatly reduced by the low B treatment but not completely eliminated. The B treatment appeared to have no effect on total shoot growth under any soil conditions.

Shoot dry weights

Analysis of total dry weight of shoots showed strong (P<0.0001) effects of soil preparation and Zn treatment (see Appendix 1 (Section 3.3) for ANOVA table). Dry weights increased in the order unwashed < washed < pure and $Zn_{1.6}$ increased weight over Zn_0 as expected (Figure 3.3a). There was no significant effect of genotype or B treatment and no significant interactions of any order.
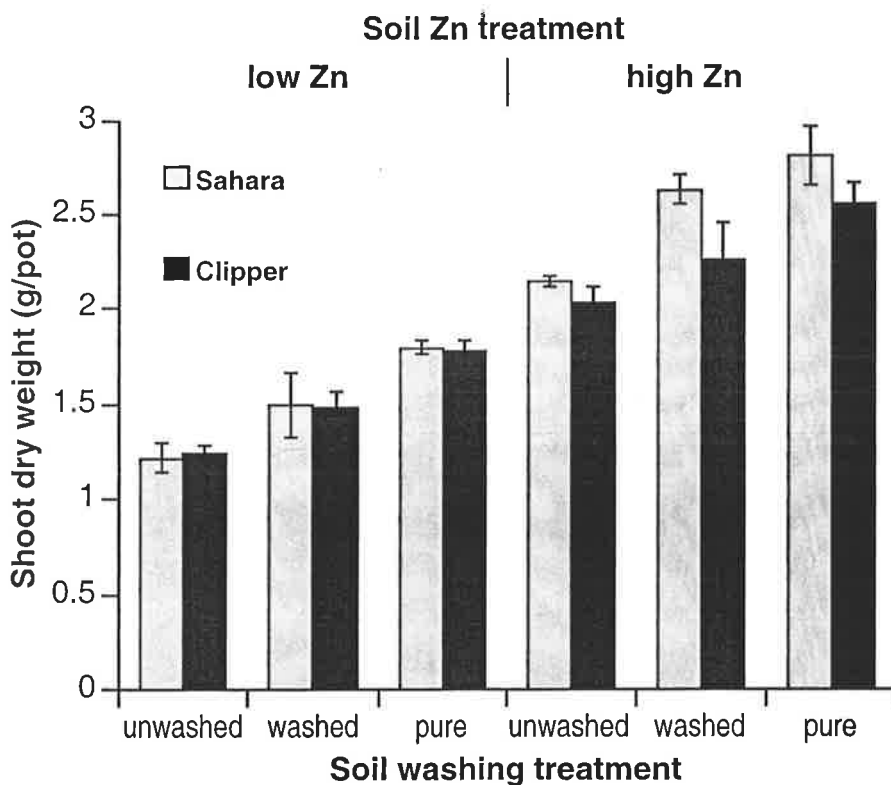
Figure 3.3a

Effect of soil washing treatment on shoot dry weight of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each treatment was grown at high and low zinc (1.6 mg Zn/kg soil and 0 mg Zn/kg soil respectively). Data were averaged over boron treatment (0.018 mg B/kg soil or 0.175 mg B/kg soil) because the main effect and all interactions involving boron treatment were non-significant.

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Shoot zinc concentrations and contents

Predictably, shoot Zn concentrations were much higher at $Zn_{1.6}$ than at Zn_0 (Figure 3.3b). There was also a significant ($P<0.01$) genotype effect with Sahara having greater concentrations than Clipper at the high level of Zn. At Zn_0, shoot Zn concentrations were approximately the same for the two varieties. These results were consistent across all soil treatments. There was also a strong ($P<0.0001$) soil treatment by Zn interaction and a strong ($P<0.0001$) Zn by genotype interaction for shoot Zn concentration (see Appendix 1 (Section 3.3) for ANOVA table of log transformed data).

Shoot Zn contents were also much higher at $Zn_{1.6}$ (Figure 3.3c). The genotype effect was much stronger ($P<0.0001$) than it was for Zn concentration due to differences in total shoot weights. In addition to the Zn and genotype effects, there was also a significant ($P<0.0001$) soil treatment effect (see Appendix 1 (Section 3.3) for ANOVA table of log transformed data). The significant interactive effects were the same as for shoot Zn concentration, ie. soil treatment x Zn treatment and genotype x Zn treatment. There were no other significant ($P<0.01$) interactions of any order.

(b)

(c)
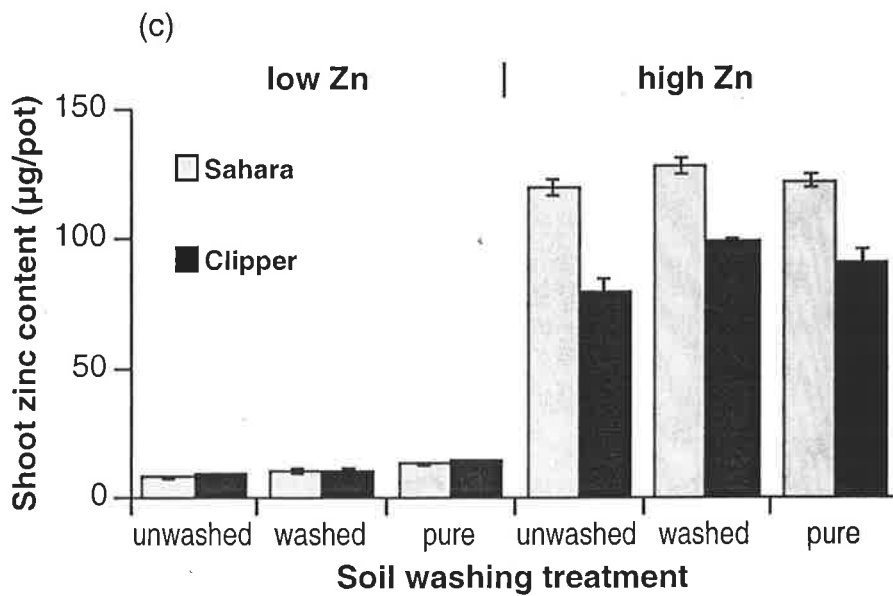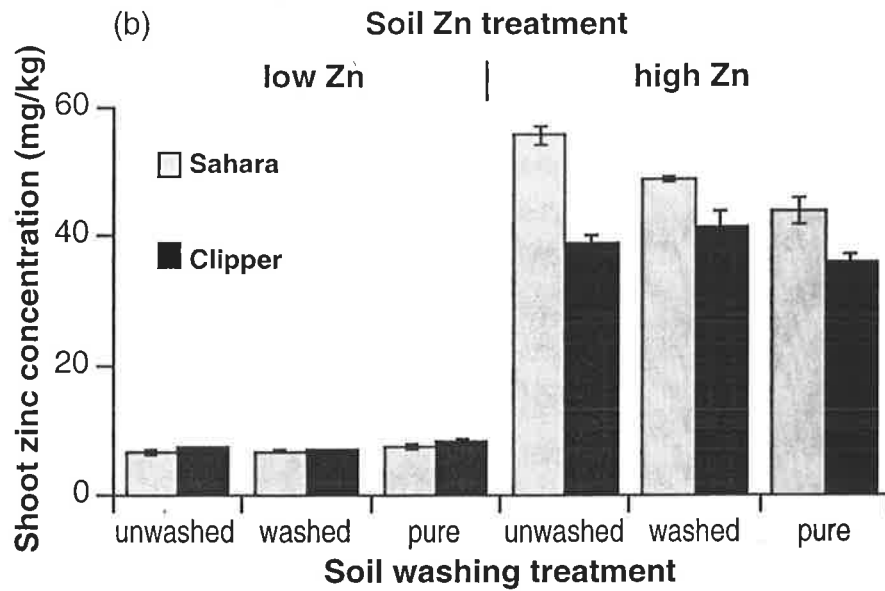
Figures 3.3b and c

Effect of soil washing treatment on shoot zinc concentration and content of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Each treatment was grown at high and low zinc (1.6 mg Zn/kg soil and 0 mg Zn/kg soil respectively). Data were averaged over boron treatment (0.018 mg B/kg soil or 0.175 mg B/kg soil) because the main effect and all interactions involving boron treatment were non-significant.

Other elements

Shoot boron concentrations (Figure 3.3d) were strongly ($P < 0.0001$) influenced by all main effects i.e. genotype, Zn level, boron level and soil treatment (see Appendix 1 (Section 3.3) for ANOVA table of log transformed data). Clipper maintained a higher range of shoot B concentrations (3.4 - 20.5 mg/kg) than variety Sahara (2.3 - 8.3 mg/kg).

In comparison to Experiments 3A and 3B, shoot B concentrations were greatly reduced. The fact that this reduction was far greater than any difference between soil treatments suggests that the quality of water used for watering was more important in affecting B levels than the quality of water used for washing soil. This is not to say, however, that at least some effect was caused by washing with impure water. Plants grown in both the washed and pure washed treatments showed higher shoot B concentrations than the unwashed treatment (Figure 3.3d).
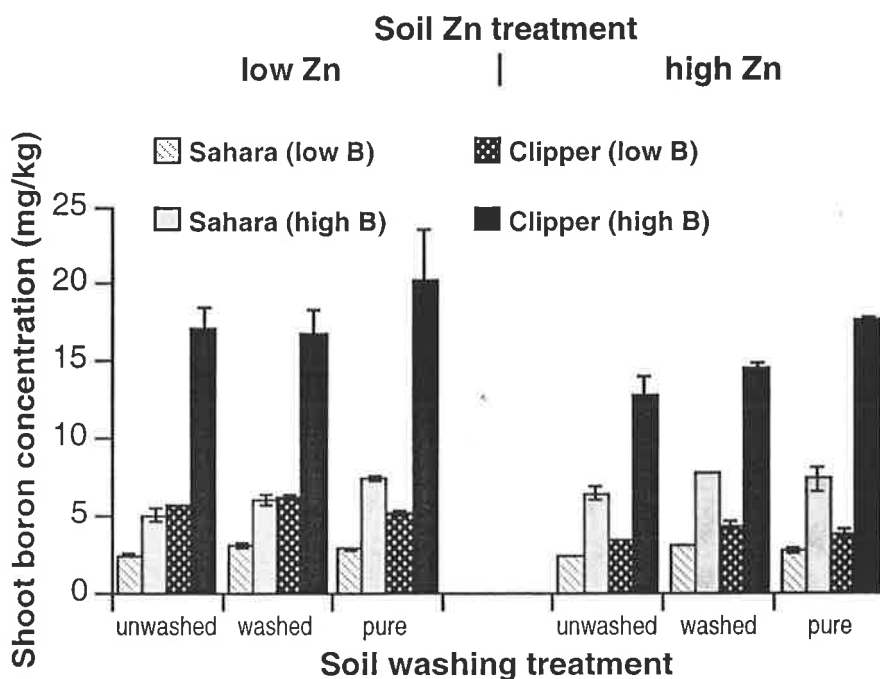
Figure 3.3d

Effect of washing treatment, applied zinc (1.6 mg Zn/kg soil or 0 mg Zn/kg soil) and applied boron (0.175 mg B/kg soil or 0.018 mg B/kg soil) on shoot boron concentrations of barley varieties Clipper and Sahara grown in Laffer sand for 42 days. Data represent the average of two replicates with standard errors shown.

As expected, shoot copper concentrations were reduced in comparison to those in plants grown in Experiments 3A and 3B (see Appendix 2). Concentrations were still high at Zn_0 but at $Zn_{1.6}$ shoot concentrations ranging from 5.5-7.9 mg/kg for Sahara and 7.0-9.8 mg/kg for Clipper were within acceptable limits (Reuter *et al.*, 1997). Shoot concentrations of all other elements measured, with the exception of calcium, were reduced compared to those obtained in the two previous experiments (see Appendix 2).

The effect of washing soil was quite dramatic as evidenced by differences in elemental shoot concentrations between unwashed soil and either of the washed treatments, particularly at low levels of Zn. Shoot concentrations of iron, copper, magnesium, sodium and potassium were all higher in plants grown in the unwashed treatment. Shoot concentrations of calcium, phosphorus and sulphur remained approximately the same between soil treatments while manganese was the exception, decreasing for the unwashed treatment.

3.5 Experiment 3D - Further refinement of the zinc efficiency bioassay

3.5.1 Materials and methods

Results of Experiment 3C suggested the level of H_3BO_3 added in this bioassay was too high. Although there was no effect of boron level on shoot dry weight, it obviously contributed to the development of foliar symptoms. For this reason the low boron treatment was considered to be a more appropriate level of H_3BO_3 to use in future testing. The fact that some symptoms persisted even at B_{low} was of concern and may have been caused by inappropriate levels of a hitherto unforeseen element.

In the time between conducting Experiments 3C and 3D, improvements in the capability of ICPAES analysis made it possible to analyse shoot tissue for the element molybdenum (Mo). A screening experiment detailed in Chapter 4 (Experiment 4A) was the first experiment in which barley shoots were analysed for Mo and revealed extremely high concentrations (40-90 mg/kg). Adequate levels for whole shoots are considered to be in the range 0.1 - 0.3 mg/kg (Reuter *et al.*, 1997), and although scant information regarding Mo toxicity in barley exists, the symptoms are generally characterised by chlorosis, yellowing and other leaf discolouration (Gupta, 1997). With this in mind, together with concern at the high concentrations of K (see Appendix 2), a final effort was made to further improve the bioassay. Potassium levels in the soil were reduced by decreasing the amounts of KH_2PO_4 and K_2SO_4 added, while Mo was eliminated from the nutrient mixture.

Soil was sieved, washed and dried as per Experiment 3C (pure washed treatment)

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before being thoroughly mixed with calcium carbonate powder (0.3% w/w). The following nutrients were then added: (mg salt/kg dry soil) NH_4NO_3, 350; KH_2PO_4, 120; K_2SO_4, 80; $MgSO_4.7H_2O$, 90 $MnSO_4.4H_2O$, 7; $CuSO_4.5H_2O$, 5; NaCl, 4.2; $CoSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 0.7; $NiSO_4.7H_2O$, 0.15; H_3BO_3, 0.1.

The soil was then divided into 3 equal portions and $ZnSO_4.7H_2O$ added to correspond to the following treatments: (mg Zn/kg dry soil) 0, 0.05 and 1.6 hereafter referred to as Zn_0, $Zn_{0.05}$ and $Zn_{1.6}$. Seeds of Sahara and Clipper, together with two of the doubled haploid lines, all with similar Zn contents, were sown as in previous experiments. DH125 and DH51 were chosen as low and high Zn accumulators, respectively (based on results of Experiment 5B). Due to limited seed reserves the experiment was set up as a randomised block with unequal replicates comprising 4 varieties x 3 Zn treatments x (3 replicates for parents and 2 replicates for doubled haploids).

Data were analysed as a two-way analysis of variance with genotype and Zn treatment forming the relevant factors. As previously, values for Zn concentration and Zn content in shoots were log transformed to overcome problems associated with non-homogeneity of variances. Pairwise comparisons of means were made using Tukey-Kramer's Honestly Significant Difference (HSD) for unequal replicates at $\alpha = 0.05$ (Zar, 1996).

3.5.2 Results

Visual symptoms

Foliar symptoms of Zn deficiency similar to those observed in previous experiments first appeared on variety Clipper and DH125 at Zn_0 at approximately 16 days after sowing, closely followed by $Zn_{0.05}$ at 18 days. Zinc deficiency symptoms on variety Sahara and DH51 appeared at 26 days and 29 days respectively for Zn_0, and 29 days and 33 days respectively for $Zn_{0.05}$. No symptoms were observed at $Zn_{1.6}$ for any genotype.

Reduction in shoot weight between $Zn_{0.05}$ and Zn_0 appeared similar for Sahara and the doubled haploid lines but less for Clipper. Both doubled haploid lines appeared to

decrease in top growth between $Zn_{0.05}$ and $Zn_{1.6}$ whereas their parents were unchanged.

The foliar symptoms initially attributed to B toxicity and later thought to involve Mo toxicity noted in Experiments 3A-C were absent from all Zn treatments.

Shoot dry weights

Total shoot dry weight was strongly ($P<0.0001$) affected by both genotype and Zn treatment (see Appendix 1 (Section 3.4) for ANOVA table). There was also a significant interaction. Shoot dry weights of all genotypes increased significantly from Zn_0 to $Zn_{0.05}$ (Figure 3.4a). The percentage increase was similar for Sahara and both doubled haploid lines, ranging from 36% to 45 %, but only 19% for Clipper, again suggesting it is more Zn efficient than Sahara. There was no significant change in shoot dry weight between $Zn_{0.05}$ and $Zn_{1.6}$ for Sahara and Clipper but in contrast to their parents, both doubled haploids recorded significant decreases.
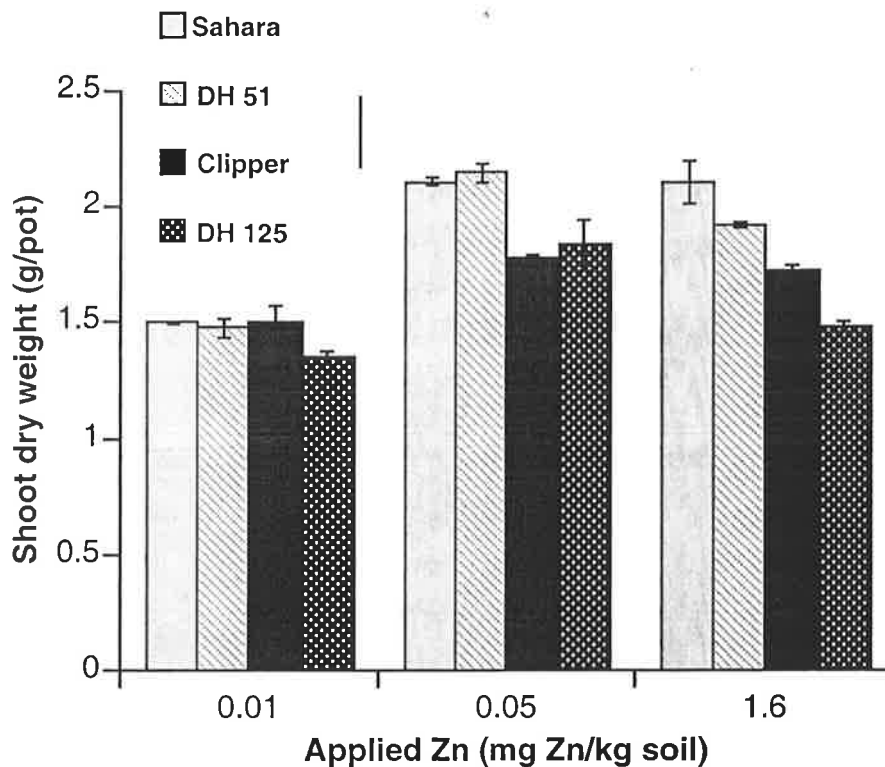
Figure 3.4a

Effect of soil applied zinc on shoot dry weight of barley varieties Clipper, Sahara and two doubled haploid lines derived from a cross between these two varieties. Plants were grown in Laffer sand for 42 days. Values are a mean of either 3 replicates (Sahara and Clipper) or two replicates (DH 51 and DH 125) with standard errors shown. The vertical line represents Tukey-Kramer's $HSD_{0.05}$ for the genotype x zinc interaction.

Shoot Zn concentrations (Figure 3.4b) increased slightly between Zn_0 and $Zn_{0.05}$ with no significant difference between genotypes within a treatment (see Appendix 1 (Section 3.4) for ANOVA table of log transformed data). At $Zn_{1.6}$, however, shoot Zn concentrations increased sharply with variation among genotypes, as shown in Table 3b.
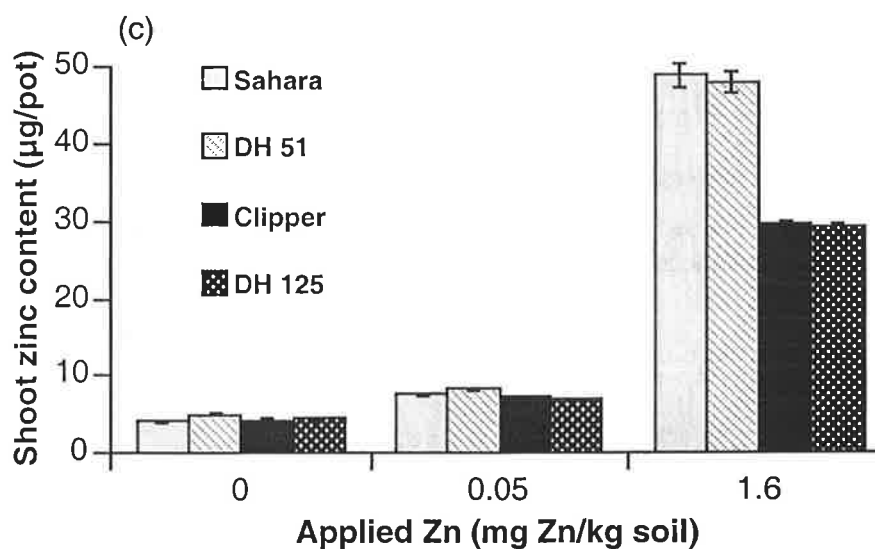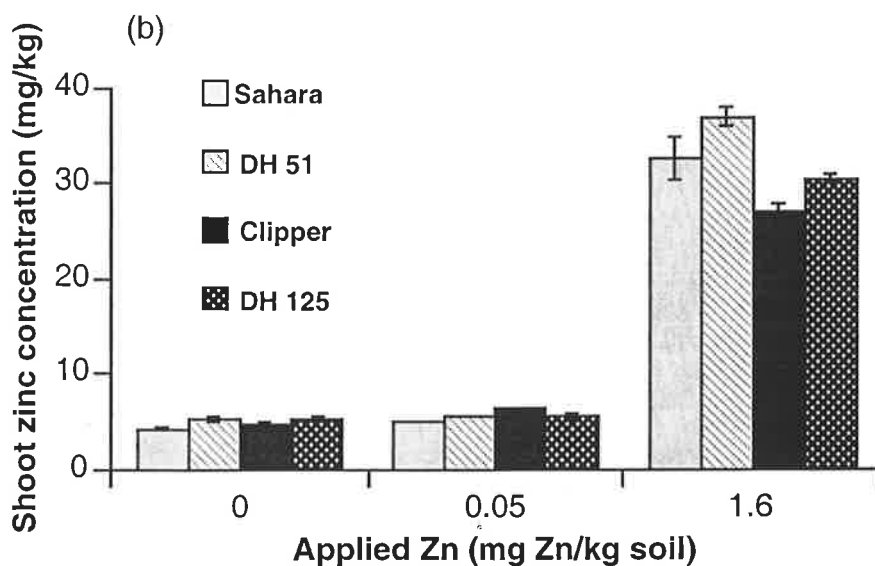
Figures 3.4b and c

Effect of applied zinc on shoot zinc concentration and content of barley varieties Clipper and Sahara and two doubled haploid lines derived from a cross between these two varieties. Plants were grown in Laffer sand for 42 days. Values are a mean of either 3 replicates (Clipper and Sahara) or two replicates (DH 51 and DH 125) with standard errors shown.

Shoot Zn contents (Figure 3.4c) were strongly ($P<0.0001$) affected by both Zn

treatment and genotype (see Appendix 1 (Section 3.4) for ANOVA table of log transformed data). There was no difference between genotypes at Zn_0 or $Zn_{0.05}$ but, at $Zn_{1.6}$, Sahara and DH51 were significantly greater in Zn content than Clipper and DH125 (Table 3c). Sahara and DH51 recorded similar values to each other as did Clipper and DH125.

Table 3b

Log transformed values of shoot zinc concentration for the 4 barley genotypes used in Experiment 3D. Values are an average of either 3 replicates (Sahara and Clipper) or 2 replicates (DH51 and DH125) with standard errors shown. Natural averages appear in brackets. The $HSD_{0.05}$ value is applicable to log-transformed data only.

Genotype	log shoot Zn concentration		
	Zn_0	$Zn_{0.05}$	$Zn_{1.6}$
DH51	0.72±0.03 (5.25)	0.75±0.01 (5.62)	1.57±0.01 (37.15)
Sahara	0.63±0.02 (4.27)	0.70±0.01 (5.01)	1.51±0.03 (32.36)
DH125	0.73±0.02 (5.37)	0.75±0.02 (5.62)	1.48±0.01 (30.20)
Clipper	0.67±0.03 (4.68)	0.81±0.01 (6.46)	1.43±0.02 (26.92)

Tukey-Kramer $HSD_{0.05}$ = 0.111 for genotype x Zn treatment

Table 3c

Log transformed values of shoot zinc content for the 4 barley genotypes used in Experiment 3D. Values are an average of either 3 replicates (Sahara and Clipper) or 2 replicates (DH51 and DH125) with standard errors shown. Natural averages appear in brackets. The $HSD_{0.05}$ value is applicable to log-transformed data only.

Genotype	log shoot Zn content		
	Zn_0	$Zn_{0.05}$	$Zn_{1.6}$
DH51	0.89±0.02 (7.76)	1.08±0.001 (12.02)	1.85±0.01 (70.79)
Sahara	0.81±0.01 (6.46)	1.03±0.01 (10.72)	1.84±0.01 (69.18)
DH125	0.86±0.01 (7.24)	1.01±0.01 (10.23)	1.65±0.01 (44.67)
Clipper	0.85±0.01 (7.08)	1.06±0.01 (11.48)	1.67±0.01 (46.77)

Tukey-Kramer $HSD_{0.05}$ = 0.055 for genotype x Zn treatment

3.6 Discussion

A perfect bioassay is one in which the variable of interest can be manipulated while maintaining all other factors at optimal levels. While this is possible in theory, it is rarely achieved in practice when operating with nutrients since they interact with many physical and chemical factors as well as with each other. Despite this fact, attempts were made to improve the bioassay employed in this study through manipulation of the growing medium. Ideally, it would have been desirable to change only one parameter at a time but due to constraints of limited time and resources this was not practical.

The most obvious problem initially, appeared to be B toxicity. The fact that only one genotype was affected compounded the problem since this was a comparative study. The diagnosis was based on the characteristic symptoms of B toxicity and the difference in response between Clipper and Sahara which had previously been classified as susceptible and tolerant respectively to high B (Jenkin, 1993; Jefferies et. al., 1999). Thus, it was important to remove B toxicity from this bioassay.

The first attempt to achieve this through leaching (Experiment 3B) was not completely successful in that B toxicity symptoms remained. This led to the suspicion that the water and not the soil was the source of B, leading to a study of the quality of water used for washing and watering (Experiment 3C). Even so, shoot B concentrations in plants grown in Experiment 3B were reduced appreciably for Sahara across all Zn treatments and for Clipper at Zn_0 and $Zn_{0.05}$. This reduction in B concentrations at low levels of soil applied Zn coincided with an increase in Clipper's shoot dry weight between Experiment 3A and Experiment 3B. It would appear that there was indeed an effect of B toxicity upon Clipper's growth but only under Zn deficiency. This is consistent with nutrient solution studies by Graham et al. (1987) with barley and Swietlik (1995) with orange seedlings where low Zn treatments enhanced B concentrations in shoots to toxic levels. The same effect was also found in soil culture studies (Singh *et al.*, 1990) where Zn deficiency increased B concentrations in wheat.

The effect of B on shoot growth was not evident in Experiment 3C, there being no

significant difference between B treatments. This was most probably due to the fact that B levels had by this time been further reduced from Experiment 3B by using a purer water source for watering plants. This decreased level was still sufficient enough to produce B toxicity symptoms but not high enough to affect shoot growth. This discrepancy between symptoms and shoot growth can be explained by studies of Jefferies et al. (1999) who found that the locus most important in controlling leaf symptom expression of B toxicity in barley was not linked to that controlling dry matter response.

It would appear that the same is true for Zn deficiency. Although Sahara was shown to be less efficient in growth under Zn deficient conditions than Clipper, its foliar symptoms did not reflect this. Typical symptoms of chlorosis followed by necrosis appeared later and were less severe than for Clipper. Although it was never intended in this study that foliar symptoms would be quantified in any way as a measure of Zn deficiency, it is interesting to note the disparity between foliar symptoms and relative growth at 42 days after sowing for these two varieties. This occurrence takes on more importance in a field situation where initial visual assessment could be quite misleading. Relative shoot growth, ie. a comparison of growth at both Zn deficiency and Zn sufficiency as proposed by Graham (1984), is a far more effective means of evaluating a genotype's response to Zn deficiency. By using this method, the genetic or maximum growth potential of a variety is taken into account. Genetic differences that would confound results if viewed only under conditions of Zn deficiency are thereby corrected for and comparisons between genotypes can easily be made. Even so, this index may not fully reflect the efficiency of a genotype based on grain yield criteria.

It appeared that Mo toxicity was also playing some role in the expression of leaf symptoms. Although Mo concentrations in shoots were not measured in Experiments 3A,B and C, the data from Chapter 4 suggest that concentrations would have been up to 300 times greater than the recommended adequate levels. Virtually no data regarding the incidence of Mo toxicity in barley exists but symptoms in other species are generalised by chlorosis and leaf discolouration that starts in the youngest leaves (Bergmann, 1992; Gupta, 1997). The similarity to B toxicity symptoms made identification impossible until

Mo concentrations were measured. The lack of symptoms in plants grown in Experiment 3D where $H_2MoO_4.H_2O$ was omitted seems to confirm that Mo toxicity had been occurring but was previously masked or compounded by B toxicity.

The consistent result across experiments is Clipper's greater Zn efficiency compared to Sahara. Apart from Experiment 3A, where results were variable and confusing, all other experiments show that shoot dry weight of Sahara decreased by a greater percentage than Clipper when plants were subject to Zn deficiency. Some clue as to the mechanism of efficiency may be provided by the relationship between shoot Zn concentration and YEB Zn concentration observed in Experiments 3A and 3B. Sahara's YEB Zn concentration increased linearly with soil applied Zn whereas Clipper increased up to a certain level before reaching a plateau. One explanation for this would be that Sahara has a greater Zn requirement than Clipper and Zn is continually transported to young leaves. Clipper, however, having a lower requirement, begins to retranslocate Zn, having reached a maximum threshold. This appears to be the case at Zn sufficiency but under Zn deficiency a different scenario emerges. Sahara's shoot weight decreased at a greater rate than Clipper as Zn supply was progressively decreased below the optimal. Graham and Rengel (1993) suggest that differences in efficiency could be explained by more efficient utilization and compartmentalization of Zn within plant cells, tissues and organs. It could be that Clipper is more efficient in Zn utilization and therefore requires less than Sahara.

The results of Experiment 3D show that each genotype potentially has its own unique response curve to changes in soil Zn status. This makes measurement of Zn efficiency of a large population extremely difficult as each individual achieves maximum yield at a different level of soil applied Zn. To use two levels of Zn only would give false indications of Zn efficiency. Such data, subjected to linkage analysis in order to map chromosomal positions of Zn efficiency genes could be misleading. This, together with the fact that the seed Zn content of individuals of the Sahara x Clipper doubled haploid population differ markedly (see Chapter 7) casts doubt on the accuracy of mapping the Zn efficiency trait. However, since the purpose of the bioassay is to provide a correlation with

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field results, it may be possible to use levels of soil applied Zn that correspond with the levels experienced in the field situation. This would require only two levels of Zn (equivalent to +Zn and nil) and results generated in this manner are more likely to relate to field Zn efficiency.

Chapter 4

Screening of Additional Parental Lines for Vegetative Zinc Efficiency and their Correlation to Yield Efficiency

4.1 Introduction

A further set of potential parents were screened for Zn efficiency in order to see if they could be used in mapping studies. Suitability was determined in the first instance by the magnitude of difference in Zn efficiency between genotypes as well as ready availability of F1 seed derived from the cross. If deemed suitable, the F1 seed could be multiplied under standard conditions to provide an F2 mapping population. In the second experiment, parents of established doubled haploid populations were chosen in order to save time on construction of a linkage map.

The vegetative screening tests described in this chapter were conducted in conjunction with those described in the latter part of Chapter 3. As a result, nutrient levels of the soil medium used were different between Experiment 4A and 4B owing to continuing refinement of the bioassay. In addition, grain yield results from field based trials were compared to vegetative Zn efficiency to determine whether the assay is a true predictor of grain yield under Zn deficiency. Field based screening has been the most popular method of measuring Zn efficiency in terms of grain yield but has a number of associated problems. These include the inherent variability of Zn deficiency across any given site, large seasonal variations and large inputs of time, labour and money in conducting large scale field trials. It is important, therefore, that any bioassay that attempts to measure Zn efficiency at the vegetative stage is closely correlated to grain yield efficiency.

4.2 Materials and methods

4.2.1 Experiment 4A - Measuring vegetative zinc efficiency of parental barley lines (F1 populations)

The varieties tested were based on the ready availability from the Adelaide University barley breeding program of a large number of F1 seeds from any particular cross. These crosses were as follows-

Amagi nijo x WI2585, Amagi nijo x Galleon, Amagi nijo x Haruna nijo, Amagi nijo x WA73S276, Amagi nijo x Weeah, WA73S276 x WI2585 and Haruna nijo x Galleon.

Due to the difficulty of obtaining seed of all varieties with similar seed Zn content, two sources of the variety Amagi nijo were used (Table 4.2a) so that reasonable comparisons could be made between varieties. This avoided confounding effects caused by different seed Zn contents (Rengel and Graham, 1995a; Genc et $al.$, 2000).

Table 4.2a

Seed zinc content of six* barley varieties screened for vegetative zinc efficiency.

Genotype	Seed zinc content (μg/seed)
*Amagi nijo 30/3	2.22
Galleon	2.14
Haruna nijo	2.34
Weeah	2.37
*Amagi nijo 3	1.62
WA 73S276	1.65
WI 2585	1.63

*2 selections of Amagi nijo were used so that comparisons could be made between genotypes with similar seed zinc content. The selection nos. '30/3' and '3' distinguish between the sources used.

The soil used was the same Laffer sand used in the experiments of Chapter 3. Soil preparation was similar to that used for Experiment 3B (see Chapter 3) except that the

amount of added H_3BO_3 was reduced in order to avoid boron toxicity. The sand was sieved through a 2mm stainless steel sieve, washed 10 times with deionised water and air dried in a glasshouse. Calcium carbonate powder (0.3% w/w) was mixed thoroughly into dry soil before the following nutrients were added: (mg salt/kg dry soil) NH_4NO_3, 350; KH_2PO_4, 150; K_2SO_4, 120; $MgSO_4.7H_2O$, 90; $CuSO.5H_2O$, 10; $MnSO_4.4H_2O$, 7; $CoSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 0.7; $H_2MoO_4.H_2O$, 0.5; $NiSO_4.7H_2O$, 0.15; H_3BO_3, 0.1.

The soil was divided into three equal portions and $ZnSO_4.7H_2O$ added to correspond to the following treatments (mg Zn/kg dry soil) 0, 0.05 and 1.6 hereafter referred to as Zn_0, $Zn_{0.05}$ and $Zn_{1.6}$.

Clear plastic sample jars (6.5cm diameter x 15cm height) covered with aluminium foil and lined with a plastic bag were filled with soil (equivalent to 650g dry weight) and allowed to equilibrate overnight. Seeds of the six barley varieties (kindly supplied by Ms M. Pallotta) were surface-sterilised by soaking in 70% ethanol for 1 minute, rinsed with deionised water and then soaked in sodium hypochlorite (1.4% v/v) for 6 minutes before rinsing again with deionised water. The seed was germinated on moistened Whatman No. 42, ashless filter paper for 36 hours at $20^{\circ}C$ before sowing (3 seeds per pot).

Plants were grown in a controlled environment growth chamber with a 12-hour light/12-hour dark photoperiod at $20^{\circ}C$ day/$10^{\circ}C$ night. Light was supplied by high-pressure mixed-metal halide lamps delivering a photon flux density of 500 $\mu mol/m^2/s$ at the surface of the pot. The experiment was set up as a completely randomised block and pots were rotated within a block every few days to minimise the effect of microenvironments caused by uneven light distribution and/or air flow. Pots were watered daily and watered to weight every 3 days to maintain the soil moisture at 12% w/w.

Due to seed availability, only two replicates of each genotype x treatment combination were possible. The design was thus 6+1 genotypes x 3 treatments x 2 replicates = 42 pots. After 6 weeks whole shoots were harvested and oven-dried at 70ºC for 24 hours. Dried samples were digested with 70% nitric acid and analysed for

concentrations of the elements Zn, Mn, Fe, Cu, Mo, B, Ca, Mg, Na, K, P and S by ICPAES.

Measurements of vegetative Zn efficiency were calculated as either

(a) [(shoot weight (Zn_0)/shoot weight $(Zn_{1.6})$) x 100] <u>or</u>

(b) [(shoot weight $(Zn_{0.05})$/shoot weight $(Zn_{1.6})$) x 100].

Data were analysed as a two-way analysis of variance using the computer program SuperANOVA^{TM}. The factors for the ANOVA model were Zn level and genotype. The least-square means for Zn efficiency were calculated using this model and pairwise comparisons made between parental genotypes. To overcome problems associated with non-homogeneity of variances, values for Zn concentration and content were log-transformed prior to statistical analysis.

4.2.2 Experiment 4B - Measuring vegetative zinc efficiency of parental barley lines (doubled haploid populations)

In order to try to save time and resources on linkage map construction it was decided to test the parents of some doubled haploid populations that had either been mapped or were in the process of being mapped by other research groups. The crosses from which parent selection was made were as follows- Chebec x Harrington, Chebec x Haruna nijo, Amagi nijo x Chebec and WA73S276 x Harrington. Seeds of the 5 genotypes with similar zinc contents (Table 4.2b) were kindly supplied by Mr J. Lewis.

Soil and seed preparation, growing conditions and harvest were identical to those described for Experiment 3D (see Chapter 3). Due to seed availability, either 2 replicates (Chebec, Haruna nijo and Harrington) or 3 replicates (Amagi nijo and WA73S276) were used. The design was thus - [(3 genotypes x 2 reps) + (2 genotypes x 3 reps)] x 3 treatments = 36 pots.

Vegetative Zn efficiency was calculated as for Experiment 4A. Data were analysed as a two-way analysis of variance with genotype and Zn treatment forming the relevant factors. The least-square means for Zn efficiency were calculated using this model and

pairwise comparisons made between parental genotypes. To overcome problems associated with non-homogeneity of variances, values for Zn concentration and content were log-transformed prior to statistical analysis.

Table 4.2b

Seed zinc content of 5 barley varieties tested for vegetative zinc efficiency.

Genotype	Seed zinc content (µg/seed)
Amagi nijo	1.85
Chebec	1.70
Haruna nijo	1.72
Harrington	1.83
WA73S276	1.79

4.2.3 Experiment 4C - Correlation of vegetative efficiency with grain yield efficiency

In order to determine the correlation between vegetative efficiency and grain yield efficiency, a selection of genotypes was examined in two field trials conducted on Zn deficient soils. The trials were split-plot designs set up as completely randomised blocks with genotype forming the main plot effect and Zn treatment the split plot effect. Each split plot consisted of 6 rows, 5m long and 15cm apart. Seed was planted with a cone seeder at a depth of approximately 25-30mm and a rate of 180 seeds/m². Both field trials were conducted during the growing season of 1998, one at Lameroo in the mallee region of South Australia and one at Birchip in the mallee region of Victoria. DTPA-extractable Zn at the Lameroo site was 0.27 mg/kg and 0.18 mg/kg at Birchip. The Lameroo trial had 4 replicates and the Birchip trial 3. The varieties chosen for observation were those used in Experiments 4A and 4B as well as the common commercial varieties Sloop and Arapiles.

At sowing, Zn was supplied to one half of the main plot as Zn oxysulphate granules at a rate of 7kg Zn/ha delivered directly with the seed. The basal fertiliser used was 'Hifert™ Special Mix' (see Appendix 3 for analysis) supplied at seeding to both halves of the main plot at a rate of 175 kg/ha. The Lameroo trial was sown on 28 May 1998 and

harvested on 27 November 1998. Weeds were controlled by spraying Hoegrass™ at a rate of 1l/ha on 24 July 1998 and 2-4-D at a rate of 1l/ha plus Lontrel™ at 100 ml/ha on 24 August 1998. The Birchip site was sown on 11 June 1998 and harvested on 2 December 1998. Weeds were controlled by spraying Bromoxynil/MCPA at a rate of 1l/ha on 28 July 1998. Foliar applications of Zincsol™ were applied to the Zn treated plots only at a rate of 334 g Zn/ha at 7 weeks after sowing and again at 14 weeks after sowing. Plots were reduced to a length of 4.76m before harvesting with a Wintersteiger™ plot harvester.

Genotypes were scored for vegetative efficiency at the time of the first foliar application. This score was based on a visual assessment of the difference in growth between treatments and had a scale of 0-5 where

0= no difference (ie. 100% efficiency)

1= 0-10%

2= 10-20%

3= 20-40%

4= 40-60%

5= >60%

The visual score for each genotype was correlated against final grain yield efficiency to observe if vegetative efficiency is a close predictor of yield efficiency. It was also possible to observe if cultivar rankings of vegetative efficiency in the field correlated to rankings in the bioassay.

4.3 Results

4.3.1 Experiment 4A

<u>Visual Symptoms</u>

Foliar symptoms of Zn deficiency including pale yellow chlorotic areas on middle leaves followed by dark brown necrosis were first observed at approximately 20 days after sowing in both sources of variety Amagi nijo at Zn_0. Similar symptoms were noticed on Haruna nijo at Zn_0 after 23 days and on Weeah and WI2585 at approximately 32 days. The symptoms were more severe at Zn_0 than $Zn_{0.05}$. Galleon and WA73S276 exhibited few symptoms other than a slight yellowing of foliage at Zn_0. By harvest Amagi nijo appeared

far more affected than any other variety and Amagi Nijo 3 appeared to be slightly worse than Amagi Nijo 30/3. Shoot growth reduction appeared similar for all genotypes.

Shoot dry weights

Shoot dry weights were strongly ($P \leq 0.0001$) affected by genotype and Zn treatment (see Appendix 1 (Section 4.1) for ANOVA table). There was also an interactive effect. All genotypes showed a significant increase in shoot weight between Zn_0 and $Zn_{0.05}$, but either increased, decreased or remained unchanged between $Zn_{0.05}$ and $Zn_{1.6}$ (Figure 4.1a). Amagi nijo 30/3, Galleon and Haruna nijo appear to have achieved maximum growth at $Zn_{0.05}$ with no significant increase in weight at the higher Zn level. WA73S276, by contrast, while also achieving maximum growth at $Zn_{0.05}$ had decreased yield at $Zn_{1.6}$. Amagi nijo 3, Weeah and WI2585 show a progressive increase in shoot weight with increasing applied Zn reaching greatest yield at $Zn_{1.6}$. It is interesting to note that the same genotype (Amagi nijo) achieves greater dry shoot weight with a higher starting Zn content, particularly at Zn_0.

Comparisons of Zn efficiency were made between parents of the various populations (Table 4.3a) to identify which parents differed and at what rate of Zn nutrition the difference was expressed. There was a highly significant ($P \leq 0.01$) difference between Amagi nijo and Galleon expressed at Zn_0. Amagi nijo was also significantly different ($P \leq 0.05$) from WA73S276, Haruna nijo and Weeah all expressed at Zn_0. The only other parental comparison that was significantly different was between WA73S276 and WI2585 expressed at $Zn_{0.05}$.

Shoot zinc concentrations and contents

Shoot Zn concentration was strongly ($P \leq 0.0001$) affected by both genotype and Zn treatment (see Appendix 1 (Section 4.1) for ANOVA table of log-transformed data). There was little change between Zn_0 and $Zn_{0.05}$ but a large increase at $Zn_{1.6}$ (Figure 4.1b). Analysis of log-transformed data showed that only 1 genotype, WI 2585, increased significantly between Zn_0 and $Zn_{0.05}$, whereas Zn concentrations for all genotypes were greatly increased between $Zn_{0.05}$ and $Zn_{1.6}$ (Table 4.3b).

Similar results were noted for shoot Zn content (Figure 4.1c). Analysis of log-transformed data showed that there was a significant increase in shoot Zn accumulation for all varieties not only between $Zn_{0.05}$ and $Zn_{1.6}$ but also between Zn_0 and $Zn_{0.05}$ (Table 4.3c) due to large differences in shoot dry weight.
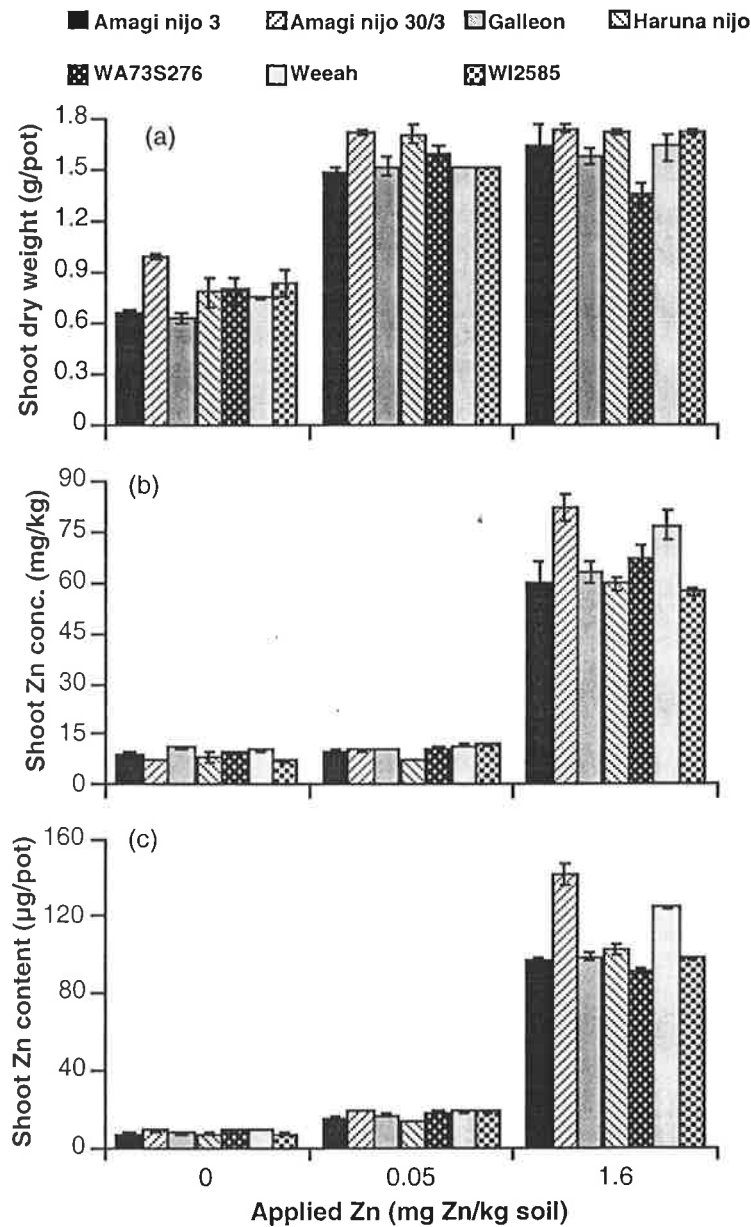
Figures 4.1a, b and c

Effects of applied zinc on (a) shoot dry weight (b) shoot zinc concentration and (c) shoot zinc content in 6* barley varieties grown in Laffer sand for 42 days. Values are an average of two replicates with standard errors shown.

* Two sources of Amagi nijo differing in seed zinc content were used.

Table 4.3a

P-values for pairwise comparisons between barley varieties for vegetative zinc efficiency expressed at soil zinc levels Zn_0 and $Zn_{0.05}$. Efficiency was calculated as either [(yield (Zn_0)/yield ($Zn_{1.6}$)) x 100] or [(yield ($Zn_{0.05}$)/yield ($Zn_{1.6}$)) x 100]. Comparisons have only been made between parents of existing doubled haploid populations.

Genotype (treatment)	Vs	P-value
Amagi nijo 3 (0)	WA73S276 (0)	0.033
	WI2585 (0)	0.110
Amagi nijo 3 (0.05)	WA73S276 (0.05)	0.060
	WI2585 (0.05)	0.521
Amagi nijo 30/3 (0)	Galleon (0)	0.002
	Haruna Nijo (0)	0.026
	Weeah (0)	0.049
Amagi nijo 30/3 (0.05)	Galleon (0.05)	0.577
	Haruna Nijo (0.05)	0.847
	Weeah (0.05)	0.174
Galleon (0)	Haruna Nijo (0)	0.252
Galleon (0.05)	Haruna Nijo (0.05)	0.455
WA73S276 (0)	WI2585 (0)	0.541
WA73S276 (0.05)	WI2585 (0.05)	0.015

Table 4.3b

Effects of applied zinc on shoot zinc concentrations of 6* barley varieties grown in Laffer sand for 42 days. Values are an average of 2 replicates. * Two sources of Amagi nijo differing in seed zinc content were used.

Applied zinc (mg/kg)	Shoot zinc concentration (mg/kg)						
	Amagi Nijo 3	Amagi Nijo 30/3	Galleon	Haruna nijo	WA73S276	Weeah	WI2585
0	9.8 (0.99)[a]	7.9 (0.90)	11.3 (1.05)	8.5 (0.92)	10.1 (1.01)	10.8 (1.03)	7.7 (0.89)
0.05	10.2 (1.01)	10.7 (1.03)	10.9 (1.04)	7.7 (0.88)	11.0 (1.04)	11.8 (1.07)	11.9 (1.08)
1.6	59.7 (1.77)	81.7 (1.91)	63.0 (1.80)	59.3 (1.77)	67.1 (1.83)	76.5 (1.88)	57.0 (1.76)

Tukey's $HSD_{0.05}$[b]

Genotype x zinc treatment   (0.15)

[a]Numbers in parentheses refer to averages of log-transformed data.

[b]The $HSD_{0.05}$ value is applicable to log-transformed data only.

Table 4.3c

Effects of applied zinc on shoot zinc contents of 6* barley varieties grown in Laffer sand for 42 days. Values are an average of 2 replicates. * Two sources of Amagi nijo differing in seed zinc content were used.

Applied zinc (mg/kg)	Shoot zinc content (µg/pot)						
	Amagi Nijo 3	Amagi Nijo 30/3	Galleon	Haruna nijo	WA73S276	Weeah	WI2585
0	6.5 (0.81)[a]	7.9 (0.90)	7.1 (0.85)	6.6 (0.82)	8.1 (0.91)	8.2 (0.92)	6.5 (0.81)
0.05	15.2 (1.18)	18.4 (1.26)	16.6 (1.22)	13.1 (1.12)	17.6 (1.25)	17.8 (1.25)	18.0 (1.26)
1.6	97.2 (1.99)	141.8 (2.15)	99.1 (2.00)	102.5 (2.01)	91.2 (1.96)	124.6 (2.10)	98.1 (1.99)

Tukey's $HSD_{0.05}$[b]

Genotype x zinc treatment   (0.12)

[a]Numbers in parentheses refer to averages of log-transformed data.

[b]The $HSD_{0.05}$ value is applicable to log-transformed data only.

Other elements

This was the first experiment in this research project to be analysed for the element molybdenum. Shoot concentrations were in the range 40 - 90 mg/kg. As reported in Chapter 3 (see Section 3.2.4) this is far greater than the sufficiency range for this element, considered to be 0.09 - 0.18 mg/kg (Gupta, 1971).

Concentrations of Fe in shoots were greatly increased under Zn deficiency, particularly for WA73S276 and Weeah (Figure 4.2a) with Haruna nijo accumulating the

least amount of Fe. Shoot Cu concentrations increased under zinc deficiency for Galleon, WA73S276 and Weeah but remained similar for the other genotypes (Figure 4.2b). Shoot concentrations of P (Figure 4.3a) and S (Figure 4.3b) increased under Zn deficiency for all genotypes.
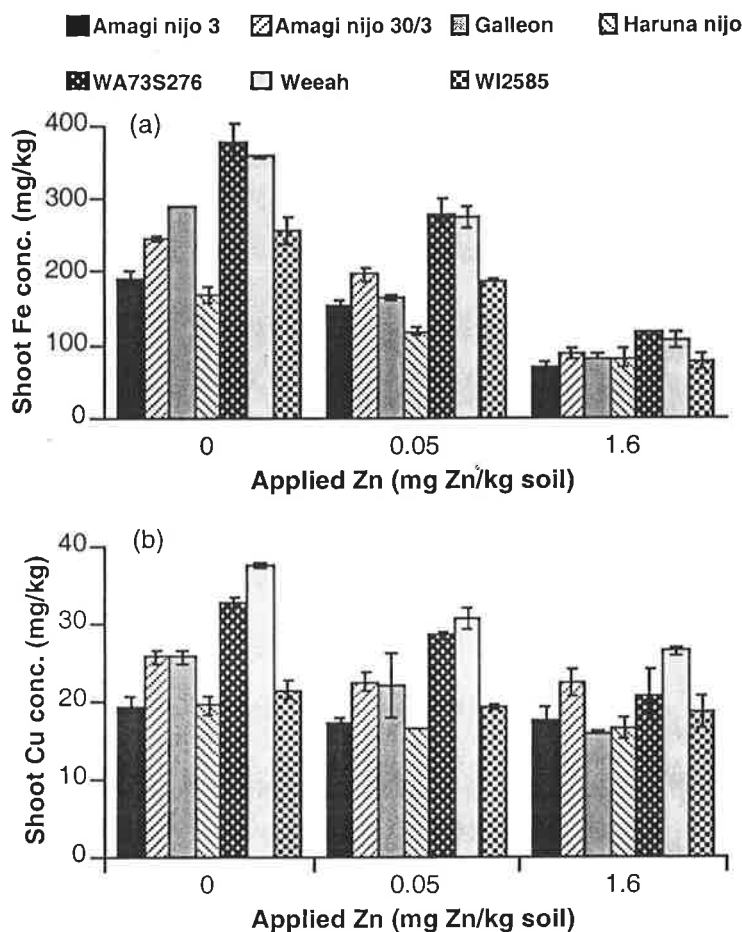
Figures 4.2a and b

Effects of applied zinc on (a) shoot iron concentration and (b) shoot copper concentration in 6* barley varieties grown in Laffer sand for 42 days. Values are an average of two replicates with standard errors shown.

* Two sources of Amagi nijo differing in seed zinc content were used.

Legend: ■ Amagi nijo 3 ▨ Amagi nijo 30/3 ▨ Galleon ▨ Haruna nijo ▨ WA73S276 □ Weeah ▨ WI2585
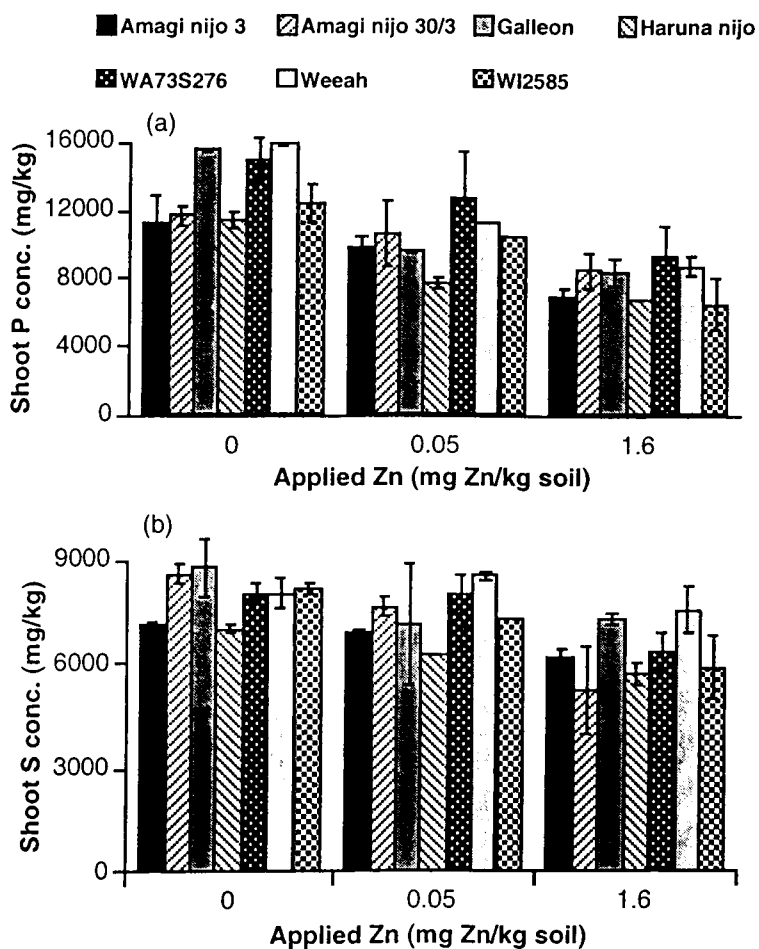
Figures 4.3a and b

Effects of applied zinc on (a) shoot phosphorus concentration and (b) shoot sulphur concentration in 6*

barley varieties grown in Laffer sand for 42 days. Values are an average of two replicates with standard

errors shown. * Two sources of Amagi nijo differing in seed zinc content were used.

4.3.2 Experiment 4B

Visual Symptoms

Foliar symptoms of Zn deficiency were most notable on Amagi nijo and Haruna

nijo at Zn_0. Symptoms started at approximately 18-20 days on the mid leaves and

progressively worsened, spreading to younger leaves. The same two varieties at $Zn_{0.05}$

exhibited foliar symptoms at approximately 30-32 days. At harvest, the symptoms

appeared to be more developed in Amagi nijo than Haruna nijo. In contrast, Chebec and

Harrington showed few foliar symptoms other than occasional chlorotic spots and a

general yellowing at the lower Zn treatments. WA73S276 showed no obvious foliar

symptoms. Although foliar symptoms varied widely, total shoot growth reduction caused

by Zn deficiency appeared similar for all genotypes (although the dry weights differed – see below).

Shoot dry weights

Shoot dry weights were strongly ($P \leq 0.0001$) affected by both genotype and Zn treatment (see Appendix 1 (Section 4.2) for ANOVA table). All genotypes showed an increase in shoot weight between Zn_0 and $Zn_{0.05}$ but either increased (Amagi nijo, Haruna nijo and WA73S276) or decreased (Chebec and Harrington) between $Zn_{0.05}$ and $Zn_{1.6}$ (Figure 4.4a). Curiously, shoot weights were similar between genotypes at $Zn_{0.05}$ but significantly different at both Zn_0 and $Zn_{1.6}$.

As previously, comparisons of Zn efficiency were made between parents of each population (Table 4.3d) to identify which parents differed from each other and at what rate of applied Zn the difference was expressed. The only significant difference was between Chebec and Haruna nijo at Zn_0.

Table 4.3d

P-values for pairwise comparisons between barley varieties for vegetative zinc efficiency expressed at soil zinc levels Zn_0 and $Zn_{0.05}$. Efficiency was calculated as either [(yield (Zn_0)/yield ($Zn_{1.6}$)) x 100] or [(yield ($Zn_{0.05}$)/yield ($Zn_{1.6}$)) x 100]. Comparisons have only been made between parents of existing doubled haploid populations.

Genotype (treatment)	Vs	P-value
Chebec (0)	Harrington (0)	0.920
	Haruna nijo (0)	0.035
	Amagi nijo (0)	0.103
Chebec (0.05)	Harrington (0.05)	0.999
	Haruna nijo (0.05)	0.103
	Amagi nijo (0.05)	0.129
Harrington (0)	WA73S276 (0)	0.096
Harrington (0.05)	WA73S276 (0.05)	0.190

Shoot zinc concentrations and contents

Shoot Zn concentrations were affected by genotype and treatment. There were significant ($P \leq 0.0001$) Zn treatment and genotype effects as well as a significant ($P \leq 0.01$) interaction (see Appendix 1 (Section 4.2) for ANOVA table of log-transformed data). There was little change between Zn_0 and $Zn_{0.05}$ but a large increase at $Zn_{1.6}$ (Figure 4.4b). Analysis of log-transformed data showed no significant increase in shoot Zn concentration between Zn_0 and $Zn_{0.05}$ but a significant increase for all genotypes between $Zn_{0.05}$ and $Zn_{1.6}$ (Table 4.3e).

Shoot Zn contents were also affected by genotype and treatment. There were significant ($P \leq 0.001$) Zn treatment and genotype effects as well as a significant ($P \leq 0.05$) interaction (see Appendix 1 (Section 4.2) for ANOVA table of log-transformed data). There was a slight increase between Zn_0 and $Zn_{0.05}$ followed by a much larger increase at $Zn_{1.6}$ (Figure 4.4c). Analysis of log-transformed data showed an increase in shoot Zn content for all varieties except Haruna nijo between Zn_0 and $Zn_{0.05}$ and for all genotypes beteen $Zn_{0.05}$ and $Zn_{1.6}$ (Table 4.3f).
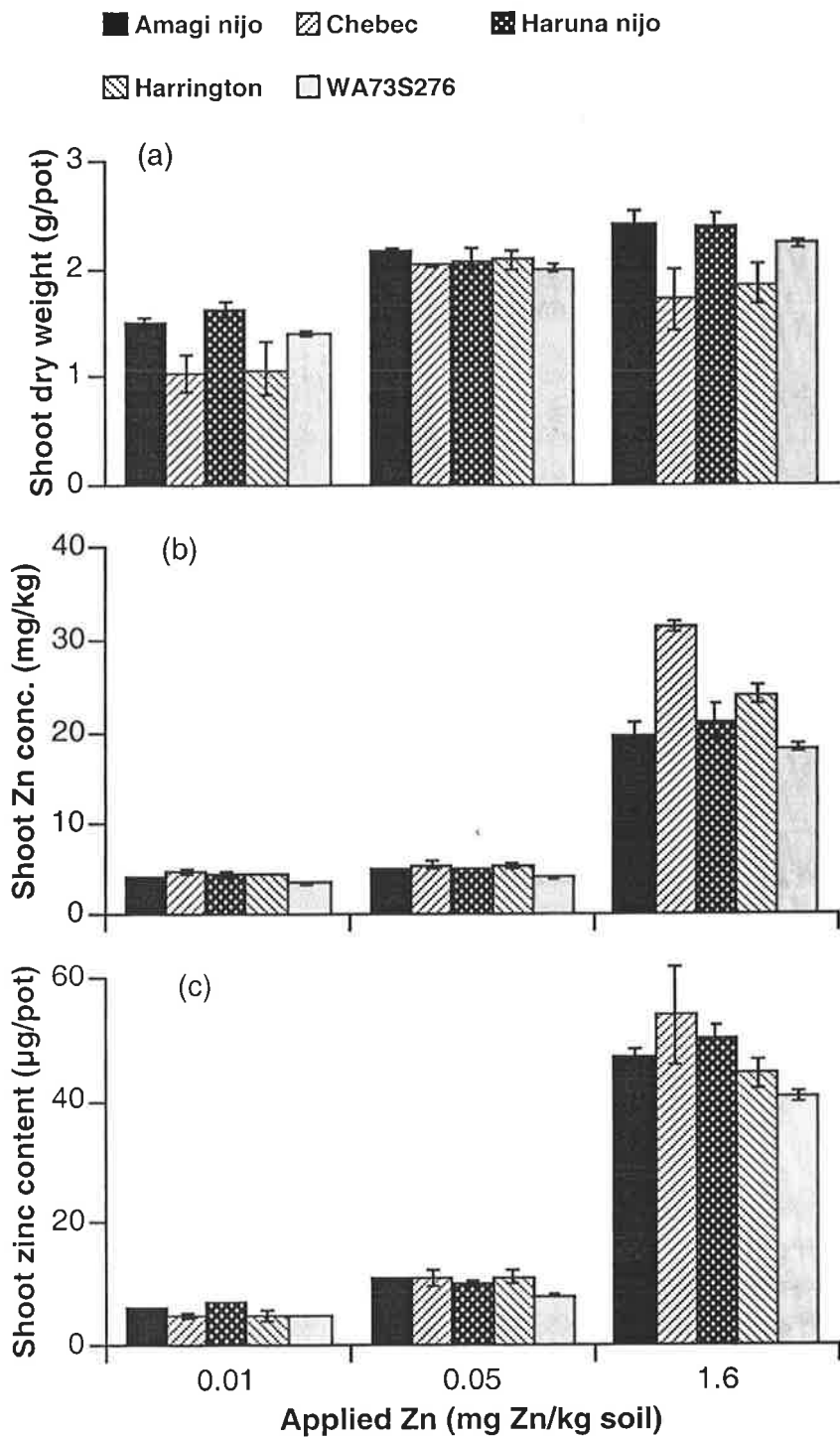
Figures 4.4a, b and c

Effects of applied zinc on (a) shoot dry weight (b) shoot zinc concentration and (c) shoot zinc content in 5 barley varieties grown in Laffer sand for 42 days. Values are an average of either two or three replicates with standard errors shown.

Table 4.3e

Effects of applied zinc on shoot zinc concentration of five barley varieties grown in Laffer sand for 42 days. Values are an average of either 2 replicates (Chebec, Haruna nijo and Harrington) or 3 replicates (Amagi nijo and WA73S276).

Applied Zinc (mg/kg)	Shoot zinc concentration (mg/kg)				
	Amagi nijo	Chebec	Haruna nijo	Harrington	WA73S276
0	4.0 (0.60)[a]	4.7 (0.67)	4.4 (0.64)	4.4 (0.64)	3.4 (0.53)
0.05	5.0 (0.70)	5.3 (0.72)	4.9 (0.69)	5.3 (0.72)	4.0 (0.60)
1.6	19.7 (1.29)	31.5 (1.50)	21.1 (1.32)	24.0 (1.38)	18.3 (1.26)

Tukey-Kramer $HSD_{0.05}$[b]

Genotype x zinc treatment   (0.12)

[a]Numbers in parentheses refer to averages of log-transformed data.

[b]The $HSD_{0.05}$ value is applicable to log-transformed data only.

Table 4.3f

Effects of applied zinc on shoot zinc contents of five barley varieties grown in Laffer sand for 42 days.

Values are an average of either 2 replicates (Chebec, Haruna nijo and Harrington) or 3 replicates (Amagi nijo and WA73S276).

Applied Zinc (mg/kg)	Shoot zinc content (µg)				
	Amagi nijo	Chebec	Haruna nijo	Harrington	WA73S276
0	6.0 (0.78)[a]	4.7 (0.67)	7.0 (0.85)	4.6 (0.65)	4.8 (0.68)
0.05	10.7 (1.03)	10.8 (1.03)	10.0 (1.00)	10.9 (1.04)	8.0 (0.90)
1.6	47.4 (1.68)	53.8 (1.73)	50.2 (1.70)	44.2 (1.64)	40.6 (1.61)

Tukey-Kramer HSD$_{0.05}$[b]

Genotype x zinc treatment   (0.19)

[a]Numbers in parentheses refer to averages of log-transformed data.

[b]The HSD$_{0.05}$ value is applicable to log-transformed data only.

Other elements

Shoot Fe concentrations again were high under zinc deficiency and rapidly decreased with increasing soil applied zinc. WA73S276 showed the largest decrease and Haruna nijo the least (Figure 4.5a). Shoot Mn (Figure 4.5b) and Cu (Figure 4.5c) concentrations also decreased as soil zinc increased. WA73S276 showed the greatest change for these elements also.
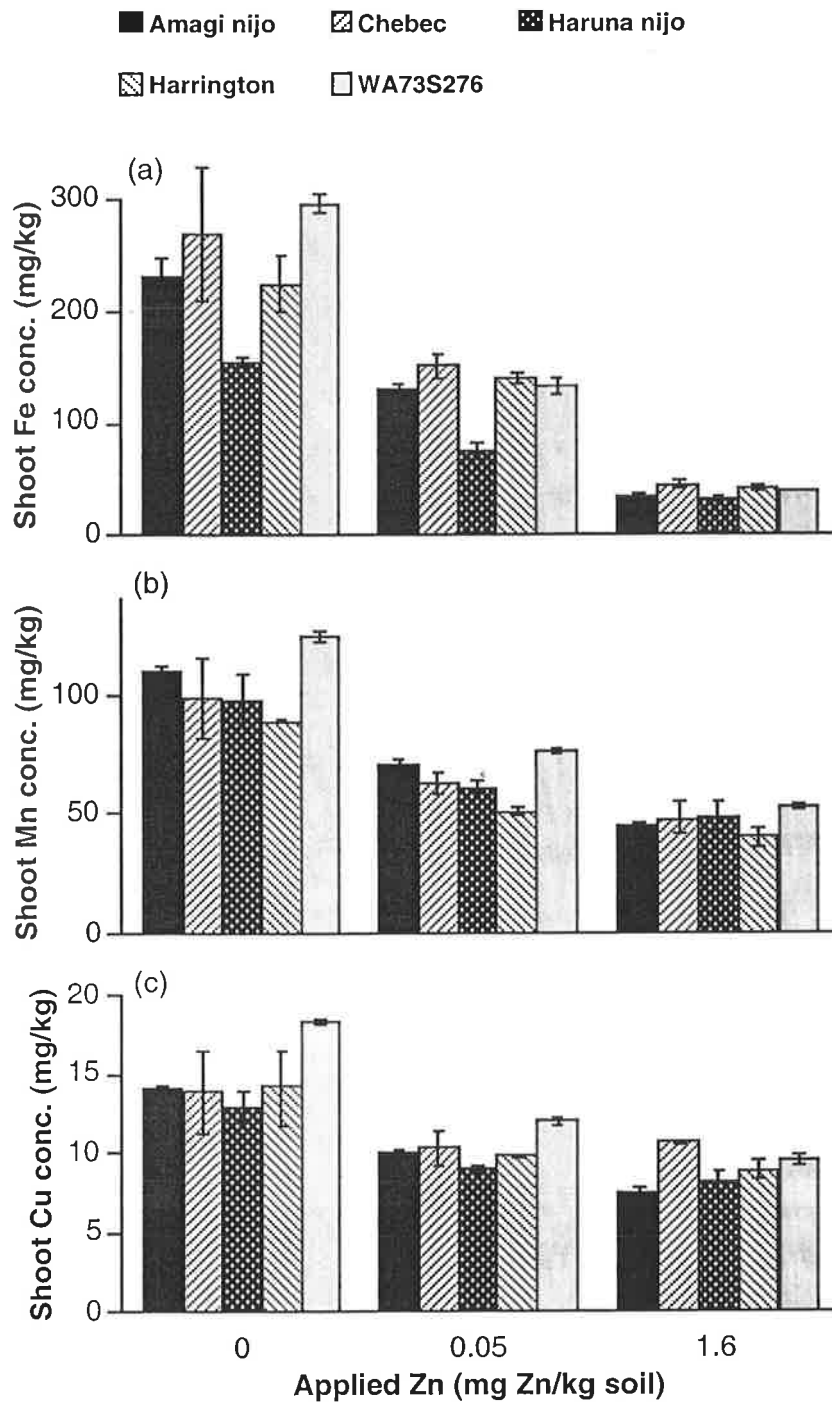
Figures 4.5a, b and c

Effects of applied zinc on (a) shoot iron concentration (b) shoot manganese concentration and (c) shoot copper concentration in 5 barley varieties grown in Laffer sand for 42 days. Values are an average of either two or three replicates with standard errors shown.

Shoot P concentrations were much higher at Zn_0 for all genotypes except Haruna nijo which exhibited only slight increase (Figure 4.6a). Concentrations of S were also higher at Zn_0, most notably for Harrington and WA73S276 (Figure 4.6b).
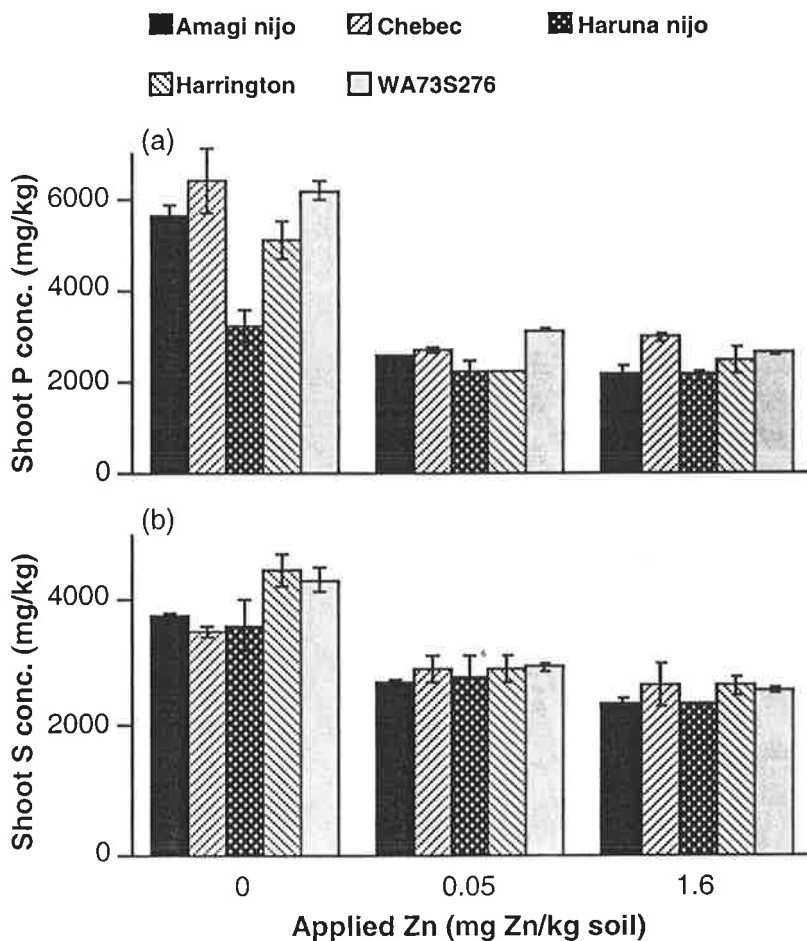
Figures 4.6a, b and c

Effects of applied zinc on (a) shoot phosphorus concentration and (b) shoot sulphur concentration in 5 barley varieties grown in Laffer sand for 42 days. Values are an average of either two or three replicates with standard errors shown.

4.3.3 Experiment 4C

Vegetative efficiency scores ranged from one to four at Birchip and from zero to three at Lameroo (Figures 4.7a and b) suggesting that Birchip was the more zinc deficient site and, consequently, the barley plants grown there exhibited a greater response to zinc application. This is further supported by the greater range of grain yield efficiency experienced at Birchip (64-129%) compared to Lameroo (77-123%).

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Simple regression of measured grain yield efficiency against visual scores of vegetative efficiency showed poor correlation at both sites (Figures 4.7a and b). At Birchip, vegetative efficiency scores of 2 were associated with the highest grain yield efficiencies but yield efficiency decreased for both higher (3-4) and lower (1) vegetative scores. At Lameroo, all vegetative scores (0-3) were associated with a similar range of grain yield efficiency. In summation, increased grain yield efficiency did not consistently correspond with a lower vegetative efficiency score.

Vegetative efficiency measured in pots (Experiment 4B) was not a reliable predictor of grain yield efficiency measured in the field at either Birchip (Figures 4.8a and b) or at Lameroo (Figures 4.9a and b). Rankings were inconsistent between the two measurements whether using vegetative efficiency calculated at 0 mg Zn/kg soil (Figures 4.8a, 4.9a) or at 0.05 mg Zn/kg soil (Figures 4.8b, 4.9b).

85

(a)

(b)
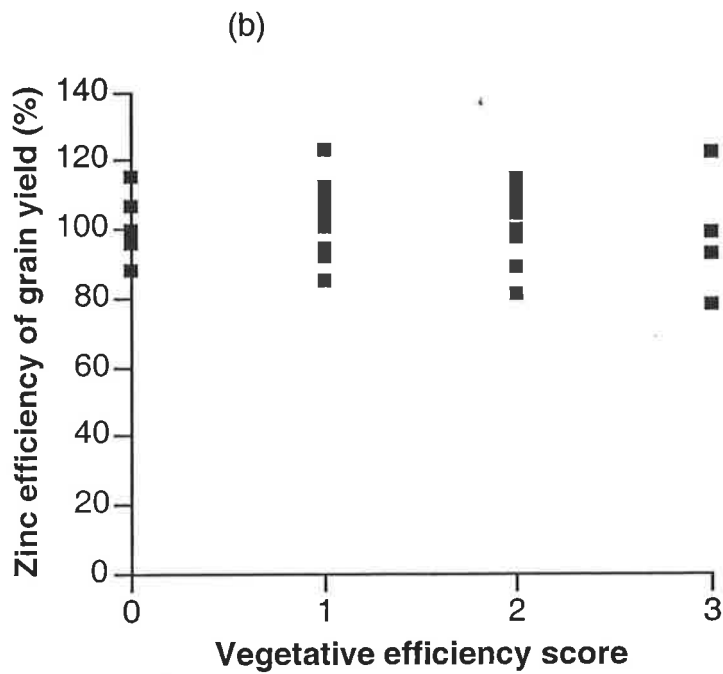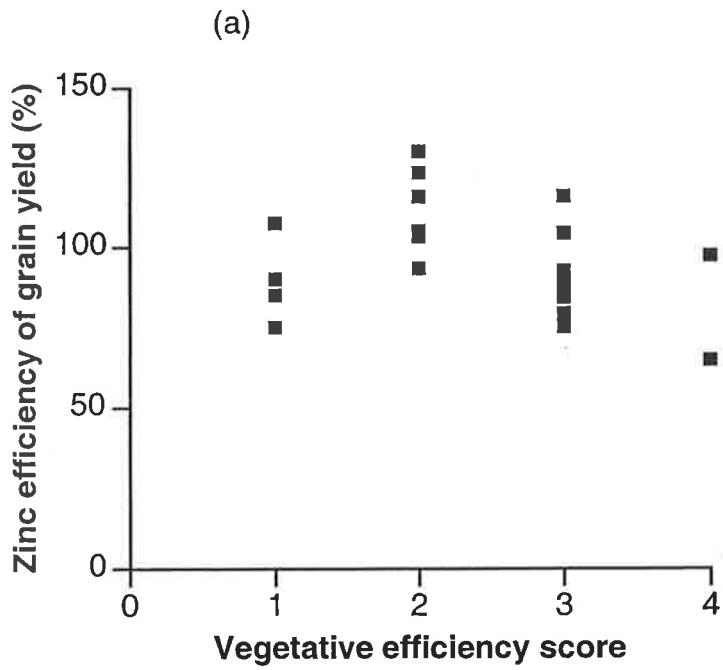
Figures 4.7a and b

Relationship between zinc efficiency of grain yield and vegetative efficiency in seven barley varieties grown at (a) Birchip and (b) Lameroo. Grain yield efficiency was calculated as [(yield (nil Zn)/yield (+Zn)) x 100]. Vegetative efficiency was a visual assessment at 49 days after sowing.

(a)

■ Grain yield □ Vegetative (0 Zn)

(b)

■ Grain yield ▨ Vegetative (0.05 Zn)
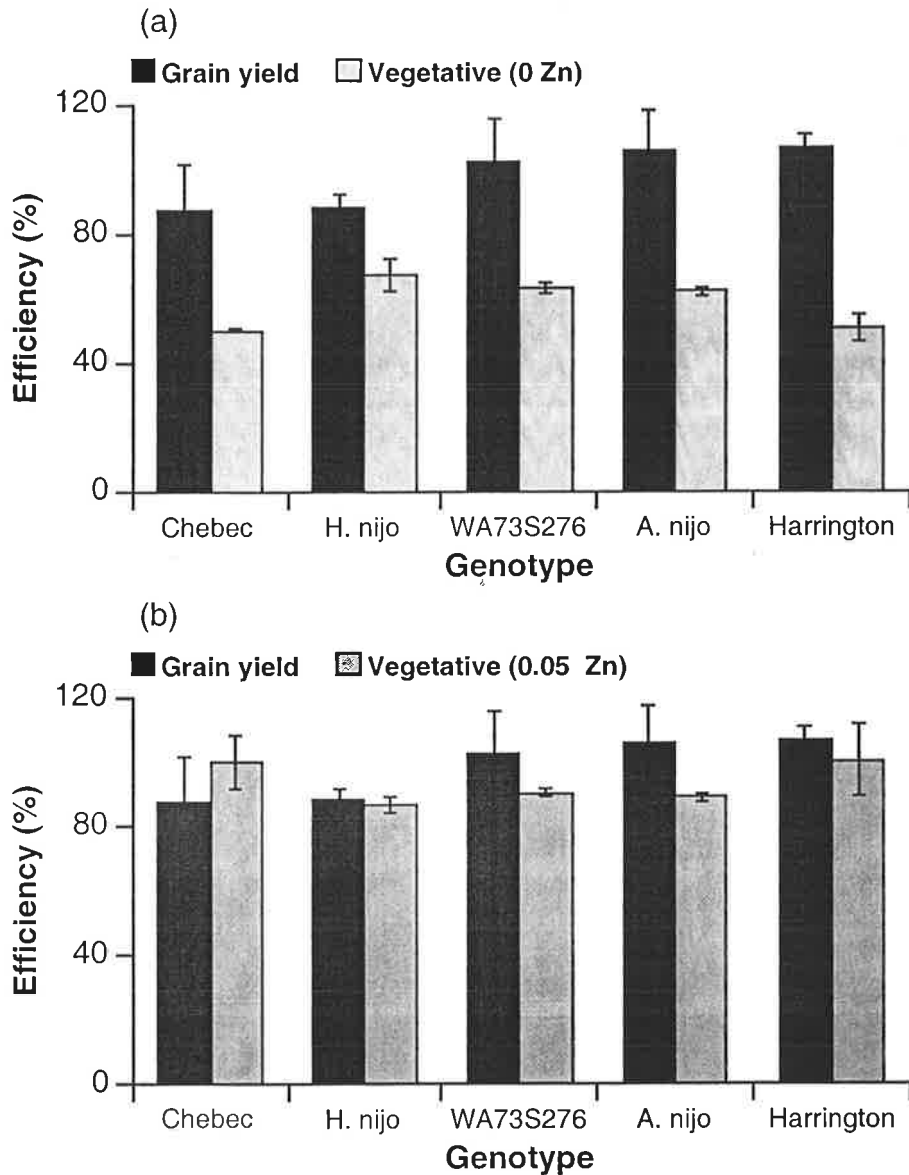
Figures 4.8a and b

Comparison of grain yield efficiency determined in the field at Birchip with vegetative efficiency determined in pots at two levels of zinc deficiency. Grain yield efficiency was calculated as [(yield (nil Zn)/yield (+Zn)) x 100]. Vegetative efficiency was calculated as either (a) [(yield (Zn_0)/yield $(Zn_{1.6})$) x 100] or (b) [(yield $(Zn_{0.05})$/yield $(Zn_{1.6})$) x 100]. Varieties appear in increasing order of grain yield efficiency.

Figures 4.9a and b

Comparison of grain yield efficiency determined in the field at Lameroo with vegetative efficiency determined in pots at two levels of zinc deficiency. Grain yield efficiency was calculated as [(yield (nil Zn)/yield (+Zn)) x 100]. Vegetative efficiency was calculated as either (a) [(yield (Zn_0)/yield $(Zn_{1.6})$) x 100] or (b) [(yield $(Zn_{0.05})$/yield $(Zn_{1.6})$) x 100]. Varieties appear in increasing order of grain yield efficiency.

Vegetative efficiency measured in pots did not correlate particularly well with vegetative field scores (Figures 4.10a, b and 4.11a, b). As pot efficiency increases, field scores should decrease. The best correlation was vegetative efficiency at Zn0.05 versus Birchip field score (Figure 4.10b).

(a)

■ Vegetative efficiency (pot 0Zn)
☐ Vegetative score (field)

(b)

■ Vegetative efficiency (pot 0.05Zn)
☐ Vegetative score (field)
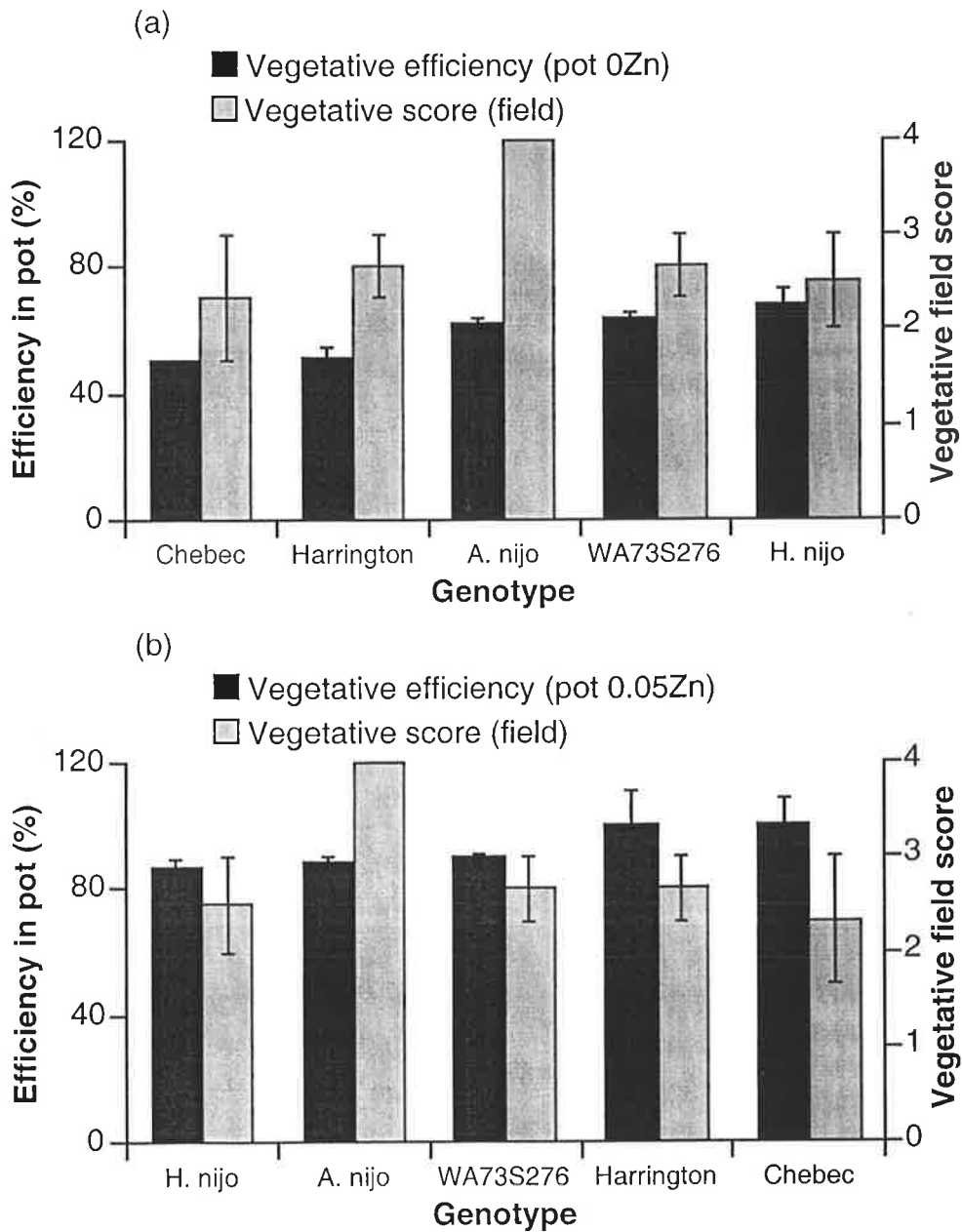
Figures 4.10a and b

Comparison of vegetative efficiency determined in pots at two levels of zinc deficiency with vegetative field scores at Birchip. Vegetative efficiency was calculated as either (a) $[(\text{yield } (Zn_0)/\text{yield } (Zn_{1.6})) \times 100]$ or (b) $[(\text{yield } (Zn_{0.05})/\text{yield } (Zn_{1.6})) \times 100]$. Varieties appear in increasing order of vegetative pot efficiency.
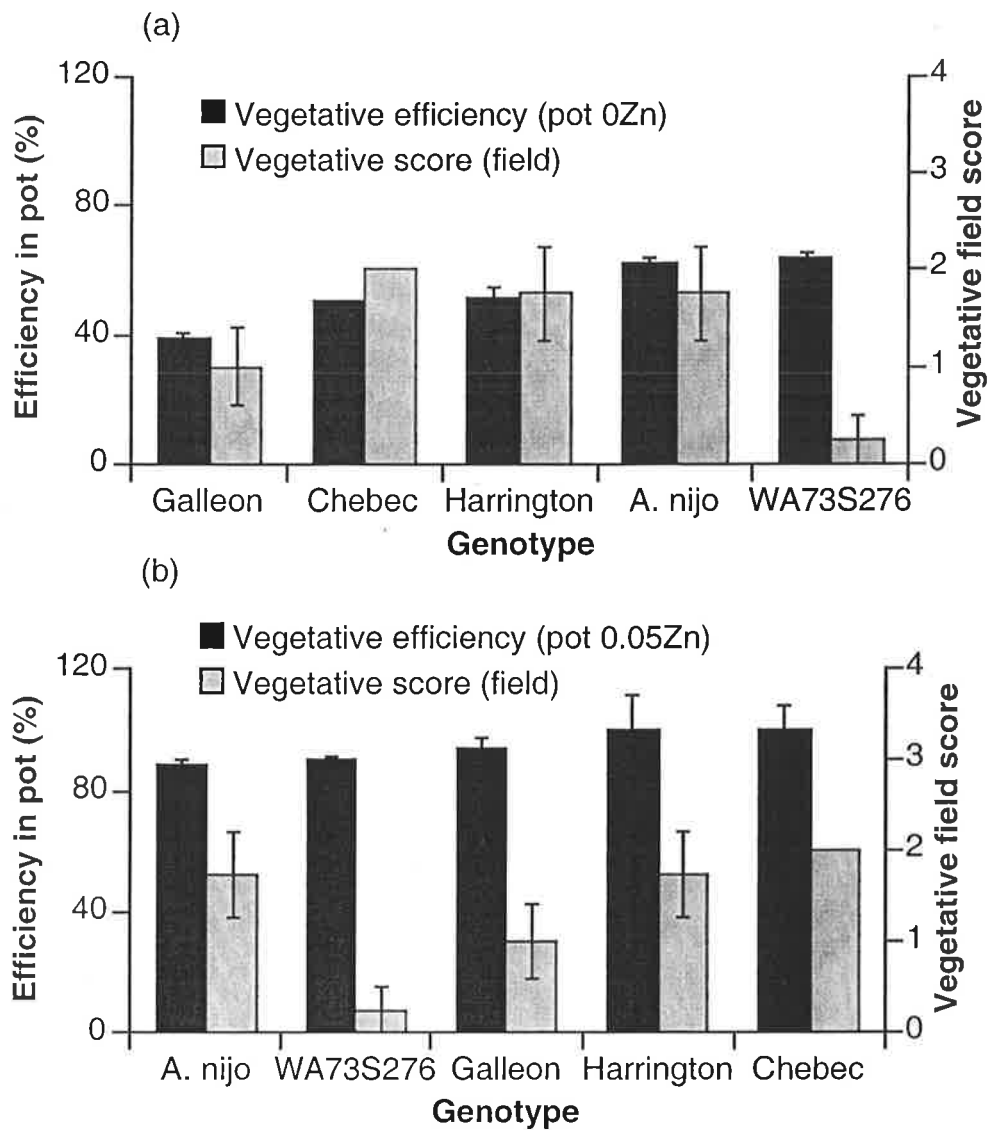
Figures 4.11a and b

Comparison of vegetative efficiency determined in pots at two levels of zinc deficiency with vegetative field scores at Lameroo. Vegetative efficiency was calculated as either (a) [(yield (Zn_0)/yield $(Zn_{1.6})$) x 100] or (b) [(yield $(Zn_{0.05})$/yield $(Zn_{1.6})$) x 100]. Varieties appear in increasing order of vegetative pot efficiency.

4.4 Discussion

Although experiments 4A and 4B differed in the amount of nutrients other than zinc supplied to the soil medium they both demonstrated that the various genotypes can have unique growth responses in relation to changes in soil zinc supply. By analogy with the work of Paull (1990), who found that the various genes controlling tolerance to B toxicity were expressed at different levels of B stress, it is reasonable to assume that zinc efficiency genes will be expressed in a similar manner. The unique responses of various

genotypes make selection of zinc levels at which to measure efficiency, extremely difficult to choose since individual genotypes achieve maximum growth at differing levels of supplied zinc and may actually decrease beyond these levels. Many rates of zinc application would have to be chosen in order to give a true indication of zinc efficiency but this is impractical, particularly if working with large populations. As alluded to in Chapter 3, however, it may be possible to use two levels of zinc that correspond with levels experienced in the field situation (viz. +Zn and nil).

For Experiments 4A and 4B, it is interesting to note that although large differences in shoot weights occur between Zn_0 and $Zn_{0.05}$, there is little difference between shoot zinc concentrations. Although there is insufficient Zn treatments to accurately calculate a critical Zn concentration for deficiency, it is obvious that it would occur somewhere between Zn_0 and $Zn_{0.05}$. Conversely, there is a large difference in shoot zinc concentrations between $Zn_{0.05}$ and $Zn_{1.6}$ but little difference in shoot dry weights. It is also interesting to note that shoot Zn concentrations in Experiment 4B were much less than those in Experiment 4A for all Zn treatments. This was presumably caused by the difference in added nutrients between the two experiments and highlights the necessity of a reliable, repeatable bioassay for which to measure Zn efficiency.

Experiments 4A and 4B represented a continuing refinement of the zinc bioassay. The problem of boron toxicity had by this time been alleviated but Experiment 4A was likely to have been affected by Mo toxicity which had been unmeasurable at that time. The effect of Mo toxicity on different varieties is unknown and so the results of Experiment 4A must be observed cautiously. It is unfortunate that the bioassay had not been further refined at this point as it appeared that some of the parental pairs, particularly Galleon and Amagi nijo may be useful in further genetic studies. Despite the overall lack of highly significant differences in zinc efficiency between genotypes, experience has shown that transgressive segregation occurs frequently for quantitative traits even when parents are not significantly different for that trait (Nelson and Sorrells, pers. comm.). This suggests promise for mapping zinc efficiency in any of the populations derived from the genotypes measured in Experiments 4A and 4B.

As was the case for the experiments detailed in Chapter 3, foliar symptoms of zinc deficiency did not correlate well with vegetative zinc efficiency based on yield. Amagi nijo exhibited the strongest foliar symptoms but was not the most inefficient. In contrast, Galleon in Experiment 4A and Chebec and Harrington in Experiment 4B showed very mild foliar symptoms but were reduced in total growth more than the other genotypes. This, together with similar observations for Clipper and Sahara (Chapter 3) further support the notion that symptoms and shoot growth are controlled at least in part by separate genetic loci.

High shoot iron concentrations associated with zinc deficiency have been reported previously for sweet corn, sugar beet (*Beta vulgaris* L.) and barley (Rosell and Ulrich, 1964; Jackson *et al.*, 1967; Schwartz *et al.*, 1987). The results measured in experiments here suggest more than just a dilution effect as iron concentrations are increased from $Zn_{1.6}$ to $Zn_{0.05}$ even though shoot dry weights do not differ greatly. Although the cause of this increased uptake is not known, it can be speculated that passive uptake of iron may be intensified by higher root cell membrane permeability (Welch *et al.*, 1982).

The poor correlation of relative grain yield with relative vegetative yield casts doubt over the usefulness of this bioassay to predict grain yield efficiency. Grain yield can be affected by many factors such as yield potential, maturity, timing of late rain relative to time of maturity, frost during grain development, etc. but even the field score measured at the vegetative stage does not correlate very well with vegetative pot efficiency. Therefore, a need still exists to develop rapid, reliable measures of determining zinc efficiency in order to service breeding programmes. It would seem that molecular biological techniques might be the most useful tool in achieving this aim. While field screening cannot be eliminated altogether, it may be used to reliably calibrate molecular marker assisted selection in early generation material.

Chapter 5

Mapping of Chromosome Regions Associated with Increased Vegetative Zinc Accumulation Using a Barley Doubled Haploid Population

5.1 Introduction

In the past, many agronomically and economically important traits have been mapped in barley. These include the *Mla* mildew resistance gene (Hilbers *et al.*, 1992), the *denso* dwarfing gene (Barua *et al.* 1993; Laurie *et al.*, 1993), grain yield, heading date, plant height (Thomas *et al.*, 1995; Backes *et al.*, 1995; Bezant *et al.*, 1996), kernel characters (Backes *et al.*, 1995) and malting quality (Thomas *et al.*, 1996). While significant effort has been directed at disease resistance and quality parameters, there has been little research on mapping nutritional characteristics, either at the vegetative or reproductive stage with a consequent lack of knowledge regarding the genetics of micronutrient uptake and transport. Recently, Pallotta *et al.* (2000) mapped a gene for Mn efficiency in barley and Jefferies *et al.* (1999) mapped several QTLs associated with tolerance of barley to B toxicity. With the ever increasing number of linkage maps and diverse mapping populations being produced by various research groups, it is anticipated that mapping of nutritional characters will become more common. The purpose of the study described in this chapter was to identify major genes or QTLs controlling Zn concentration and content in barley shoots at the vegetative stage of development. As well as providing basic information about the process involved in Zn accumulation, this will also provide the starting point for development of molecular markers to assist breeding programs in efficient selection of high Zn accumulating genotypes. Such genotypes are likely to be more resistant to pathogenic attack (Huber and Graham, 1999) and possibly able to transport more Zn to the grain.

Two experiments were conducted under controlled conditions. Experiment 5A was designed to confirm the results of Pallotta (unpublished, see Section 3.1), while at the same time investigating the possibility that varying soil Mn and Zn fertility may affect the differential in Zn concentration/accumulation between Clipper and Sahara. The results of this experiment were used to determine the most suitable levels of these nutrients for screening the doubled haploid population (Experiment 5B).

5.2 Materials and methods

5.2.1 Experiment 5A - Effect of zinc and manganese on vegetative zinc accumulation in varieties Clipper and Sahara

The procedures used followed those of Pallotta et al. (2000) who first demonstrated the differential in Zn accumulation between Sahara 3771 and Clipper. The soil used was a calcareous aeolian sand (Uc 1.11, Northcote 1979) collected from a severely Mn-deficient site at Wangary, on the lower Eyre Peninsula of South Australia (Wilhelm et al., 1988). The soil was collected in 1996 and contained approximately 67% $CaCO_3$ with a pH (in water) of 8.63. Preparation was similar to that of Huang et al. (1994). Topsoil (0-10cm) and subsoil (10-20cm) were air-dried, passed through a 1mm stainless steel sieve and stored separately. A bulk of topsoil and subsoil (1:1, w/w) was well combined before adding deionised water to bring the moisture content to 20% (w/w). The soil was then incubated for 3 weeks in the dark at temperatures of $15^{\circ}C$ (16h) and $10^{\circ}C$ (8h). Such preincubation was required to decrease available Mn released from endogenous Mn sources during the soil drying process and storage (Uren et al., 1988). The following nutrients were added and mixed well with the incubated soil: (mg salt/kg dry soil) $Ca(NO_3)_2.4H_2O$, 918; KH_2PO_4, 179.5; $MgSO_4.7H_2O$, 140; K_2SO_4, 28.4; $FeSO_4.7H_2O$, 17.2; H_3BO_3, 5.6; NaCl, 4.16; $CuSO_4.5H_2O$, 3.9; $CoSO_4.7H_2O$, 0.585; $H_2MoO_4.H_2O$, 0.13.

The soil was then divided into 4 equal portions and the following treatments applied-

treatment 1 -low Mn, low Zn

treatment 2 -low Mn, high Zn

treatment 3 -high Mn, low Zn

treatment 4 -high Mn, high Zn

where low Mn = 10 mg Mn/kg dry soil as $MnSO_4.4H_2O$

high Mn = 50 mg Mn/kg   "   "   "

low Zn = 0.02 mg Zn/kg dry soil as $ZnSO_4.7H_2O$

high Zn = 3.9 mg Zn/kg   "   "   "

Clear plastic sample jars (6.5cm diameter x 15cm height) covered with aluminium foil and lined with a plastic bag were filled with soil (equivalent to 450g dry weight) and allowed to equilibrate overnight. Barley seeds of the varieties Clipper and Sahara with similar Zn contents (Clipper 2.71 μg/seed and Sahara 2.75μg/seed) were surface-sterilised by soaking in 70% ethanol for 1 minute, rinsed with deionised water and then soaked in sodium hypochlorite (1.4% v/v) for 6 minutes before rinsing again with deionised water. The seed was germinated on moistened Whatman No. 42, ashless filter paper for 36 hours at 20^oC before sowing (3 seeds per pot).

Plants were grown in a controlled environment growth chamber with a 10 h light/14 h dark photo period at 15^oC day/10^oC night. Light was supplied by high-pressure mixed-metal halide lamps delivering a photon flux density of 500 μmol/m^2/s at the surface of the pot. There were five replicates for each genotype x treatment combination amounting to-:

2 genotypes x 4 treatments x 5 replicates = 40 pots

The experiment was set up as a completely randomised block and pots were rotated within a block every few days to minimise the effect of microenvironments caused by uneven light distribution and/or air flow. Pots were watered daily and watered to weight every 3 days to maintain the soil moisture at 20% w/w.

After 28 days all leaves except the oldest two were harvested according to the method of Pallotta et al. (2000) and oven-dried at 70ºC for 24 hours. Dried material was digested with 70% nitric acid and analysed for concentrations of the elements Zn, Mn, Fe, Cu, B, Ca, Mg, Na, K, P and S by ICPAES. The remaining two leaves and stem material were also harvested and analysed separately.

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Data were analysed as a three-way analysis of variance using the computer program SuperANOVA™. The factors for the ANOVA model were Zn level, Mn level and genotype. In addition, simple linear regressions were performed in order to assess whether the ICPAES results for young tissue were indicative of the Zn status of whole plants. This was done by regressing Zn concentration and accumulation of young tissue against Zn concentration and accumulation for the whole plant.

5.2.2 Experiment 5B - Screening of Clipper x Sahara doubled haploid lines for vegetative zinc accumulation

The genetic material used was a population of 150 doubled haploid lines derived from a cross between Clipper and Sahara. The Clipper x Sahara population was produced by the *Hordeum bulbosum* method (Islam and Shepherd, 1981) using embryo culture followed by chromosome doubling through colchicine treatment. Original seed was obtained from Dr. A.K.M.R. Islam at the University of Adelaide and grown through one generation to obtain seed produced under standard conditions. Seed Zn content of the doubled haploids ranged from 1.07 µg/seed to 6.30 µg/seed. Seed Mn content of the doubled haploids ranged from 0.43 µg/seed to 2.49 µg/seed. Due to the number of lines to be screened and limited growth cabinet space, the experiment was conducted in 4 stages. Each stage was set up as a randomised complete block with 4 replicates. The parents together with two of the doubled haploid lines (DH 79 and DH 106) were used as controls. Clipper and Sahara were included 12 times for each experiment and DH 79 and DH 106 included four times. This amounted to

[(37 DH lines + DH 79 + DH 106) x 4 replicates] + [(Clipper + Sahara) x 12 replicates]

= 180 pots per experiment.

Plants were grown under conditions described for Experiment 5A using treatment 4 (high Zn, high Mn).

After 28 days all leaves except the oldest two were harvested according to the method of Pallotta *et al.* (2000) and oven-dried at 70°C for 24 hours. Dried material was digested with 70% nitric acid and analysed for concentrations of the elements Zn, Mn, Fe,

Cu, B, Ca, Mg, Na, K, P and S by ICPAES.

In order to carry out the linkage analysis it was necessary to obtain an adjusted value for a particular variable such that all 150 doubled haploids could be reasonably compared without bias. This was done by analysis of variance using the computer software BMDP5V (BMDP Statistical Software Inc., 1992). The model incorporated unbalanced repeated measures with structured covariance matrices. By employing this form of analysis it was possible to remove any 'between experiments' effect.

Single point linkage analysis (P<0.001) was performed using the computer program Map Manager QT version b16 (Manly and Cudmore, 1997) employing a total of 202 RFLP markers (Langridge *et al.*, 1995). These markers covered all barley chromosomes. A minimum LOD score (logarithm of odds score) of 3.0 was used to detect significant associations between markers and Zn concentration/content. Analysis was also performed on shoot dry weight data.

5.3 Results

5.3.1 Experiment 5A

Visual symptoms

Both genotypes exhibited obvious Mn deficiency at the low Mn treatments. Symptoms included reduced growth and a limp, wilted appearance with foliage becoming pale green, particularly on younger leaves. Young leaves also developed grey flecking which later formed necrotic patches. There were no symptoms of Zn deficiency.

Shoot dry weights

Shoot dry weights were strongly ($P \leq 0.0001$) affected by Mn treatment and to a lesser degree ($P \leq 0.05$) by genotype (see Appendix 1 (Section 5.1) for ANOVA table). This was true for both young shoot tissue and remaining shoot tissue (Figures 5.1a and b). There was no significant effect of Zn treatment. This is not surprising since Zn concentrations for young tissue were above the accepted critical range of 10-15 mg/kg for
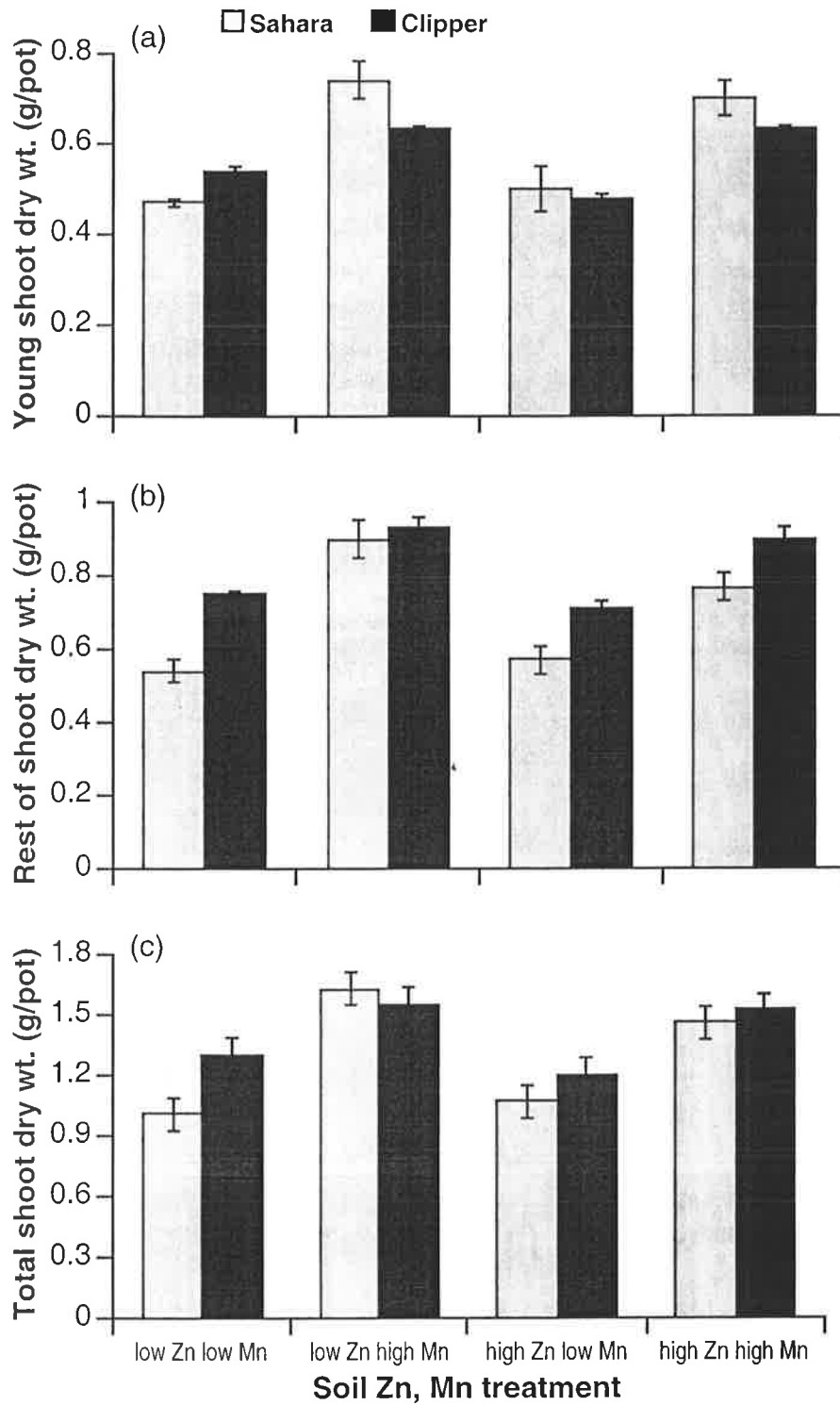
Figures 5.1a, b and c

Effect of Zn and Mn treatments on shoot dry weights of barley varieties Clipper and Sahara grown in Wangary soil for 28 days. Data has been partitioned into (a) young shoot tissue (b) rest of shoot and (c) total shoot. Values represent an average of five replicates with standard errors shown.

most graminaceous species (Marschner, 1993) ranging from 31-52 mg/kg for Clipper and 46-78 mg/kg for Sahara. Manganese deficiency resulted in an average total shoot dry weight reduction of 20% for Clipper at high Zn and 16% at low Zn (Figure 5.1c). The reduction for Sahara, by contrast, was 27% at high Zn and increased to 39% at low Zn, as indicated by a strong genotype x Mn interaction (see Appendix 1 (Section 5.1) for ANOVA table). There was also a significant ($P \leq 0.05$) genotype x Mn x Zn interaction.

Shoot zinc concentrations and contents

Zinc concentrations and contents in young shoot tissue showed high correlation with total shoot Zn concentration and content for both genotypes at all treatments (Figures 5.2a and b). Total shoot Zn concentrations were strongly ($P \leq 0.0001$) affected by Zn treatment, Mn treatment and genotype (see Appendix 1 (Section 5.1) for ANOVA table). Concentrations in shoots of Sahara were significantly higher than those in Clipper for all treatments (Figures 5.3a,b and c) with the largest difference exhibited at treatment 1 (low Zn, low Mn).

Shoot Zn contents were also higher for Sahara than Clipper at all treatments but the largest difference occurred at treatment 4 (high Zn, high Mn) (Figures 5.4a,b and c). The level of applied Zn had a highly significant ($p<0.001$) effect on Zn content while Mn level had a less significant ($p<0.05$) effect (see Appendix 1 (Section 5.1) for ANOVA table).
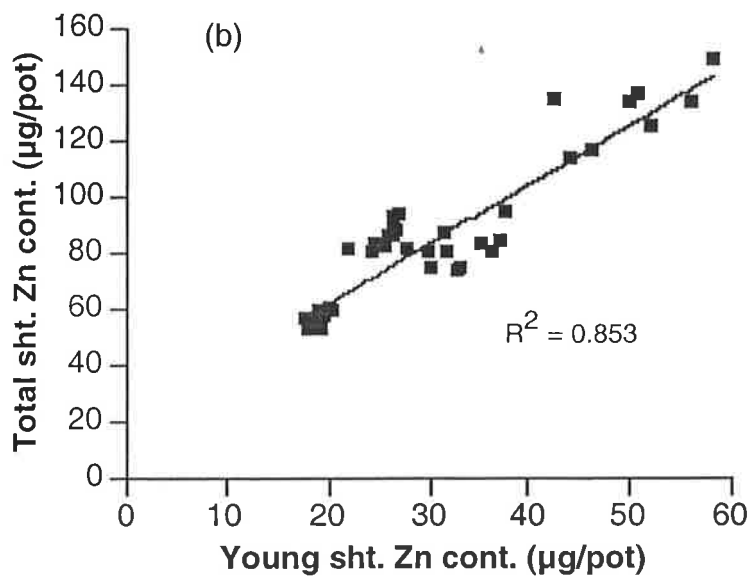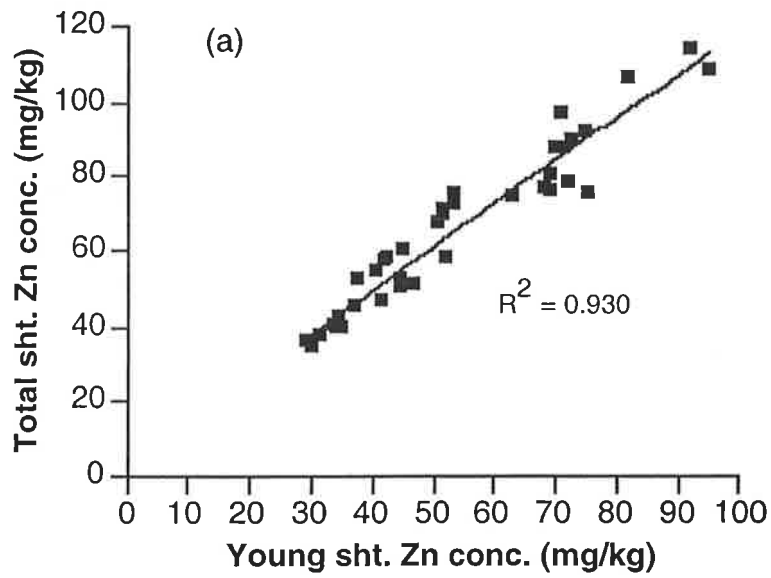
Figures 5.2a and b

Correlation of young shoot tissue with total shoot for (a) zinc concentration and (b) zinc content for barley

varieties Clipper and Sahara grown in Wangary soil for 28 days. Each symbol represents a single replicate.
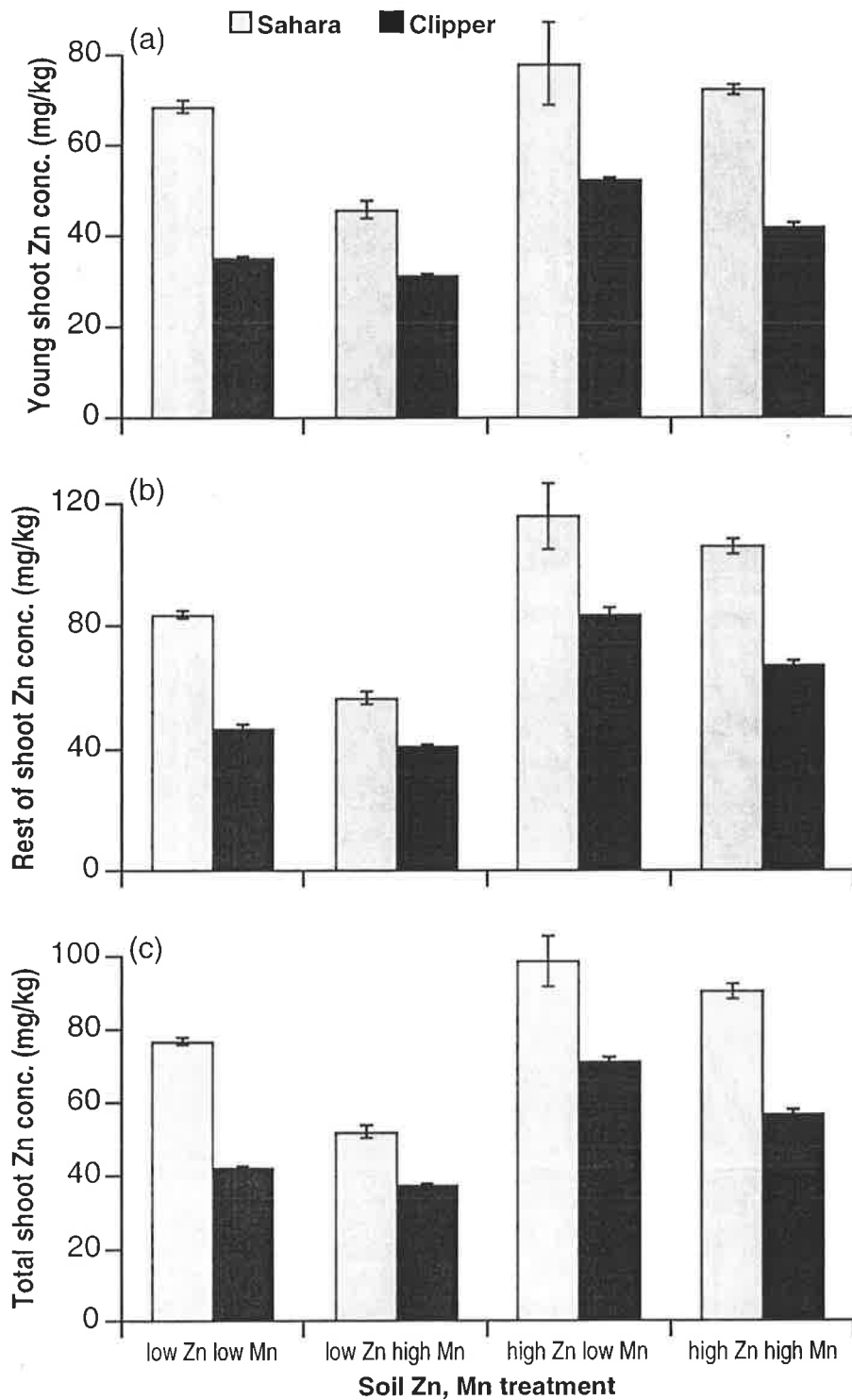
Figures 5.3a, b and c

Effect of Zn and Mn treatments on shoot zinc concentrations of barley varieties Clipper and Sahara grown in Wangary soil for 28 days. Data has been partitioned into (a) young shoot tissue (b) rest of shoot and (c) total shoot. Values represent an average of five replicates with standard errors shown.
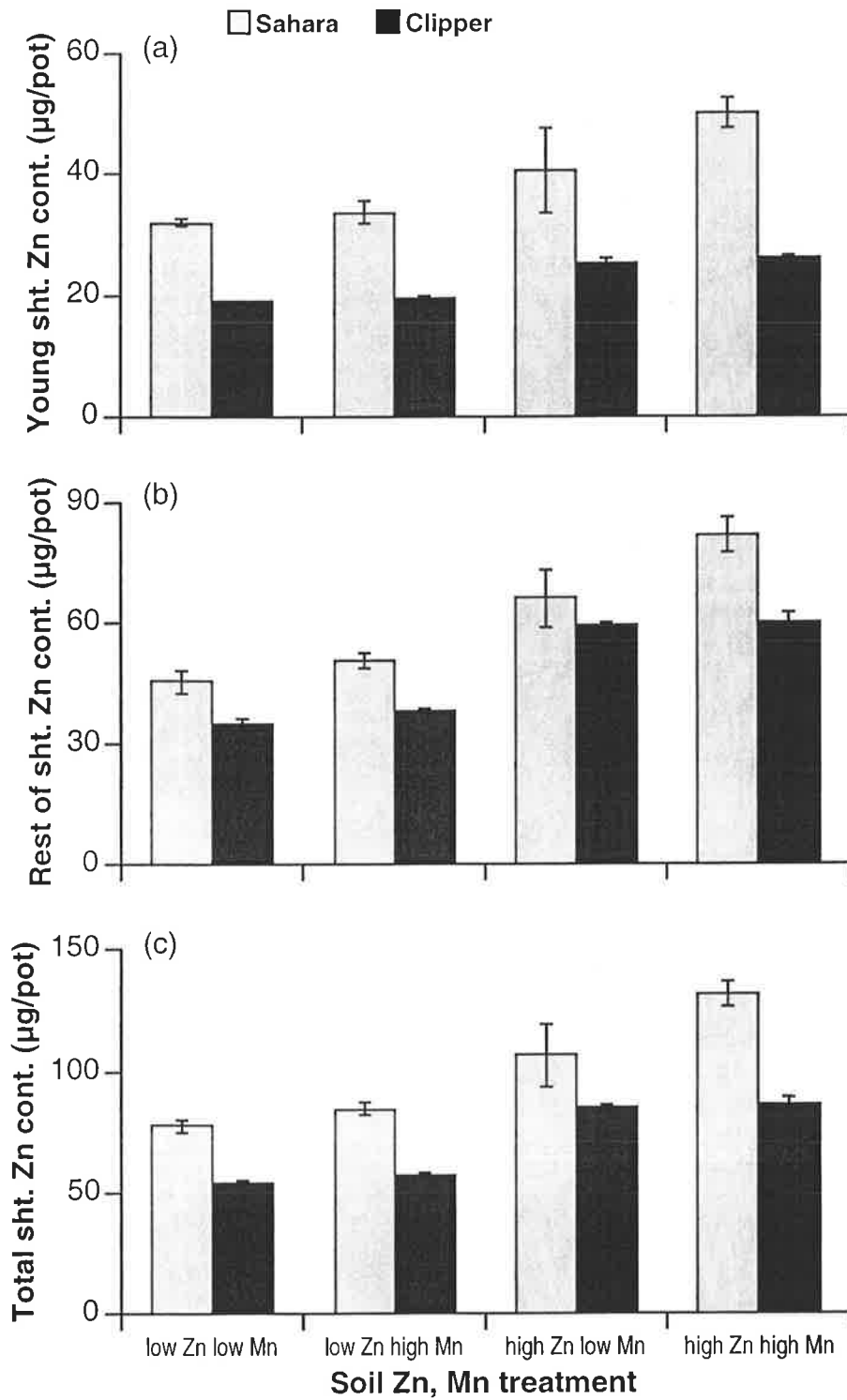
Figures 5.4a, b and c

Effect of Zn and Mn treatments on shoot zinc contents of barley varieties Clipper and Sahara grown in Wangary soil for 28 days. Data has been partitioned into (a) young shoot tissue (b) rest of shoot and (c) total shoot. Values represent an average of five replicates with standard errors shown.

5.3.2 Experiment 5B

Shoot zinc concentrations and contents

Adjusted values for Zn concentration of the doubled haploid lines ranged from 36 mg/kg to 86 mg/kg with Clipper having a Zn concentration of 38 mg/kg and Sahara 66 mg/kg (Figure 5.5a). Adjusted values for shoot Zn content ranged from 16 ug/pot to 45 ug/pot with Clipper containing 18 μg/pot and Sahara 37 μg/pot (Figure 5.5b). The distributions for Zn concentration and accumulation were slightly skewed towards the lower values contrasting with shoot dry weight (Figure 5.5c) which displayed a more normal distribution.

Other elements

The population was also found to be segregating for shoot sodium concentration and shoot boron concentration. Adjusted values for Na concentration ranged from 2200 mg/kg to 8000 mg/kg (Figure 5.6) and for B concentration from 2.5 mg/kg to 11.5 mg/kg (Figure 5.7).
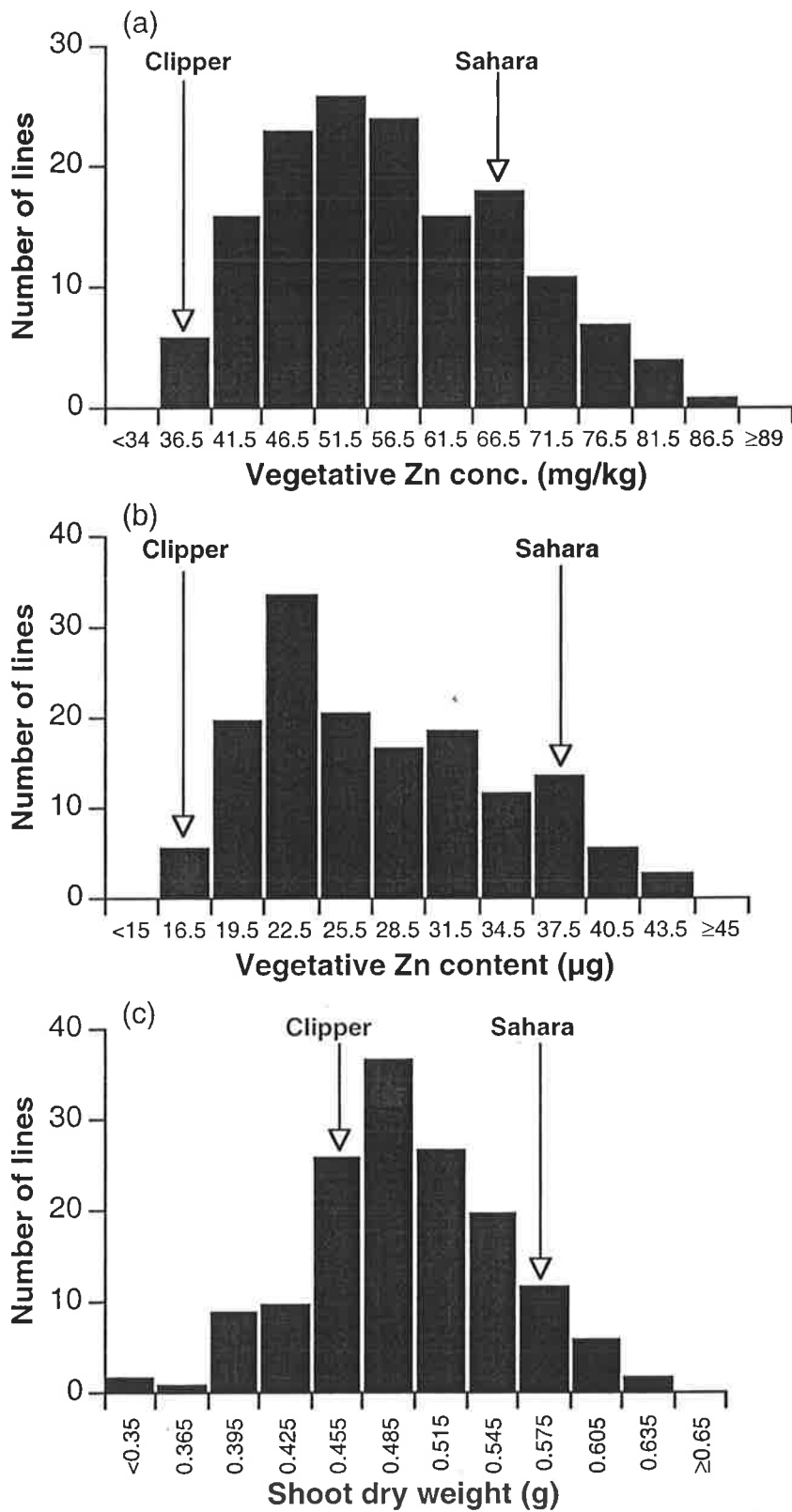
Figures 5.5a, b and c

Frequency distributions for (a) shoot zinc concentration, (b) shoot zinc content and (c) shoot dry weight of 150 doubled haploid barley lines and their parents, Clipper and Sahara, grown in Wangary soil for 28 days.
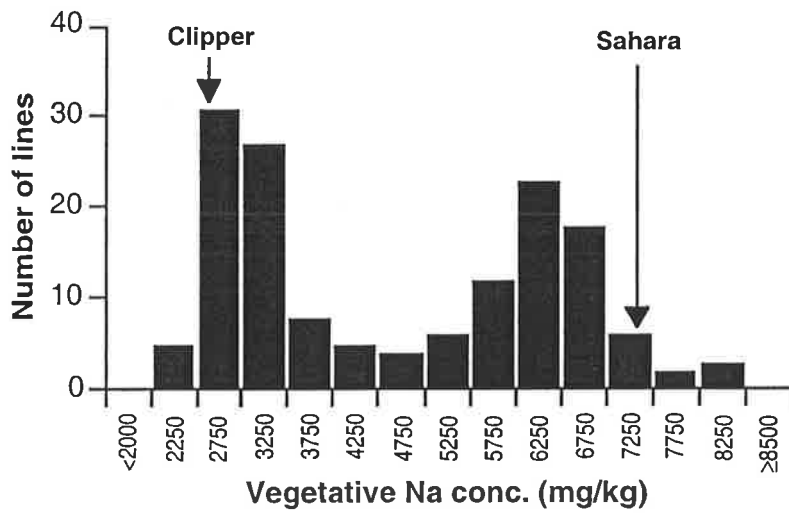
Figure 5.6

Frequency distribution for shoot sodium concentration of 150 doubled haploid barley lines and their parents, Clipper and Sahara, grown in Wangary soil for 28 days.
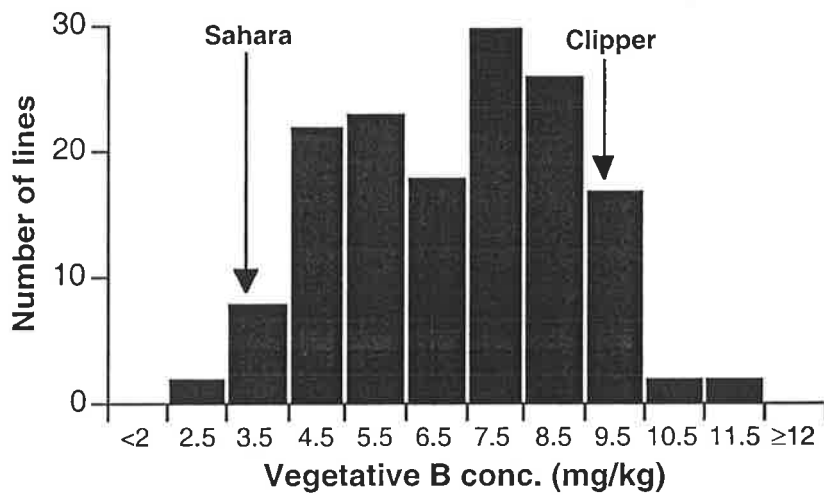
Figure 5.7

Frequency distribution for shoot boron concentration of 150 doubled haploid barley lines and their parents, Clipper and Sahara, grown in Wangary soil for 28 days.

5.3.3 Mapping

Marker analysis of Zn concentration and Zn content identified the same region on the long arm of chromosome 4H (Figure 5.8) as being associated with both of these traits. The LOD score for Zn concentration is presented in Figure 5.8, while the maximum LOD score for Zn content was 14.85. This region coincided with Mne-1(B), one of three homologous positions of a cDNA clone, Mne-1, isolated by Huang (1996) from the Mn-efficient cultivar Weeah as being associated with differential expression of Mn efficiency. Interval analysis showed that this locus accounted for approximately 60% of the total variation in Zn concentration and approximately 59% of the total variation in Zn content. Marker analysis of shoot dry weight failed to show any significant association with this region or with any other markers across the genome.

The bimodal distribution of shoot Na concentration of the doubled haploid lines is characteristic of segregation at a single gene. Marker analysis identified a highly significant region on the short arm of chromosome 1H associated with this trait (Figure 5.9). Shoot B concentration was associated with a region on the short arm of chromosome 4H (Figure 5.10). Jefferies *et al.* (1999) also detected this QTL in a B toxicity bioassay using the same Clipper x Sahara doubled haploid population. Analysis of all other elements showed no significant association with any markers across the genome.

GERMIN
AWBMA30
WG876
BCD808(B)
BCD265(A)
AWBMA29
PSR163
Mne(B)
WG232
CDO795
CDO358
PSR141(A)
WG464
ABC152(B)
CDO541
ABC152(A)
Mne-1(B)
WG719
ksuG10
WG114
BSP
CDO669
CDO1312°
CDO63
BCD1130
ksuE2
ABC305
HVM67

15.10

3.0   0.0

LOD
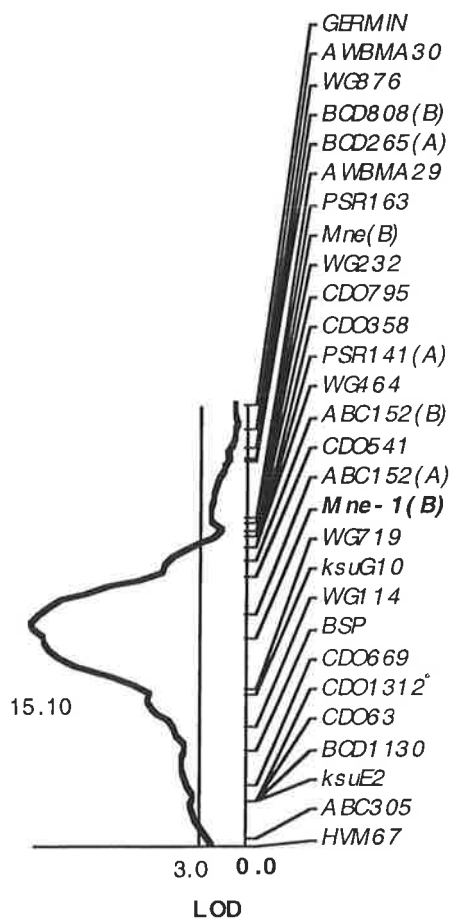
Figure 5.8

Location of a region on chromosome 4H associated with shoot zinc concentration expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is toward the top of the chromosome. LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

ksuD14
BCD402
HOR1
PSR381
ksuE18(A)
AWBMA8
AWBMA12
ksuA3(C)
BCD340(A)
AWBMA24
CDO673
BCD147
BCD340(B)
CDO105
WG789(A)
WG789(D)
BCD351(B)
AWRM1
PSR158
PSR121
El(PCR)
El
HVM2
AWBMA34
BCD454
AWWM3
CDO473
WG184
BCD808 (C)
BCD304
BCD265(C)
X7 GLOB
AWBMA4
ABG373

32.04

3.0 0.0

LOD
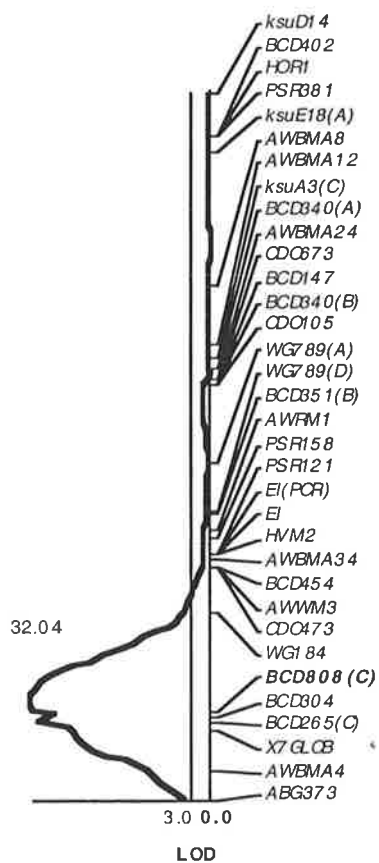
Figure 5.9

Location of a region on chromosome 1H associated with shoot sodium concentration expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is toward the top of the chromosome. LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

GERMIN
AWBMA30
WG876
BCD808(B)
BCD265(A)
AWBMA29
PSR163
Mne(B)
WG232
CDO795
CDO358
PSR141(A)
WG464
ABC152(B)
CDO541
ABC152(A)
Mne-1(B)
WG719
ksuG10
WG114
BSP
CDO669
CDO1312
CDO63
BCD1130
ksuE2
ABC305
HVM67

34.73

3.0 0.0

LOD
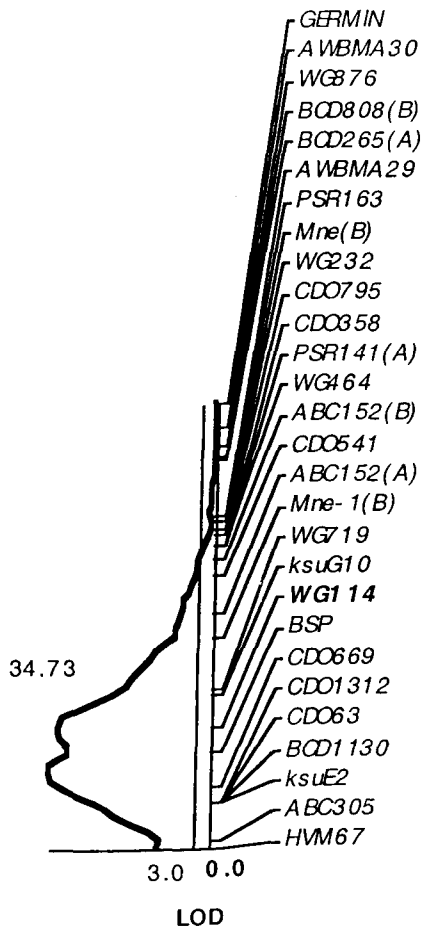
Figure 5.10

Location of a region on chromosome 4H associated with shoot boron concentration expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is toward the top of the chromosome. LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

5.4 Discussion

In determining an appropriate bioassay for the screening of 150 doubled haploid lines, it was necessary to adopt a method that would result in significant differences between lines. Although shoot Zn concentration and content of young tissue underestimated the total value of these parameters, particularly Zn content, the relative difference between genotypes remained reasonably constant, suggesting that analysis of young leaves was a suitable method for screening the doubled haploid lines.

Using the parents as a measure of the spread of the population, the highest and lowest values of a particular variable can be predicted. This assumes that the population is segregating for that variable and no transgressive segregation is occurring. Since gene mapping relies on variation in expression of a trait among individuals of a population, the larger the variation the greater is the chance of separating lines into discrete groups and therefore locating QTLs or major genes. For the variables of interest, namely Zn concentration and Zn content, the greatest differences occurred at treatment 1 (low Zn, low Mn) and treatment 4 (high Zn, high Mn) respectively. The use of treatment 1 was rejected on the basis of confounding effects caused by differences in Mn efficiency between the parents. Experiment 5A showed Sahara to be less Mn-efficient than Clipper. The reduction in shoot dry weight of Sahara under Mn-deficiency was greater than that of Clipper, resulting in an inflated difference in Zn concentration. If this treatment had been used in the screening procedure, there would be an immediate disadvantage to the Mn-inefficient lines which affects the measurement of Zn concentration. For this reason treatment 4 was chosen as the level of soil fertility under which the doubled haploid lines would be screened.

Furthermore, treatment 4 reduced the confounding effects of different starting seed nutrient reserves. Studies of wheat (Singh and Bharti, 1985; Marcar and Graham, 1986) found that lower Mn content of seeds resulted in depressed growth of seedlings, particularly at deficient levels of soil Mn. Similarly, Rengel and Graham (1995a) and Genc *et al.* (2000) found that differences in vegetative growth of seedlings derived from high-Zn seed and low-Zn seed are more pronounced at lower levels of soil Zn, becoming negligible as soil Zn approaches sufficiency. As the Clipper x Sahara doubled haploid population used here varied widely for both Zn and Mn content in the seed, despite being produced under similar conditions, sufficient supply of nutrients ensured that all lines achieved their full growth potential.

The distribution of Zn concentration of the doubled haploids suggests that positive transgressive segregation may be occurring; however only one putative gene was identified

from the mapping studies. This, together with the fact that no line was significantly lower than Clipper, indicates an alternate explanation. Inspection of the distribution of shoot weight shows it to be more indicative of true transgressive segregation with several lines greater than Sahara and several lines less than Clipper. Since concentration is affected by weight it is likely that the segregation of this character was responsible for the apparent positive transgressive segregation of Zn concentration.

The co-segregation of the Zn concentration/content character with a member of the gene family encoding the cDNA clone of Huang (1996) is interesting as this clone encodes a protein with a single Zn finger structure that is increased in abundance in Mn-efficient cultivars grown in Mn-deficient conditions. The role of Zn in such proteins is to stabilise folded conformations of domains in order to facilitate interactions between the proteins and other macromolecules such as DNA (Berg and Shi, 1996). In this way, Zn finger proteins can be involved in DNA repair (Culp et al., 1988; Tanaka et al., 1990) and transcription regulation (Berg, 1990). Until this study, no association between Mne-1 and Zn accumulation was known. Huang (1996) postulated that the Mne-1 protein may be able to bind Zn ions from studies using in vitro expressed protein and ^{65}Zn uptake. In his studies, however, the protein was accumulated at an equal level in both Mn-efficient and Mn-inefficient genotypes at high Mn supply (viz treatment 4) (although that analysis did not discriminate between different gene family members). This finding, together with the protein's likely function as a Zn-finger transcription factor probably precludes a direct role as a Zn carrier. More likely, the gene family members may act as transcriptional regulators of micronutrient accumulation. The expression pattern of the gene Mne 1B could be examined to determine if a correlation exists with the expression of enhanced Zn accumulation. Alternatively, the Zn concentration/content locus may correspond to a gene that is very close to but not actually at the Mne-1 locus. This could be tested genetically by screening for recombinants between the two traits. Such a study requires a large doubled haploid population, which is unfortunately not available at present.

The Na accumulation locus on chromosome 1H could possibly play a role in salt tolerance as salt inclusion or salt exclusion have been demonstrated as mechanisms of tolerance to salinity (Maas and Nieman, 1978; Collander, 1941). It should be noted that this locus is different to Kna1, a K^+/Na^+ discrimination locus on the long arm of chromosome 4D in wheat which confers higher salt tolerance (Dubcovsky *et al.*, 1996).

The B accumulation locus was in the same region as that identified by Jefferies *et al.* (1999). In their study they found this locus to be significantly associated with tolerance to B toxicity as measured by four different parameters (whole-shoot B concentration, realative root length, leaf-symptom score and whole-shoot dry weight). A significant association was identified between B tolerance and an RFLP marker XKsuG10 on chromosome 4A of wheat (Paull *et al.*, 1993). It is possible that wheat and barley may possess a common gene on chromosomes 4A and 4H respectively (Jefferies *et al.*, 1999).

Chapter 6

Effect of Growth Medium on Zinc Accumulation Character

6.1 Introduction

In the previous chapter a differential in vegetative zinc accumulation between the varieties Sahara and Clipper was detected under certain growth conditions and the character mapped to a region on the short arm of chromosome 4H. The existence of a suitable cDNA marker for this region, tentatively called *Mne-1-2* (Huang, unpublished) encouraged attempts to further understand the nature of this zinc accumulation character and determine its applicability to a breeding program.

There are several aspects relating to successful incorporation of a character into a breeding program. According to Graham *et al.* (1999) there must be genetic variation for the trait, phenotype or genotype must be measurable efficiently and expression of the trait should be relatively stable across a range of environments. Genetic variation and, to some degree, efficiency of measurement was demonstrated in Chapter 5. In order to test the stability of expression of the differential zinc accumulation trait, Sahara and Clipper were grown under a range of conditions with respect to the growth medium. Other environmental conditions such as light intensity, temperature and relative humidity were kept constant. In the first instance plants were grown in solution culture at high and low levels of zinc. Solution culture techniques are useful in that they provide experimental root material that is free of mineral coatings and soil particles (Kochian, 1993). This allows accurate ICP analysis, providing important information regarding the partitioning of zinc between roots and shoots. It has been shown that zinc-efficient wheat genotypes will transport more zinc from roots to shoots under zinc deficiency than zinc-inefficient genotypes (Cakmak *et al.*, 1996; Rengel *et al.*, 1998) so that partitioning may be an

important mechanism in determining zinc efficiency.

As the second component of determining stability of expression, the parents Sahara and Clipper were grown in a range of soil types. Zinc supply and uptake by roots are affected by soil factors such as pH (Barber, 1984; Moraghan and Mascagni, 1991) soil organic matter (Brennan, 1992; Sharma and Deb, 1988) and interactions with other nutrients (comprehensively reviewed by Loneragan and Webb, 1993). Measurement of zinc accumulation in plants grown in these soils will identify if the allele for the character is likely to consistently provide a phenotypic advantage.

Another requirement for successful incorporation into a breeding program is a reliable bioassay. In order to determine the repeatability of the observed zinc differential, the parents Clipper and Sahara, together with a selection of the doubled haploids, were grown under the same conditions described in Chapter 5. This is an important factor in consideration of marker assisted breeding. If this character was determined to be a desired agronomic trait and included in a breeding program, the expense devoted to marker assisted selection must be justified by certainty of the result. Finally, a preliminary RNA analysis was performed on the parents to investigate *Mne-1-2* expression in roots. As this marker cosegregated with the zinc accumulation locus (Figure 5.8), it was of interest to determine whether analysis of expression of the gene might identify a role in the zinc accumulation trait.

6.2 Materials and methods

6.2.1 Experiment 6A - Solution culture

Plants were grown using a chelate-buffered nutrient solution system similar to that described by Webb et al. (1993). The basal nutrient solution was composed of the following macronutrients (mM)-: KNO_3, 1.5; $Ca(NO_3)_2$, 1.0; $NH_4H_2PO_4$, 0.2; $MgSO_4$, 0.25 and micronutrients (μM); KCl, 50; H_3BO_3, 12.5; H_2MoO_4, 0.1; FeHEDTA, 20; CuHEDTA, 1.0; MnHEDTA, 0.4; NiHEDTA, 0.1 and K_3HEDTA, 25 (buffered to pH 6.0 with 5mM MES buffer). Zinc treatments were applied by adding ZnHEDTA at two concentrations, 0.2μM and 4μM, hereafter referred to as $Zn_{0.2}$ and Zn_4. The solution was

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changed every 5 days to limit nutrient depletion and pH fluctuations.

Barley seeds of the varieties Clipper and Sahara were surface-sterilised by soaking in 70% ethanol for 1 minute, rinsed with deionised water and then soaked in sodium hypochlorite (1.4% v/v) for 6 minutes before rinsing again with deionised water. The seed was germinated on moistened Whatman No. 42, ashless filter paper for 36 hours at 20^0C before transferring to seedling cups (hollow polyethylene stoppers with bottoms severed and replaced with polyethylene mesh). Three seeds were sown per cup and covered with black polyethylene beads to support seedlings and prevent entry of light into the rooting medium. Six seedling cups (3 Clipper, 3 Sahara) were located in the tops of each of four 6 litre black polyethylene pots (2 $Zn_{0.4}$, 2 Zn_4). One replicate of both genotypes from each pot was harvested at 21 days, 28 days and 35 days hereafter referred to as $Harv_1$, $Harv_2$ and $Harv_3$. The design was therefore -:

2 genotypes x 2 zinc treatments x 3 harvests x 2 replicates = 24 combinations in 4 pots.

Plants were grown in a controlled environment growth chamber with a 12-hour light/12-hour dark photoperiod at 20^0C day/10^0C night. Light was supplied by high-pressure mixed-metal halide lamps delivering a photon flux density of 500 $\mu mol/m^2/s$ at the surface of the pot.

At each harvest plants were divided into roots and shoots, rinsed in deionised water for 30 s, blotted dry on paper towelling, then oven-dried at 70°C for 24 hours. Dried samples were digested with 70% nitric acid and analysed for concentrations of the elements Zn, Mn, Fe, Cu, B, Ca, Mg, Na, K, P and S by ICPAES.

Data were analysed as a three-way analysis of variance using the computer program SuperANOVA™. The factors for the ANOVA model were genotype, Zn treatment and harvest date. To overcome problems associated with non-homogeneity of variances, values for zinc concentration and content were log transformed prior to statistical analysis.

6.2.2 Experiment 6B - Soil culture

The soils used were-:

1- the top layer (0-20cm) of a sodic, red duplex soil (Dr 2.23, Northcote 1979) collected from near Two Wells on the Northern Adelaide Plains (pH in water = 7.1; ESP= 8.8) hereafter referred to as Sod_{top}.

2- the sub layer (20-50cm) of the same soil (pH in water = 9.0; ESP= 33.1) hereafter referred to as Sod_{sub}.

3- a black cracking clay (Ug 5.11, Northcote 1979) collected from near Millicent in the south east of South Australia (pH in water = 6.9) hereafter referred to as Clay.

4- a calcareous sand (Gc 1.2, Northcote 1979) collected from near Mannum in the Murray Mallee region of South Australia (pH in water = 7.2) hereafter referred to as Sand

5- a recycled loam (see Appendix 4 for content) produced at the Waite Campus (pH in water = 6.3) hereafter referred to as RL.

6- a U.C. (University of California) mix (see Appendix 4 for content) produced at the Waite Campus (pH in water = 6.8) hereafter referred to as UC.

Clear plastic sample jars (6.5cm diameter x 15cm height) covered with aluminium foil and lined with a polyethylene bag were filled with soil. Due to differences in soil properties soil dry weights per pot varied considerably (Table 6.1). Seeds of the varieties Clipper and Sahara were surface sterilised and germinated as in Experiment 6A and sown at a rate of three per pot. Due to variation in the amount of soil available, the number of replicates was either 2 (Sod_{top} and Sod_{sub}), 3 (UC and RL) or 4 (Sand and Clay). Growth chamber conditions were the same as for previous experiments. Pots were watered to weight daily to maintain field capacity (see Table 6.1) and rotated within a block to minimise the effect of microenvironments caused by uneven light distribution and/or air flow. After 3 weeks, all pots were fertilised with $Ca(NO_3)_2.4H_2O$ (918 mg/kg dry soil) and KH_2PO_4 (179.5 mg/kg dry soil).

After 6 weeks, whole shoots were harvested and oven-dried at 70°C for 24 hours. Roots were washed free of soil and also oven-dried. Dried shoot samples were digested with 70% nitric acid and analysed for elemental concentrations as in previous experiments.

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Data were analysed as a two-way analysis of variance using the computer program SuperANOVA™, with genotype and soil forming the relevant factors.

<u>Table 6.1</u>

Soil dry weights per pot and water added to achieve field capacity for 6 different soils in Experiment 6C.

	Soil Type					
	Sod$_{top}$	Sod$_{sub}$	Sand	Clay	RL	UC
dry soil/pot (g)	440	390	520	295	600	520
water added (%)	21	25	19	27	29	32

6.2.3 Experiment 6C - Repeatability of Wangary soil bioassay and RNA analysis

6.2.3.1 Soil preparation and experimental design

The soil used was the same calcareous sand from Wangary, South Australia, described in Chapter 5. Pot size and methods of soil preparation were the same as that used in Section 5.2.2 for the mapping study, as were light and temperature regimes.

Seeds of Sahara, Clipper and 20 of the doubled haploid lines (10 high zinc accumulators, 10 low zinc accumulators) were surface-sterilised and germinated as for previous experiments before sowing (3 per pot). The experiment was set up as a randomised complete block with 2 replicates. The parents Sahara and Clipper appeared 3 times in each block. Thus, there were

(20 doubled haploids + 3 Sahara + 3 Clipper) x 2 replicates = 52 pots

After 28 days, whole plant shoots were harvested and oven-dried at 70°C for 24 hours before undergoing ICP analysis. Shoot zinc concentrations obtained from this experiment were regressed against those obtained from Chapter 5 for the same genotypes in order to test the repeatability of the bioassay.

6.2.3.2 Root RNA extraction

In order to conduct an RNA analysis on the parents, roots of all replicates of Clipper and Sahara were washed free of soil with water prepared by reverse osmosis, blotted dry to remove excess water, then placed in plastic tubes and snap frozen in liquid nitrogen. To isolate total RNA, root tissue was ground to a powder under liquid nitrogen

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using a mortar and pestle. Approximately 100mg of the powder was transferred to a 1.5mL Eppendorf tube, 1mL of TRIZOL® reagent added, vortexed and left at room temperature for 5-15 minutes. The tube was then centrifuged at 12,000 g and 4°C for 10 minutes to pellet polysaccharides. The supernatant was transferred to a new tube, 200 μL of chloroform added, then shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. The tube was then centrifuged at 11,200 g and 4°C for 15 minutes. The aqueous phase was collected and transferred to a fresh Eppendorf tube and RNA precipitated with 500 μL of isopropyl alcohol at room temperature for 10 minutes. The tube was then centrifuged at 12,000 g and 4°C for 10 minutes. The supernatant was removed and the pellet washed with RNAse-free 75% EtOH (1mL). This was vortexed, then centrifuged at 9,000 g and 4°C for 5 minutes. The pellet was dried and then resuspended in 10 μL of nanopure water. Absorbance of RNA samples was measured at several wavelengths (220-320nm at 20nm intervals) using a UV spectrophotometer. RNA concentration was calculated using the relationship-: 1 unit A_{260}=40μL/mL RNA (Table 6.2).

Table 6.2

Absorbance (λ=260nm) and RNA concentration of samples used in Experiment 6C.

(The numbers 2 or 3 refer to the replicate of either Clipper or Sahara used).

	Sahara 2	Sahara 3	Clipper 2	Clipper 3
Absorbance (λ=260nm)	0.118	0.125	0.148	0.105
RNA conc. (μL/mL)	4.72	5.00	5.92	4.20

6.2.3.3 RNA analysis to determine Mne-1-2 gene expression

For RNA electrophoresis, an agarose gel (1.2% Seakem GTG agarose) containing MOPS buffer and 3% formaldehyde was prepared. To ensure an even loading of RNA samples, an aliquot containing 10 μL of total RNA (calculated from concentrations for each sample) was mixed with nanopure water to make a total volume of 3.0 μL (Table 6.3). This was mixed with 2.0 μL of 10x MOPS, 10 μL deionised formamide and 3.5 μL formaldehyde, heated at 65°C for 10 minutes and chilled on ice. Two μL of 10x loading

buffer was added to each sample. The RNA samples were loaded into the gel and run in MOPS buffer at 60 V for 2-3 hours. Then, the gel was stained in 1 µg/mL ethidium bromide for 15 minutes followed by destaining in water for 30 minutes.

Table 6.3

Calculation of volume of RNA sample used in Experiment 6C.

	Sahara 2	Sahara 3	Clipper 2	Clipper 3
volume containing 10µL RNA (µL)	2.1	2.0	1.7	2.4
H$_2$O (µL)	1.4	1.5	1.8	1.1
total volume (µL)	3.0	3.0	3.0	3.0

To transfer the RNA to a membrane, the gel was washed with water and soaked in high efficiency transfer solution (Biotecx) for 15 minutes. Capillary transfer of RNA from gel to Hybond-N$^+$ membrane was carried out using the high efficiency transfer solution overnight, followed by briefly rinsing in H$_2$O and blotting dry with Whatman 3MM paper. The membrane was dried under vacuum at 80^{0}C for 1 hour.

The membrane was then prehybridised overnight at 42^{0}C in a solution containing 45% deionised formamide, 5x SSPE, 7.5x Denhardt's reagent, 0.5% SDS and 500 µg/mL denatured salmon sperm DNA. The membrane was then hybridised for 48 h at 42^{0}C in a solution containing 45% deionised formamide, 5x SSPE, 7.5x Denhardt's reagent, 0.5% SDS, 2.5% dextran sulphate, 250 µg/ml denatured salmon sperm DNA and a denatured ^{32}P-labelled probe. The probe was synthesised by random labelling of the cDNA insert *Mne-1-2* amplified by PCR. Following hybridisation, the membrane was washed sequentially in 2x SSC + 0.1% SDS at 65^{0}C for 15 minutes, in 1x SSC + 0.1% SDS at 65^{0}C for 15 minutes, 0.5x SSC + 0.1% SDS at 65^{0}C for 15 minutes and 0.2 SSC + 0.1% SDS at 65^{0}C for 15 minutes until no background signals could be detected. Then, the membrane was blotted dry, wrapped in a polyethylene bag and exposed to X-ray film in a cassette at -80^{0}C for 6 days. The film was developed in Curix 60 developer (AGFA).

6.3 Results

6.3.1 Experiment 6A

Visual Symptoms

There were no gross zinc deficiency symptoms as experienced in earlier soil culture experiments using Laffer sand (Chapters 3 and 4). The only noticeable effect was a slight yellowing of overall leaf colour in the low zinc treatment for both genotypes.

Shoot dry weights

Shoot dry weights were strongly ($P \leq 0.0001$) affected by harvest date and more weakly ($P \leq 0.05$) by genotype (see Appendix 1 (Section 6.1) for ANOVA table). Shoot dry weight increased with each harvest as expected (Figure 6.1a) with Clipper being greater than Sahara at all treatments except for Zn_4 $Harv_3$ at which there was no significant difference. There was no effect of zinc treatment on shoot dry weight and no interactive effects of any order.

Root dry weights

Root dry weights were strongly ($P \leq 0.0001$) affected by harvest date and genotype (see Appendix 1 (Section 6.1) for ANOVA table). There was an increase with each harvest and Clipper was consistently heavier than Sahara (Figure 6.1a). There was no effect of zinc treatment on root dry weight; however, there were some significant interactive effects. For Clipper, root dry weights at $Zn_{0.4}$ were heavier than at Zn_4 whereas for Sahara, the trend was reversed, resulting in a significant ($P \leq 0.01$) genotype x Zn effect. Also, Clipper root dry weights increased at a greater rate than Sahara between successive harvests resulting in a significant ($P \leq 0.001$) genotype x harvest effect. These interactions also contributed to a less significant ($P \leq 0.05$) genotype x Zn x harvest effect.
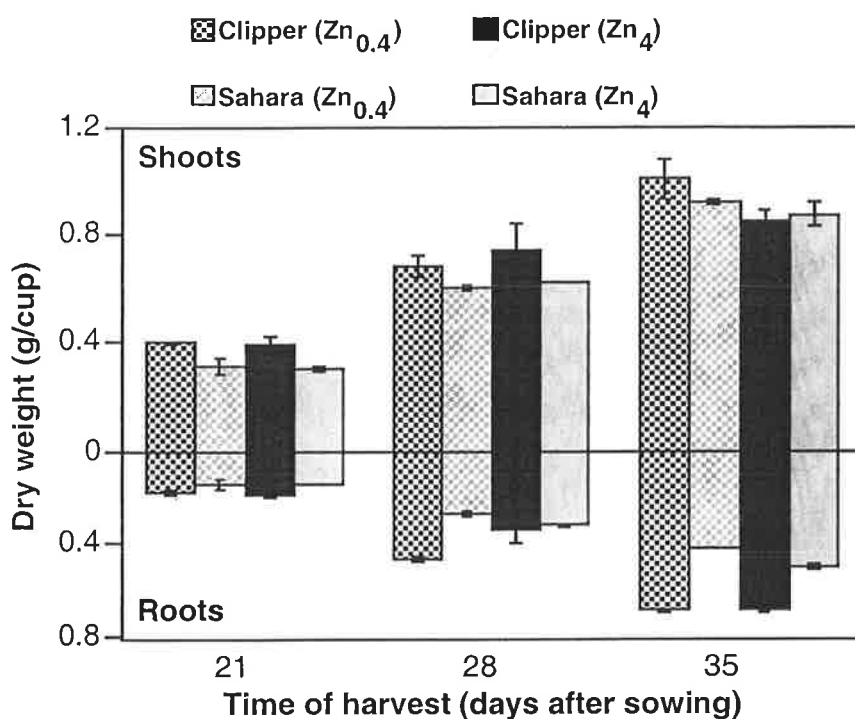
Figure 6.1a

Shoot and root dry weights of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc levels. Harvests were taken at 21, 28 and 35 days after sowing. Values are an average of two replicates with standard errors shown.

Shoot zinc concentrations and contents

Shoot zinc concentrations were significantly affected by Zn treatment, harvest and genotype (see Appendix 1 (Section 6.1) for ANOVA of log-transformed data). As expected, there was a large difference between treatments with the addition of zinc increasing shoot zinc concentrations by up to four times (Figure 6.1b). Shoot zinc concentrations decreased from the first to the third harvest and at a greater rate under $Zn_{0.4}$ than Zn_4 resulting in a significant ($P \le 0.0001$) Zn x harvest effect. There was no difference in shoot zinc concentration between Sahara and Clipper at Zn_4 but at $Zn_{0.4}$ the shoot zinc concentration in Sahara was greater than that in Clipper resulting in a significant ($P \le 0.01$) genotype x Zn effect.

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Figure 6.1b

Shoot and root zinc concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values are an average of two replicates with standard errors shown. The dotted line represents the critical zinc concentration for deficiency in shoots of barley (Reuter *et al.*, 1997).

Shoot zinc content was strongly ($P \leq 0.0001$) affected by zinc treatment and more weakly ($P \leq 0.05$) by harvest date (see Appendix 1 (Section 6.1) for ANOVA of log-transformed data). There was no genotype effect. The addition of zinc increased shoot zinc contents approximately fourfold at the later harvest dates (Figure 6.1c). Shoot zinc contents increased over time at Zn_4 but showed no significant change at $Zn_{0.4}$ as reflected in a significant Zn x harvest interaction. While there was no other significant interaction, it is interesting to note that shoot zinc contents of Clipper were consistently slightly higher than Sahara at Zn_4 while the trend was reversed at $Zn_{0.4}$.
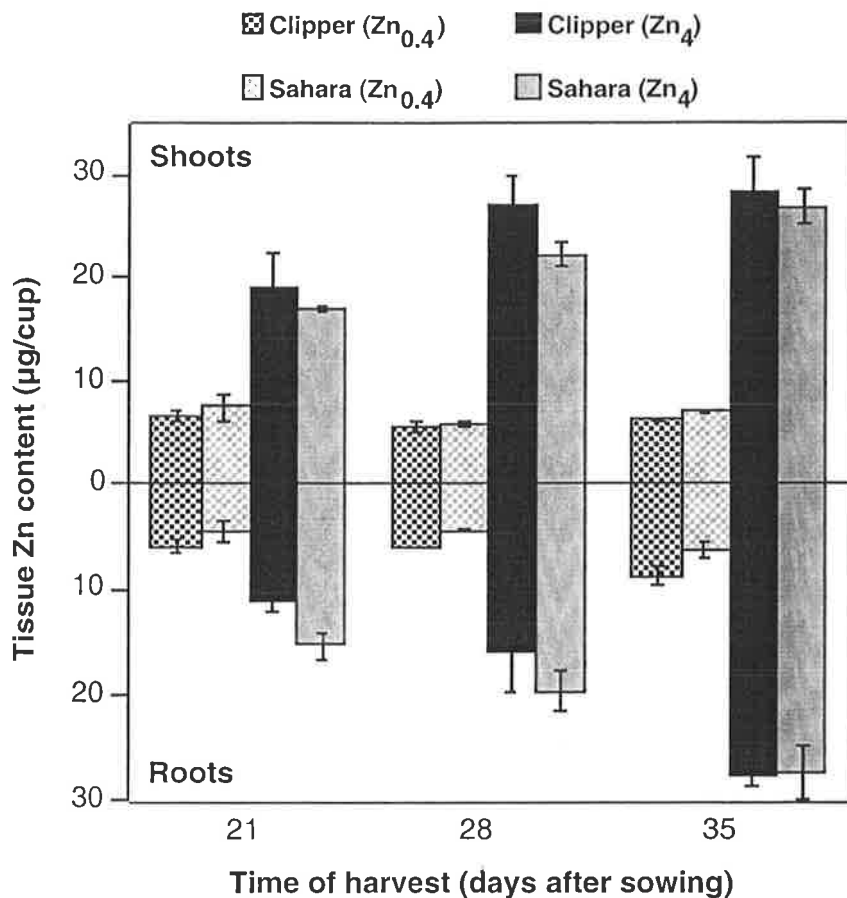
Figure 6.1c

Shoot and root zinc contents of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values are an average of two replicates with standard errors shown.

Root zinc concentrations and contents

Root zinc concentrations were strongly ($P \leq 0.0001$) affected by all three factors (see Appendix 1 (Section 6.1) for ANOVA of log-transformed data). In a similar manner to shoot zinc results, root zinc concentrations were greater at Zn_4 and decreased over time (Figure 6.1b). As was the case for shoot zinc concentrations, root zinc also decreased at a greater rate under $Zn_{0.4}$ than under Zn_4 resulting in a significant ($P \leq 0.01$) Zn x harvest effect. The difference in root zinc concentrations between Sahara and Clipper was larger at Zn_4 than at $Zn_{0.4}$ resulting in a significant ($P \leq 0.01$) genotype x Zn effect. These interactions also contributed to a significant ($P \leq 0.05$) genotype x Zn x harvest effect.

Root zinc contents were strongly (P≤0.0001) affected by zinc treatment and harvest date (see Appendix 1 (Section 6.1) for ANOVA of log-transformed data). There was no genotypic effect. Addition of zinc increased root zinc contents by approximately 2-4 times (Figure 6.1c). There was a rapid increase over time at Zn_4 while at $Zn_{0.4}$ root zinc contents increased only slightly. In direct contrast to shoot zinc contents, Clipper showed consistently higher root zinc contents at $Zn_{0.4}$ while Sahara was greater at Zn_4, $Harv_1$ and $Harv_2$ and approximately equal to Clipper at $Harv_3$. This observation is reflected in a significant value for the genotype x Zn interaction. There was no other significant interaction of any order.

Other elements

Shoot concentrations of manganese were most notably affected by genotype with values for Clipper much higher than those for Sahara (Figure 6.1d). Shoot concentrations of manganese were deficient for Sahara at both zinc treatments. Values for Clipper increased slightly over time while those for Sahara remained virtually unchanged. Root manganese concentrations remained similar over time (Figure 6.1d) except for Clipper grown at $Zn_{0.4}$ which decreased over time. Its value at $Harv_1$ was much higher than that of the other three genotype by treatment combinations but, by $Harv_3$, it was only slightly higher.

Root concentrations of iron were maintained at 2-3 times that of the shoot value (Figure 6.1e). At $Harv_1$, zinc treatment affected root iron concentration with $Zn_{0.4}$ exhibiting much larger values than Zn_4. At $Harv_3$, however, the genotype effect was more noticeable with Sahara having greater root iron concentrations than Clipper. Shoot iron concentrations were also affected by genotype at $Harv_3$ but, in contrast to the root concentrations, shoot concentrations were greater in Clipper than in Sahara.
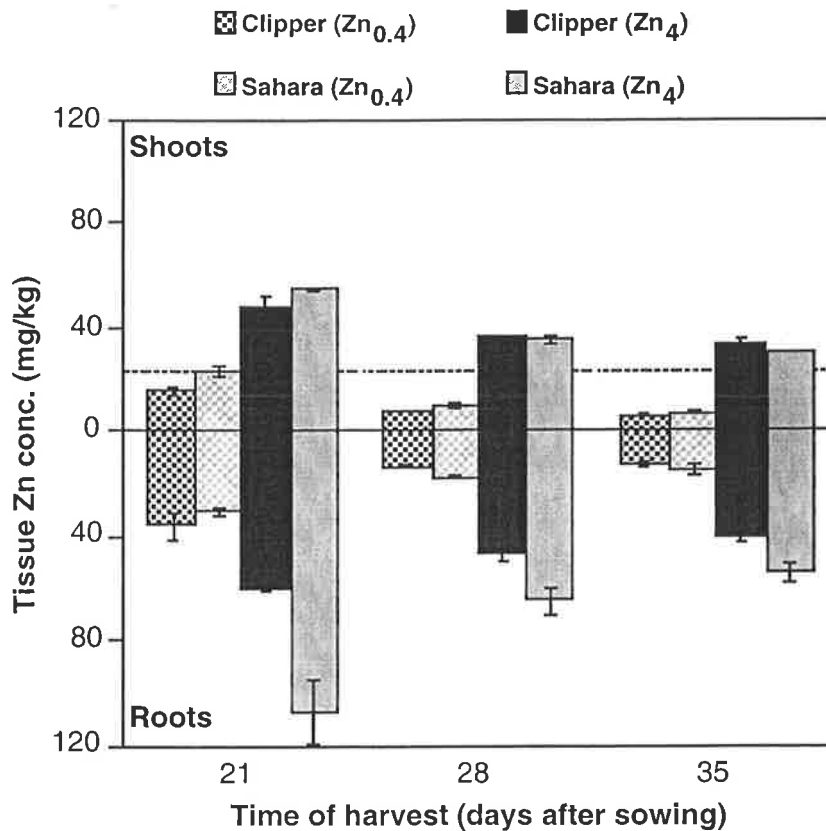
Figure 6.1d

Shoot and root manganese concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values represent an average of two replicates with standard errors shown. The dotted line represents the critical manganese concentration for deficiency in barley shoots (Reuter *et al.*, 1997).
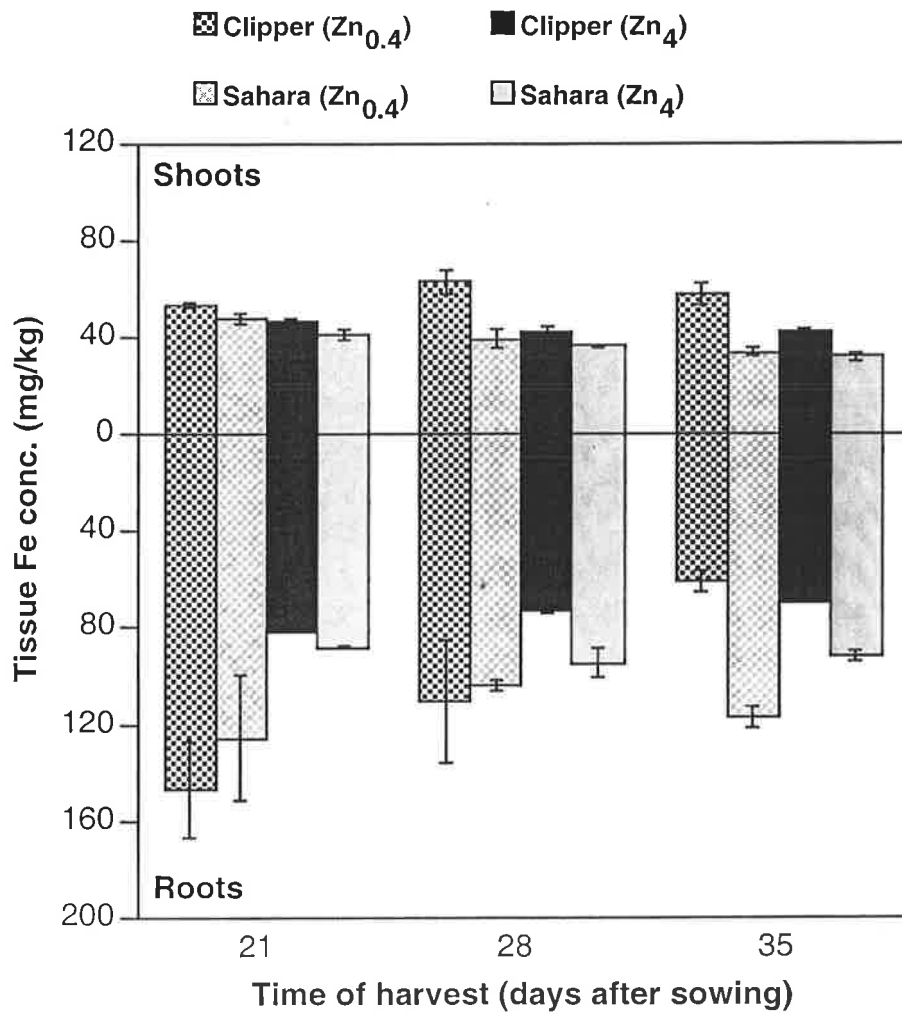
Figure 6.1e

Shoot and root iron concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values represent an average of two replicates with standard errors shown.

Tissue concentrations of copper followed a similar trend to iron with root concentrations maintained at 2-4 times higher than shoot concentrations in general, but as much as 9 times higher in the case of Clipper $Zn_{0.4}$, $Harv_1$ (Figure 6.1f). Similarly to iron, there was a zinc treatment effect at $Harv_1$ but this gave way to an overriding genotype effect by $Harv_3$. In contrast to iron, copper concentrations in both roots and shoots decreased over time.
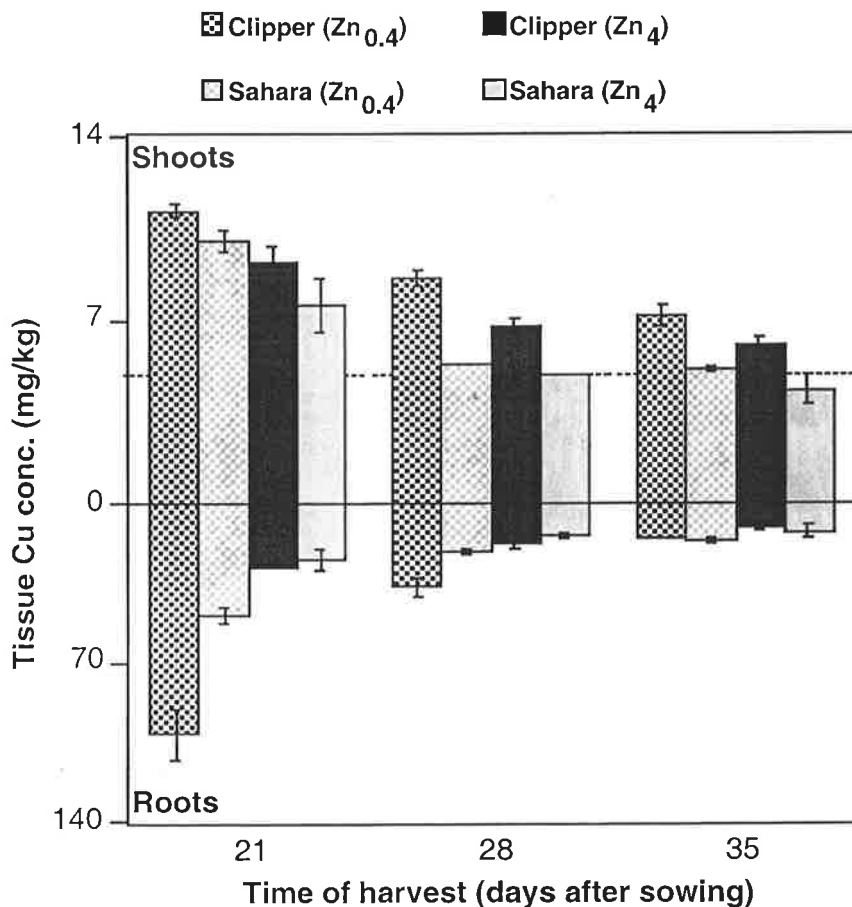
Figure 6.1f

Shoot and root copper concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values represent an average of two replicates with standard errors shown. (Note the scale difference between root and shoot tissue). The dotted line represents the critical copper concentration for deficiency in shoots of barley (Reuter *et al.*, 1997).

Shoot boron concentrations were less than adequate at $Harv_1$ and continued to decrease over time, showing an obvious zinc treatment effect with plants grown at $Zn_{0.4}$ having greater concentrations than at Zn_4 (Figure 6.1g). Root boron concentrations showed a similar treatment effect except for plants grown at Zn_4 which increased between $Harv_1$ and $Harv_2$ before decreasing again by $Harv_3$.
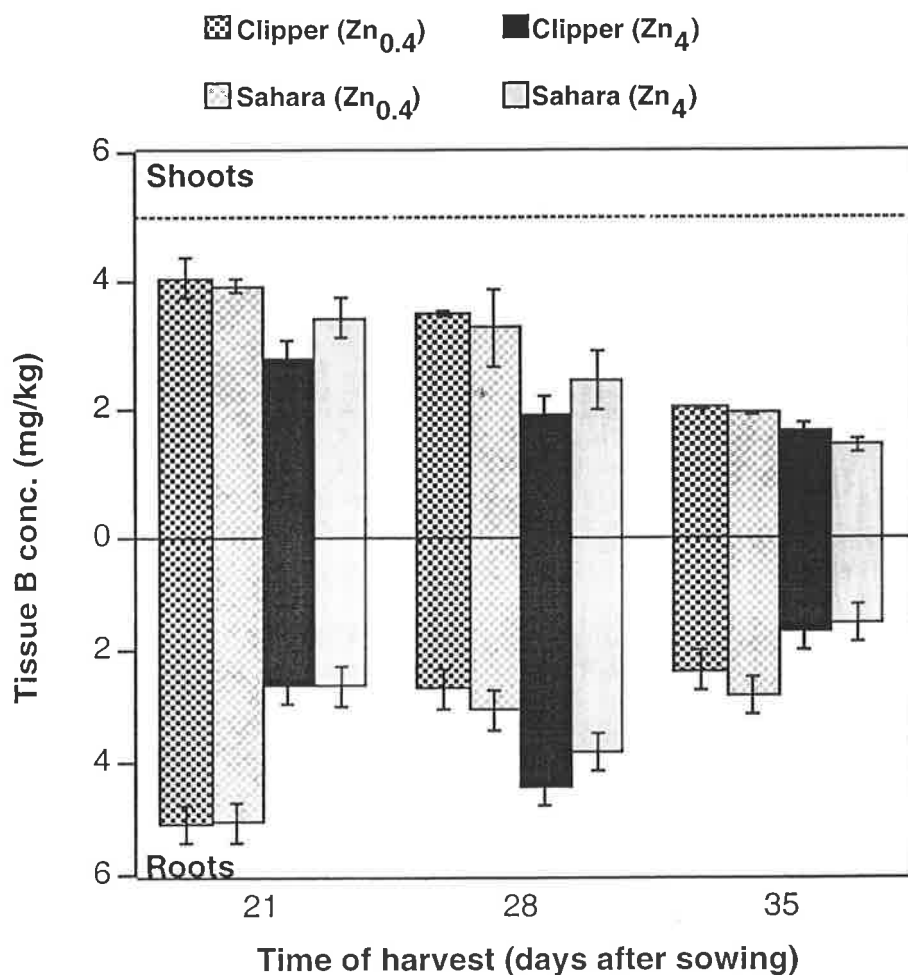
Figure 6.1g

Shoot and root boron concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values represent an average of two replicates with standard errors shown. The dotted line represents the lower limit of adequate boron concentration in shoots of barley (Reuter *et al.*, 1997).

Shoot phosphorus concentrations decreased significantly between $Harv_1$ and $Harv_2$ with no difference between $Harv_2$ and $Harv_3$ (Figure 6.1h). There was a genotype effect with Clipper having higher concentrations than Sahara although both had concentrations below the critical concentration for deficiency. There was also a treatment effect with higher phosphorus concentrations maintained at $Zn_{0.4}$. Root phosphorus concentrations followed the same trend as shoot phosphorus concentrations (Figure 6.1h).

Figure 6.1h

Shoot and root phosphorus concentrations of barley varieties Clipper and Sahara grown in chelate buffered nutrient solution at two zinc concentrations. Harvests were taken at 21, 28 and 35 days after sowing. Values represent an av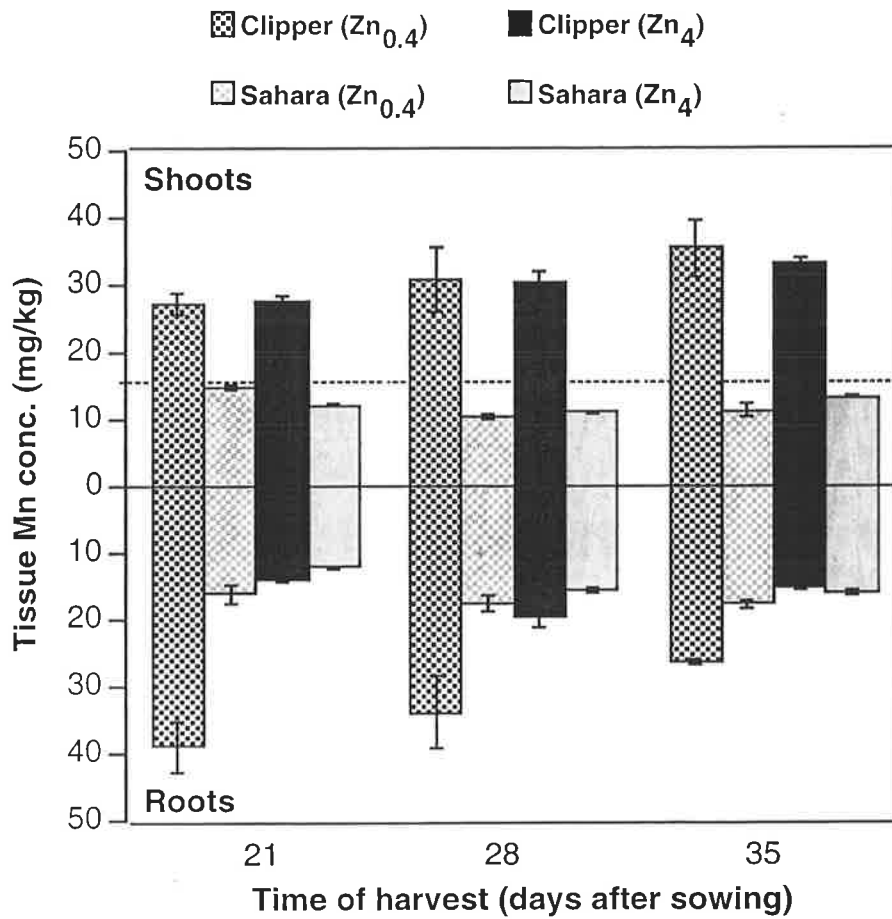erage of two replicates with standard errors shown. The dotted line represents the lower limit of critical phosphorus concentration for deficiency in shoots of barley (Reuter *et al.*, 1997).

6.3.2 Experiment 6B

Visual Appearance

Growth of both Clipper and Sahara differed noticeably among soil types, with plants grown in RL and UC showing the best growth and those grown in Sod_{top} showing

the least. Plants grown in UC and RL displayed heavy tillering and a deep green colour. There were no obvious symptoms of nutrient deficiency or toxicity. Plants grown in Clay were of a similar height to those grown in UC and RL but less tillered. Clipper grown in Sand was shorter and less tillered than those grown in UC and RL but Sahara, while appearing shorter, was heavily tillered. Again, there was no obvious indication of nutrient deficiency or toxicity in plants grown in either Clay or Sand, despite the fact that they were a lighter green in colour.

Clipper grown in the sodic soils and, in particular, the subsoil, was affected by boron toxicity. Symptoms included chlorosis and necrosis of older leaves, extending backward from leaf tips. Surprisingly, although Clipper grown in Sod_{sub} showed more extensive boron toxicity symptoms, plants grown in Sod_{top} were the shortest, weakest plants of those grown in any soil with the mid leaves approximately half the length of those grown in Sod_{sub}. Sahara, too, although exhibiting no symptoms of boron toxicity, was far more vigorous in Sod_{sub} than Sod_{top}.

Shoot dry weights

Shoot dry weights were strongly ($P \leq 0.0001$) affected by both genotype and soil type (see Appendix 1 (Section 6.2) for ANOVA table). Plants grown in UC mix and RL recorded the largest weights (Figure 6.2a). Plants grown in Sod_{sub}, Clay and Sand had similar shoot dry weights with Sod_{top} recording the lowest for both genotypes. The shoot dry weight of Sahara was greater than that of Clipper grown in RL, UC, Sand and Sod_{sub}, not significantly different when grown in Clay, and lower when grown in Sod_{top}. This observation was reflected in a significant genotype x soil interaction.
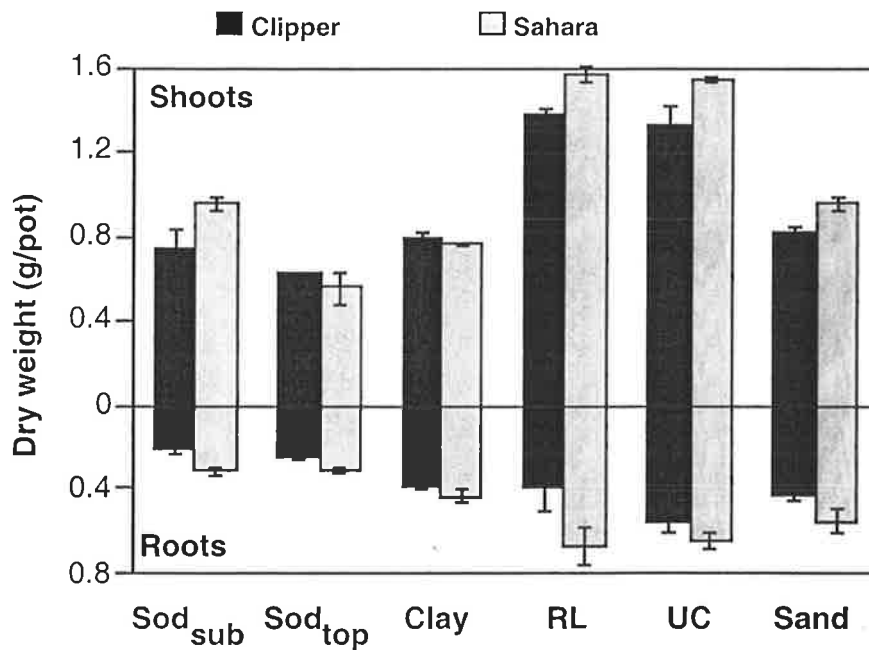
Figure 6.2a

Shoot and root dry weights of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown.

Shoot zinc concentrations and contents

Shoot zinc concentrations were strongly ($P \leq 0.0001$) affected by both genotype and soil type (see Appendix 1 (Section 6.2) for ANOVA table). Plants grown in RL showed the highest zinc concentrations, closely followed by those grown in Clay, with those grown in Sod_{sub} showing the lowest concentrations (Figure 6.2b). Concentrations in Sahara were greater than Clipper in all cases with the greatest difference occurring for the soil treatments that resulted in the higher shoot zinc concentrations. The difference between genotypes was not significant in the soil treatments with low shoot zinc concentrations and this resulted in a strong genotype x soil treatment interaction.

Shoot zinc contents were also strongly affected by both genotype and treatment (see Appendix 1 (Section 6.2) for ANOVA table). Plants grown in RL had the highest zinc content with Clay and UC accumulating only about one-half as much as those grown in

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RL. Plants grown in the other soils accumulated approximately one-half as much zinc as those grown in Clay and UC (Figure 6.2c). Sahara's zinc content was greater than that of Clipper in all cases except for Sod_{top} where there was no significant difference. Again the difference between genotypes decreased with lower zinc content resulting in a strong interaction effect.

<u>Other elements</u>

Shoot boron concentrations varied widely between soil types and ranged from adequate to extremely toxic (Figure 6.2d). There was also a genotype effect with Clipper having a greater concentration than Sahara in all soil treatments. Nable (1988) showed that shoot boron concentration correlated to boron toxicity tolerance with tolerant varieties accumulating less boron in shoots and roots than sensitive varieties and that Sahara is tolerant to boron toxicity. Shoot boron concentrations in plants grown in the sodic soil ranged from 93 mg/kg in the topsoil to 510 mg/kg in the subsoil for Clipper and 19 mg/kg to 139 mg/kg for Sahara. This is well above the adequate range of 5-12 mg/kg for whole shoots of barley (Bergmann, 1992). Shoot boron concentrations acquired from the other soils were adequate, ranging from 9.5 mg/kg -13.5 mg/kg for Clipper and 5.5 mg/kg - 8 mg/kg for Sahara.

Shoot sodium concentrations also varied widely among soils with severe toxicity occurring in both varieties when grown in Sod_{sub} and mild toxicity occurring in Sahara when grown in Clay (Figure 6.2e). Concentrations greater than 8000 mg/kg in youngest mature leaf blades of barley are considered to be toxic (Reuter *et al.*, 1997). Sahara had generally higher shoot sodium concentrations than Clipper except in the highly sodic subsoil where Clipper had a higher concentration than Sahara.
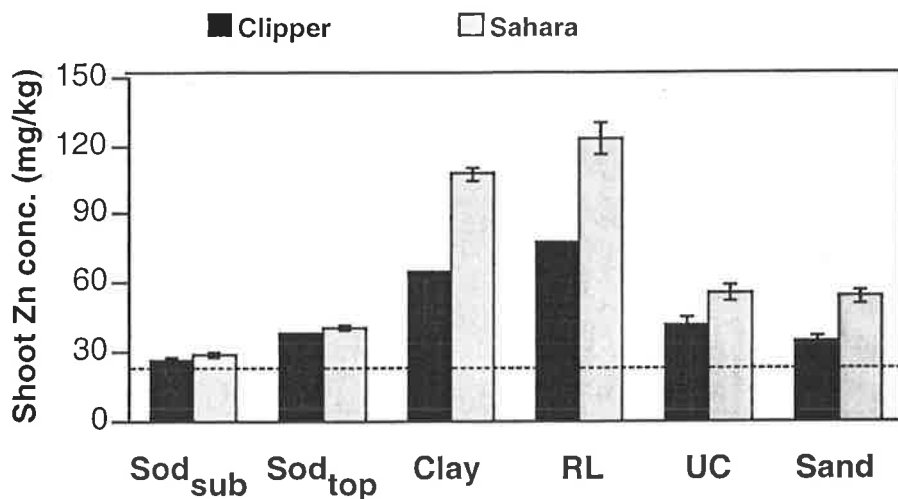
Figure 6.2b

Shoot zinc concentrations of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown. The dotted line represents the critical zinc concentration for deficiency in shoots of barley (Reuter *et al.*, 1997).
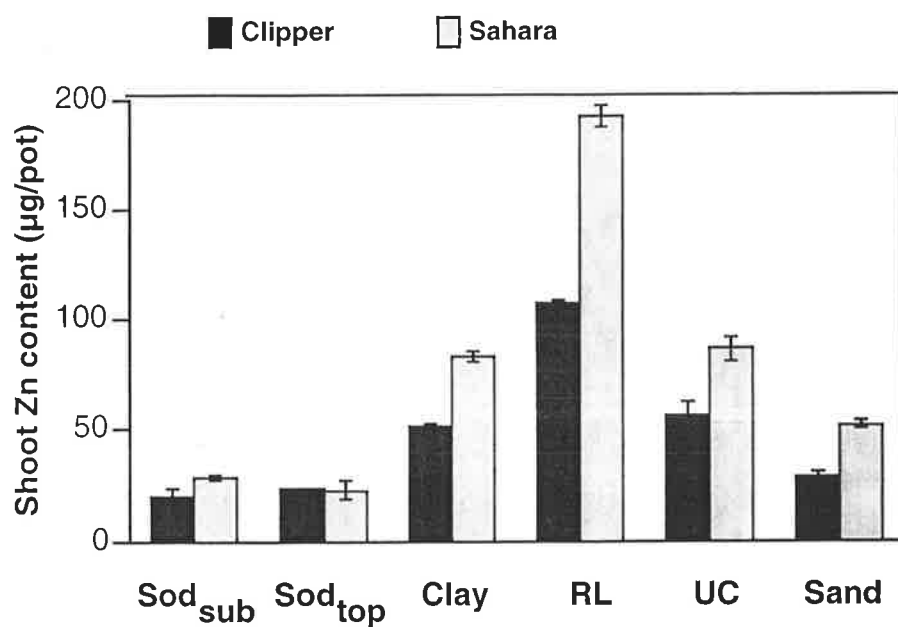
Figure 6.2c
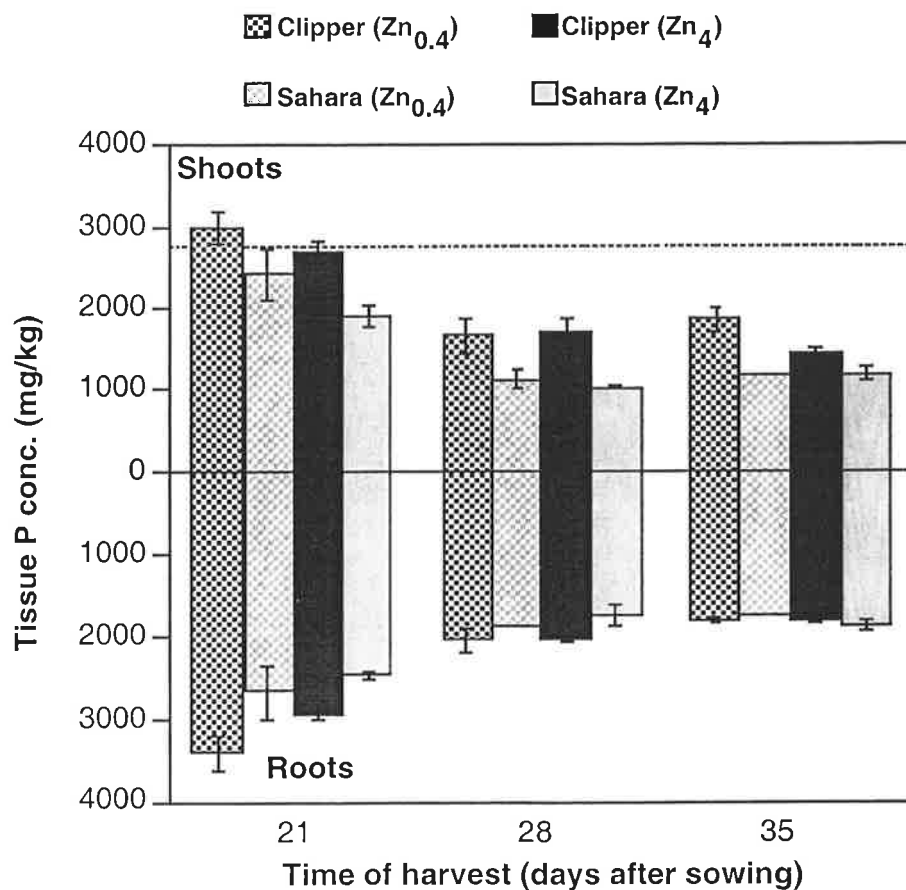
Shoot zinc contents of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown.
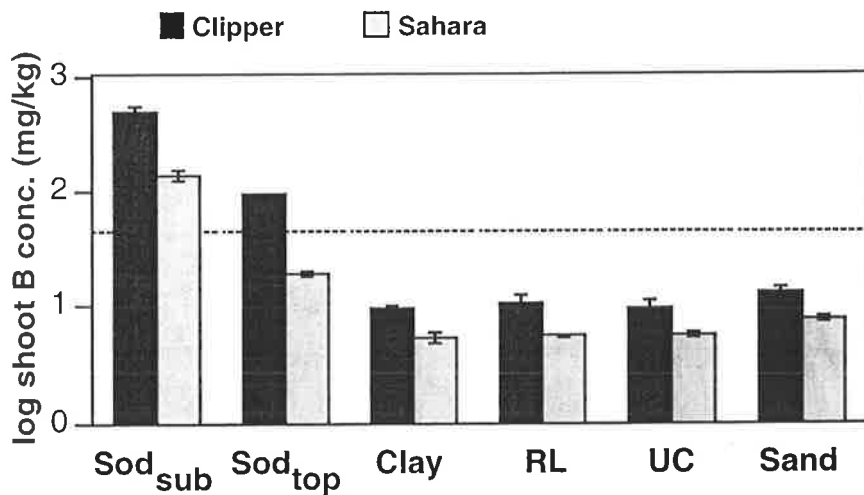
Figure 6.2d

Shoot boron concentrations of barley varieties Clipper and Sahara grown in various soils for 42 days. Values have been log-transformed due to large differences between treatments. Values represent an average of either 2, 3 or 4 replicates with standard errors shown. The dotted line represents the critical boron concentration for toxicity in shoots of barley (Reuter et al., 1997).
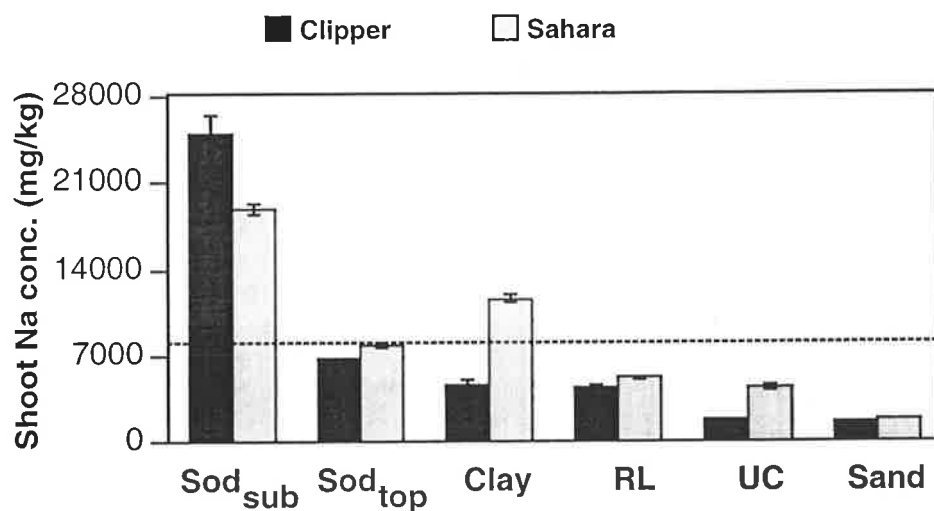
Figure 6.2e

Shoot sodium concentrations of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of 2, 3 or 4 replicates with standard errors shown. The dotted line represents the critical sodium concentration for toxicity in shoots of barley (Reuter et al., 1997).

Shoot calcium concentrations varied widely between soils, ranging from 3100 mg/kg - 12,700 mg/kg for Clipper and 1800 mg/kg - 10,700 mg/kg for Sahara (Figure

6.2f). The calcium concentration was highest in the shoots of plants grown in Clay and least when plants were grown in Sod_{sub}. Clipper had consistently higher shoot calcium concentrations than Sahara across all soils.
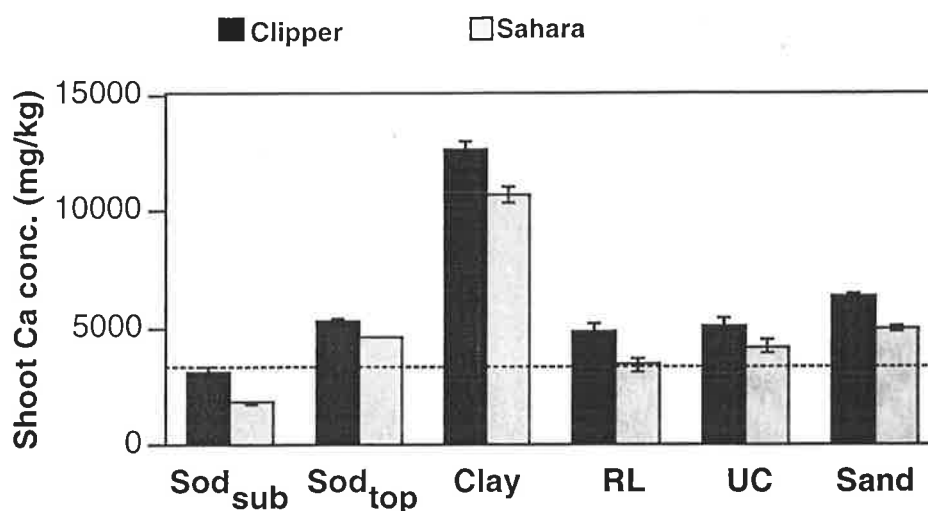
Figure 6.2f

Shoot calcium concentrations of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown. The dotted line represents the lower limit of adequate calcium concentrations in shoots of barley (Reuter *et al.*, 1997).

Shoot manganese concentrations exhibited a highly significant genotype x soil effect (Figure 6.2g) with those for Clipper either slightly higher than those for Sahara (Sod_{top}, Clay, UC and Sand), much higher than those for Sahara (Sod_{sub}) or less than those for Sahara (RL).

Shoot phosphorus concentrations (Figure 6.2h) were either high (RL, UC and Sand) or deficient (Sod_{sub}, Sod_{top} and Clay) according to published critical concentrations (Reuter *et al.*, 1997). Phosphorus concentrations for Clipper were consistently higher than those for Sahara.
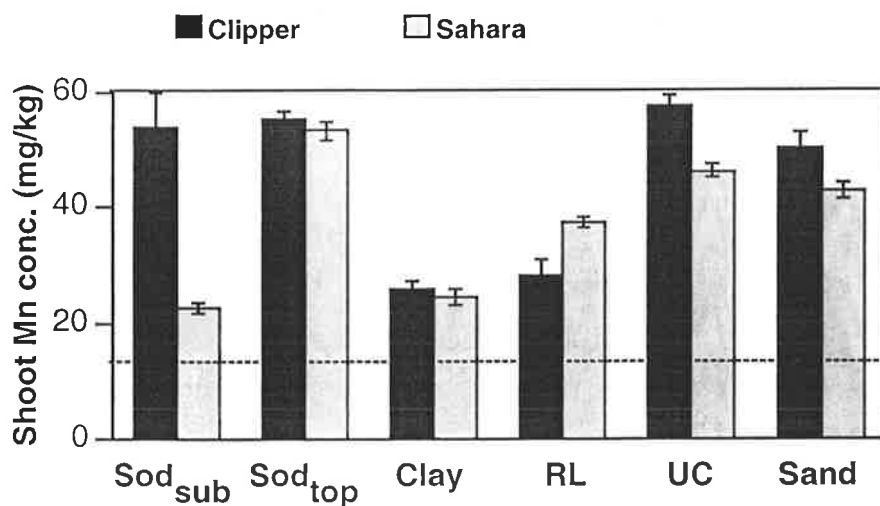
Figure 6.2g

Shoot manganese concentrations of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown. The dotted line represents the critical manganese concentration for deficiency in barley shoots (Reuter *et al.*, 1997).
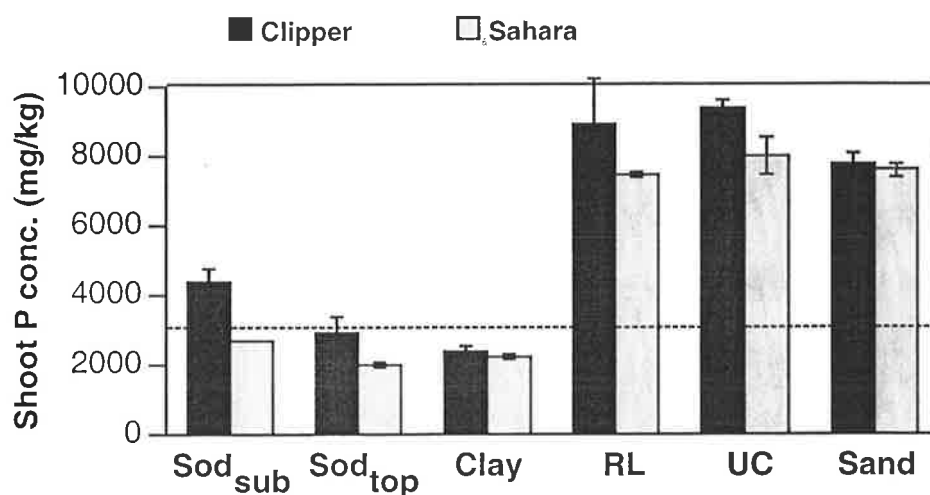
Figure 6.2h

Shoot phosphorus concentration of barley varieties Clipper and Sahara grown in various soils for 42 days. Values represent an average of either 2, 3 or 4 replicates with standard errors shown. The dotted line represents the critical phosphorus concentration for deficiency in shoots of barley (Reuter *et al.*, 1997).

6.3.3 Experiment 6C

Shoot zinc concentrations and contents

Average shoot zinc concentrations ranged from 46.0 mg/kg to 85.5 mg/kg for the doubled haploid lines while the values for Clipper and Sahara were 39.7 mg/kg and 67.5 mg/kg respectively. There was a significant difference between groups according to allele

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type at the locus Mne-1B (Figure 5.8) with Clipper-like lines having an average shoot zinc concentration of 47.4 mg/kg and Sahara types an average of 71.5 mg/kg (Figure 6.3a).

Average shoot zinc contents ranged from 49.1 µg/pot to 95.8 µg/pot for the doubled haploid lines while the values for Clipper and Sahara were 38.9 µg/pot and 80.4 µg/pot respectively. Again there was a significant difference between Mne1-B allelic groups with Clipper types having an average zinc content of 51.1 µg/pot and Sahara types an average of 78.4 µg/pot (Figure 6.3b). There was no significant difference in dry weight between allelic groups (Figure 6.3c); thus, the difference in zinc concentration and content between the two allelic groups could not be attributed to differences in plant growth.
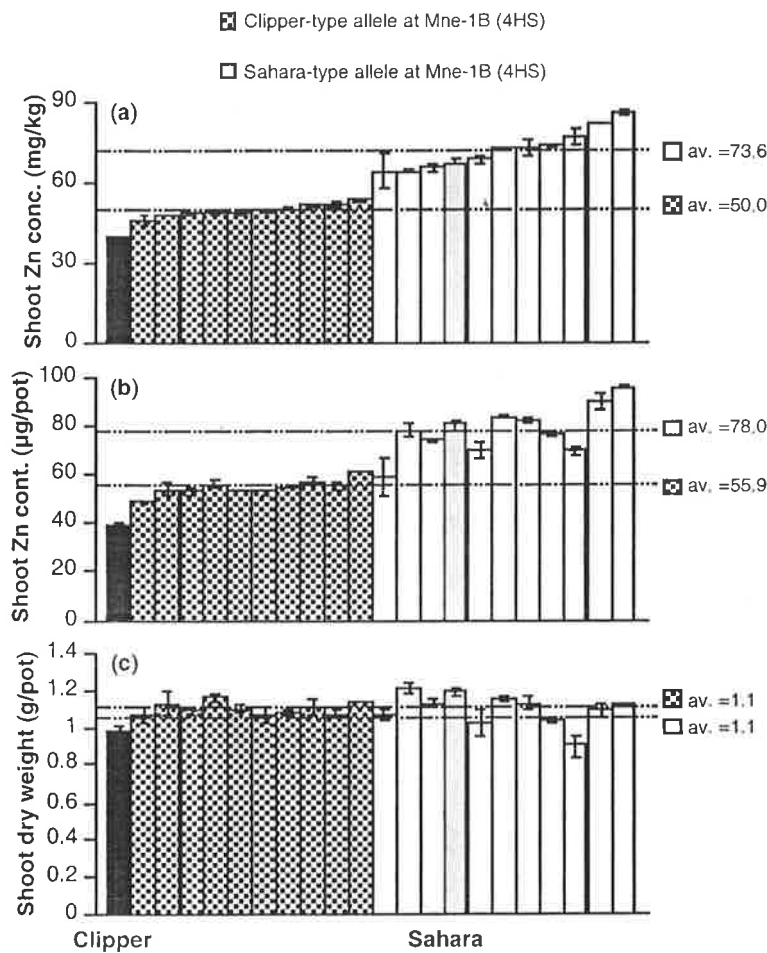
Figure 6.3a,b and c

Shoot zinc concentration, content and dry weight of barley varieties Clipper, Sahara and 20 selected doubled haploid lines grown in Wangary soil for 28 days. Genotypes are shown in increasing value of shoot zinc concentration. Values are an average of two replicates with standard errors shown. The dotted lines show the averages of the two allelic groups at the Mne-1B locus.

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Regression of zinc concentrations from separate experiments

Regression of the shoot zinc concentrations from Experiment 6C on those of Experiment 5B (Figure 6.3d) showed a strong linear correlation, indicating a high degree of repeatability for the bioassay.

RNA analysis

RNA gel blot analysis showed a differential accumulation of *Mne-1-2* between Sahara and Clipper (Figure 6.3e). Interestingly, it was the low zinc accumulator Clipper that showed a greater expression indicating a negative association.
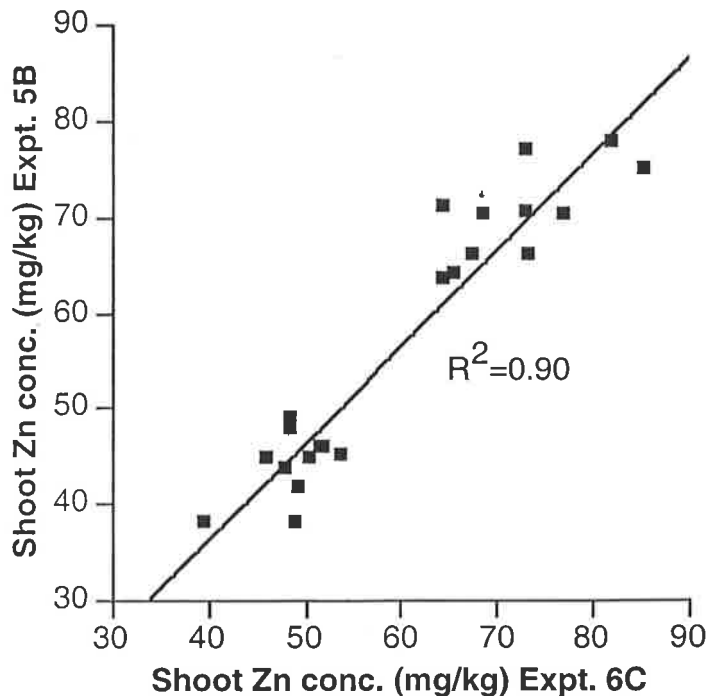
Figure 6.3d

Regression of shoot zinc concentrations obtained from Experiment 6C on those of Experiment 5B for 20 doubled haploid barley lines and their parents. Plants were grown in Wangary soil for 28 days. Symbols represent the mean adjusted values for plants grown in Experiment 5B and mean value for Experiment 6C.

Sahara Clipper Sahara Clipper
2   2   3   3
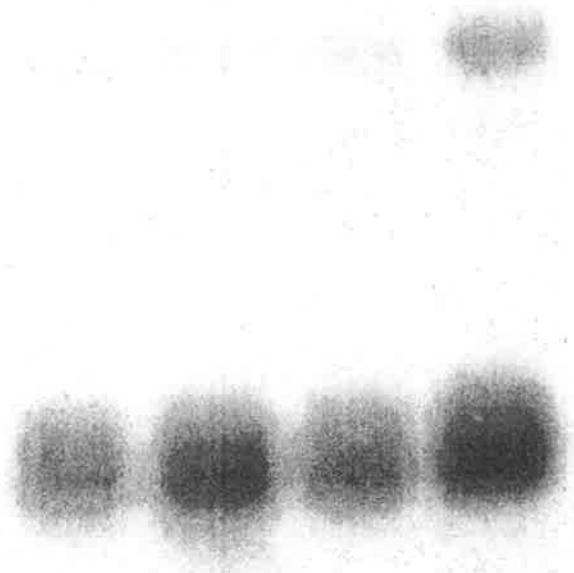
Figure 6.3e

Mne-1-2 RNA accumulation in roots of Clipper and Sahara grown in Wangary soil for 28 days. Numbers refer to replicates. (Note: replicate 1 of each variety was not used due to soil contamination resulting in poor quality of RNA).

6.4 Discussion

The behaviour of Clipper and Sahara in solution culture was quite different to that experienced in earlier soil based studies. Although shoot zinc concentrations were low for both varieties when grown in the low zinc treatment (6.1 - 9.6mg/kg at days 28 and 35) there was no expression of zinc deficiency symptoms. There was, however, a slight yellowing of leaves which could have been due to a number of factors. Shoot elemental concentrations indicated boron and phosphorus deficiency for both genotypes and manganese and copper deficiency for Sahara. The yellowing observed could have possibly been due to one or a combination of these deficiencies or different deficiencies in the two genotypes. Additionally, nutrient interactions may be expressed to a different degree in the two genotypes and thus influence the level of zinc efficiency (Rengel, 1999).

Surprisingly, none of these deficiencies was severe enough to affect yield since there was no significant difference in shoot or root dry weights between treatments. This contrasts strongly with soil culture (Chapter 3) where similar shoot zinc concentrations to

those measured under $Zn_{0.4}$ were associated with obvious zinc deficiency symptoms and reduction in shoot weights. This disparity between systems could be explained by different mechanisms operating between soil and solution. Mobilisation of zinc in the rhizosphere and differential mycorrhizal infection operate only in soil (Graham and Rengel, 1993) while differential uptake kinetics, compartmentalization, transport and translocation may be operating in both soil and solution (Rengel, 1999). These results have important implications for the use of a solution based screening system, raising doubt as to its correlation with soil based bioassays or indeed, field performance.

The use of solution culture allowed for examination of zinc partitioning between roots and shoots. The behaviour of the two varieties was quite different between treatments. Under low zinc conditions, Sahara partitioned a greater proportion of the total plant zinc into the shoots than Clipper suggesting greater translocation efficiency. Under high zinc, however, the trend was reversed with Clipper appearing to be the more efficient translocator possibly indicating an inducible rather than a constitutive mechanism. Alternatively, this result appears to be specific for zinc with none of the other elements studied displaying this trend. Zinc efficient wheat genotypes grown under zinc deficiency have been found to transport larger amounts of zinc from roots to shoots than zinc-inefficient genotypes (Cakmak et al., 1996; Rengel et al., 1998). It suggests, therefore, that Sahara is more efficient than Clipper but this is the opposite of findings in soil culture studies (Chapter 3) where Sahara was found to be less efficient than Clipper with respect to shoot growth. The solution assay employed in this study obviously requires refinement to adjust for nutrient deficiencies but such conflict with soil based results again raises doubts over the usefulness of a solution assay to predict field results.

The soil study clearly shows that genotypes can react differently to various types of soils. This raises questions as to the validity of extrapolating results gained from screening in one particular soil to a wider range of soils that might be experienced in an agricultural context. In only six soils studied, shoot dry weights varied by more than twofold and shoot zinc concentrations by up to fourfold. The relationship between the two genotypes with respect to zinc accumulation, however, appears to remain reasonably constant and it is this

relationship that is important for genetic studies. Although absolute values may alter, the ranking of the two genotypes does not. Sahara maintained a higher shoot zinc concentration than Clipper, particularly in treatments that resulted in larger shoot zinc concentrations. As the shoot zinc concentration decreased, so did the difference between Sahara and Clipper. This is similar to results in Chapter 3 although the difference between genotypes at higher soil zinc treatments was not as great. Shoot boron, calcium and phosphorus concentrations were also consistent across all soils with Clipper having greater concentrations than Sahara for all three elements. Other elements, however, show definite genotype x soil interactions. Although only a small number of soils was used, it appears that the zinc accumulation character is a stable one, likely to operate over a wide range of soil types.

Regression of shoot zinc concentrations of Experiment 6C on those of Experiment 5B indicated a high degree of repeatability, a desirable property of any bioassay that is to be used routinely and on which decisions will be based. There was a distinct separation into two discrete groups in both experiments and these classes corresponded to allelic differences at the Mne1-B locus. Such a separation would be more than adequate for genotype selection purposes, ensuring efficient identification of desired alleles. The high degree of repeatability further enhances this bioassay as a selection tool.

The expression of *Mne-1-2* in roots suggests that the differential zinc accumulation is due to differences in root zinc uptake or translocation from roots to shoots. The negative association between gene expression and zinc accumulation suggests that the action of the gene, or genes, involved is one of inhibition. A further study is required on how this differential reacts to conditions of zinc deficiency and whether or not its operation is temporal or constant. It is also possible that the cosegregation of this gene with zinc accumulation is merely coincidental. Evaluation of Mne1-B expression in doubled haploids selected on the basis of the Sahara or Clipper allele at that locus should show whether the expression pattern also cosegregates with zinc accumulation status. In addition, RNA analysis of roots grown in solution culture, where the trait is not expressed, would be of interest. Together, those data would provide more confidence in the role that

this gene plays in zinc accumulation.

The experiments presented in this chapter have further enhanced the understanding of the differential in zinc accumulation between Clipper and Sahara that was mapped in Chapter 5. In terms of expression, the character was shown to be stable across a range of soil types although the magnitude of difference was decreased in soils that resulted in lower shoot zinc concentrations. It was also demonstrated that the character is not expressed in solution culture, thereby making solution techniques unsuitable as a selection tool. Furthermore, it was shown that the character can be selected for, using either the bioassay described in Chapter 5 or by molecular marker technology.

Chapter 7

Mapping of Chromosome Regions Conferring Increased Zinc Accumulation in Barley Grain Using a Doubled Haploid Population

7.1 Introduction

Zinc is an essential micronutrient for plant and animal health forming an essential catalytic component of over 300 enzymes and a structural component of many proteins (Grotz *et al.*, 1998). The amount of Zn in seeds of crop plants is therefore important in maintaining the health and vigour of the subsequent plant generation by enabling critical biological processes to proceed unhindered (Welch, 1999). Similarly the health of animals that derive their nutrition from these plants is dependent on adequate concentrations of Zn in the grain.

For barley, efforts have been made to increase the amount of Zn in seeds through fertiliser application (Graham *et al.*, 1992; Ascher *et al.*, 1994) but there is scant information regarding genetic diversity for seed Zn. Significant diversity for seed Zn content has been observed for other species such as rice (Graham *et al.*, 1997), wheat (White *et al.*, 1981; Graham *et al.*, 1997) and beans (Tohme *et al.*, 1994) and it is reasonable to assume that similar variation exists for barley.

There has been some research into the mechanisms involved in the transport of nutrients into developing grains of wheat (Pearson and Rengel, 1994; Pearson *et al.*, 1995, 1996) but none of these studies focussed on genetic differences in seed Zn loading. Seed Zn can be considered as being comprised of two components, Zn concentration and Zn content which are related by the simple equation-:

Zn content (µg/seed) = [Zn concentration (mg/kg) x seed weight (g)] ÷ no. of seeds

Zinc content is more important than Zn concentration for seedling vigour of subsequent generations. This is reflected in increased vegetative growth and ultimately grain yield, particularly when plants are grown under Zn deficient conditions (Rengel and Graham, 1995a,b; Genc *et al.,* 2000).

Zinc concentration is more important than grain Zn content for human and animal health since food grains are generally bought and consumed by weight. The ability to increase Zn concentration, therefore, has important implications for alleviation of Zn deficiency in people reliant on these grains as their major dietary source. Zinc deficiency results in decreased infant birth weights (Neggers *et al.*, 1990; Tamura and Goldenberg, 1996) and irreversible impairment of cognitive function (Gibson and Ferguson, 1998). Although diagnosis in humans is complicated (Shrimpton, 1993) the general view held by specialists is that Zn deficiency, in a global context, is as important as iron deficiency which affects some 2.15 billion people (Welch and Graham, 1999).

In the study outlined in this chapter, a barley doubled haploid population was screened for seed Zn concentration and content and the results used to map the position of chromosomal regions associated with these traits. Although barley is not used as a major food source for humans, it is genetically related to the major food cereals, wheat and rice. The fact that genes on chromosomes often occur in a similar order (synteny) (Paterson *et al.*, 1995) should make it possible to develop molecular markers that can aid in breeding for increased Zn density in other cereals. Failing this, the developments in map-based cloning and transformation technology make it possible to introduce barley DNA into the genomes of wheat or rice for the same aim of increasing seed Zn density.

7.2 Materials and methods

7.2.1 Experiment 7A - Preliminary mapping of seed zinc concentration and content

The genetic material used was the same Clipper x Sahara doubled haploid population detailed in Chapter 5. The seed used for Experiment 5B was in fact the grain harvested from the experiment described here. Although primarily aimed at multiplying lines in order to have sufficient seed grown under standard conditions for future

experiments, this experiment was also used to gain information on the Zn concentration and content of seed and to attempt to map the chromosomal regions controlling these traits. This information was used to develop a more sophisticated subsequent experiment (Experiment 7B).

The soil used was a recycled loam produced at the University of Adelaide, Waite Campus. Eight litre black plastic pots were filled with soil and moistened with water prepared by reverse osmosis. Seeds of the 150 doubled haploid lines, together with their parents Clipper and Sahara, were surface sterilised by soaking in 70% ethanol for 1 minute, rinsed with deionised water and then soaked in sodium hypochlorite (4% Cl) for 6 minutes before rinsing again with deionised water. The seed was germinated on moistened Whatman No. 42, ashless filter paper for 36 hours at $20^{\circ}C$ before sowing (4 seeds of each line per pot). Due to limitations of space, only one pot per line was sown. Plants were grown in a glasshouse at the Waite Campus from August 1996 until harvest in January 1997. Pots were watered by volume daily to approximately 90% of field capacity until seed filling was complete at which time watering ceased.

At maturity, seed from the 4 plants in a pot was harvested and bulked. Sub-samples of approximately 20 seeds per line were oven-dried at $70^{\circ}C$ for 24 hours. Dried samples were digested with 70% nitric acid and analysed for concentrations of the elements Zn, Mn, Fe, Cu, B, Ca, Mg, Na, K, P and S by ICPAES. Zinc content per seed was calculated using the equation-:

...Zn content (µg/seed) = [Zn concentration (mg/kg) x seed weight (g)] ÷ no. of seeds

Single point linkage analysis (P<0.001) was performed using the computer program Map Manager QT version b16 (Manly and Cudmore, 1997) employing a total of 202 RFLP markers (Langridge *et al.*, 1995). These markers covered all barley chromosomes. A minimum LOD score of 3.0 was employed to detect significant associations between markers and Zn concentration and/or content. A similar analysis was also performed on seed size data.

7.2.2 Experiment 7B - Mapping of zinc concentration and content in vegetative and reproductive tissues of barley

Following the results of Experiment 7A, it was decided to conduct an experiment using only the 2 row doubled haploids. This would remove the confounding effect that head type has on seed Zn content. Reducing the number of lines also enabled the use of replication which increased the power of the experiment.

The soil used was the same calcareous sand from Wangary, South Australia as used in Experiments 5A and 5B. Methods of soil preparation were the same as for Experiment 5B, in order that reasonable comparisons could be made between vegetative and reproductive Zn accumulation. Topsoil (0-10cm) and subsoil (10-20cm) were air-dried, passed through a 1mm stainless steel sieve and stored separately. Topsoil and subsoil (1:1, w/w) were well combined before adding deionised water to bring the moisture content to 20% (w/w). The soil was then incubated for 3 weeks in the dark under a temperature regime of 15°C (16h) and 10°C (8h). The following nutrients were added and mixed well with the incubated soil: (mg salt/kg dry soil) $Ca(NO_3)_2.4H_2O$, 918; $MnSO_4.4H_2O$, 203; KH_2PO_4, 179.5; $MgSO_4.7H_2O$, 140; K_2SO_4, 28.4; $FeSO_4.7H_2O$, 17.2; $ZnSO_4.7H_2O$, 17.16; H_3BO_3, 5.6; NaCl, 4.16; $CuSO_4.5H_2O$, 3.9; $CoSO_4.7H_2O$, 0.585; $H_2MoO_4.H_2O$, 0.13.

Two litre cardboard milk cartons (95mm x 95mm x 270mm) were lined with a plastic bag and filled with soil (equivalent to 1.80 kg dry weight). Barley seeds of 78 doubled haploid lines together with their parents Clipper and Sahara were surface-sterilised and germinated as for Experiment 7A before sowing (4 seeds per carton).

Plants were grown in a controlled environment growth chamber with a 10-hour light/14-hour dark photo period at 20°C day/15°C night. Light was supplied by high pressure mixed-metal halide lamps delivering a photon flux density of 500 $\mu mol/m^2/s$ at the surface of the pot. Up until anthesis, plants were watered by volume daily and watered to weight every 3 days to maintain the soil moisture at 20% w/w. After this plants were watered by volume only to avoid damaging elongating plants through movement.

Due to limitations of growth chamber size and amount of soil available, only 2 replicates of each doubled haploid line were possible. With this in mind, the experiment was designed in order to satisfy properties of both neighbour and balance as much as possible. Replicates were blocked into row-column arrangements (8 rows and 12 columns for each replicate) and randomised to ensure that between replicates each line did not have the same neighbour or that the same line did not occur twice in corner pots or on an edge. Clipper and Sahara were used as check lines and included in each replicate nine times. They were assigned in such a way that each check line occurred on each edge and at least one check line occurred in each row and column. This amounted to-

(78 DH lines + (2 check lines x 9 entries)) x 2 reps = 192 pots

At anthesis, two plants from each pot were harvested at 1 cm above the soil surface and partitioned into developing head and remainder. At maturity, main culms of the remaining two plants were separated from the rest of the plant and partitioned into seed, chaff (palea, lemma, glumes and rachis) and stem (including leaves). All plant material was dried and analysed by ICPAES as for Experiment 7A.

All data were analysed for spatial variation (Gilmour *et al.*, 1997). Spatial analysis is considered, in most cases, to be far superior to analysis of variance in accounting for, and explaining, sources of variation. It allows for sources of variation that could possibly bias the estimates of effects and possibly inflate error. This variation is partitioned into smooth spatial trend, termed local and global variation, and extraneous variation (Gilmour *et al.*, 1997). Spatially adjusted values of all groups of measurements were subject to single point linkage analysis, as in Experiment 7A, in an attempt to map chromosomal regions associated with Zn concentration and content in different tissues of barley at two different stages of development.

7.3 Results

7.3.1 Experiment 7A

<u>Seed zinc concentrations and zinc contents</u>

Seed Zn concentrations for the doubled haploid population ranged from 35 mg/kg to 133 mg/kg with Clipper having a concentration of 54 mg/kg and Sahara 56 mg/kg. Figure 7.1a shows the histogram for this trait. Zinc content per seed ranged from 1.1 μg to 6.3 μg with Clipper containing 2.6 μg Zn/seed and Sahara 1.7 μg Zn/seed (Figure 7.2a). The amount of Zn per seed was strongly affected by the head row number (6 row or 2 row) as shown in Figures 7.2b and c. This effect could be attributed to differences in seed weight with the two row group (Clipper type) having significantly greater seed weight than the six row group (Sahara type) (Figures 7.3a,b and c). On the other hand, the frequency distributions for Zn concentration for the two row and six row groups have a similar median and range (Figures 7.1b and c).
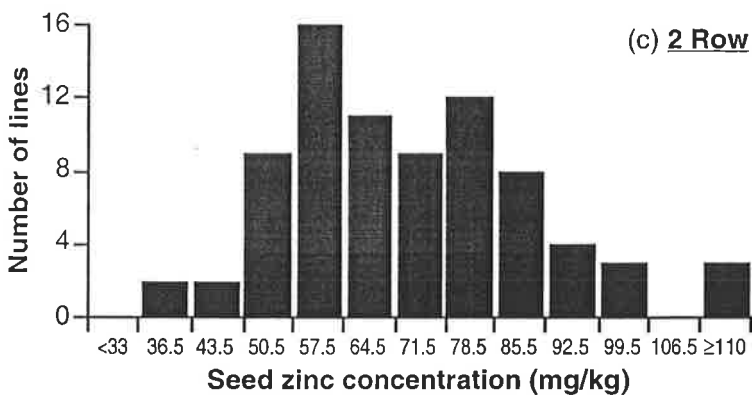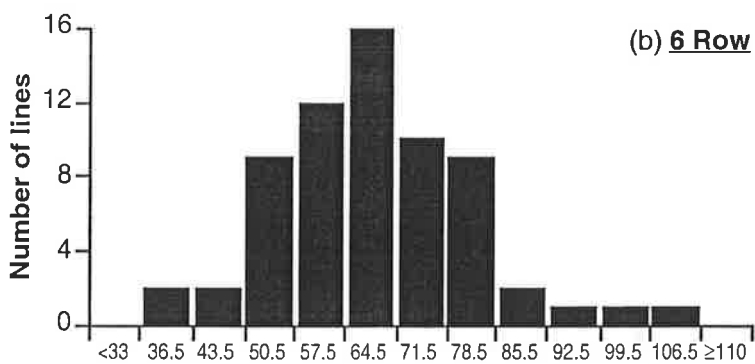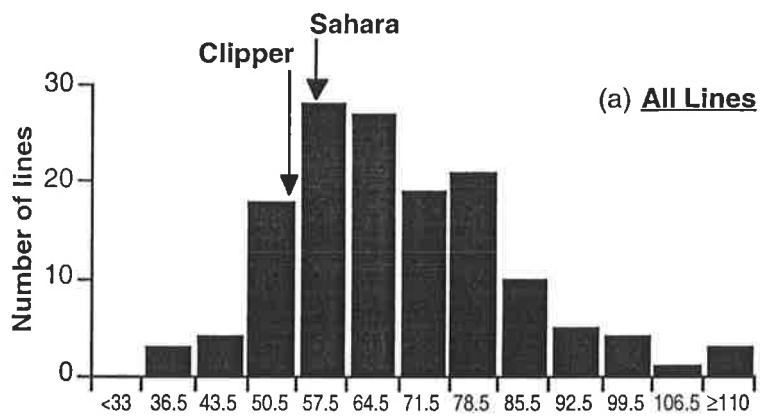
Figures 7.1a, b and c

Frequency distribution of seed zinc concentration expressed in 150 doubled haploid barley lines and their parents Clipper and Sahara. Figure 7.1a shows the total population while 7.1b and 7.1c show subgroups based on head row type.
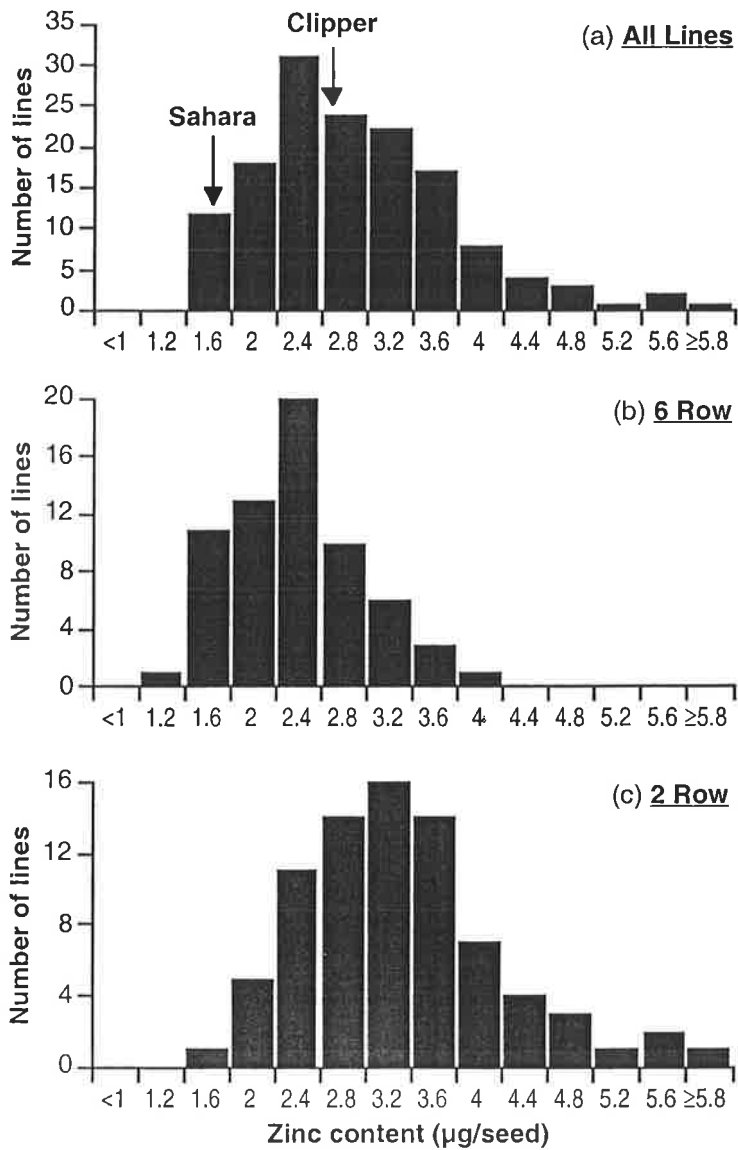
Figures 7.2a, b and c

Frequency distribution of seed zinc content expressed in 150 doubled haploid barley lines and their parents Clipper and Sahara. Figure 7.2a shows the total population while 7.2b and 7.2c show subgroups based on head row type.
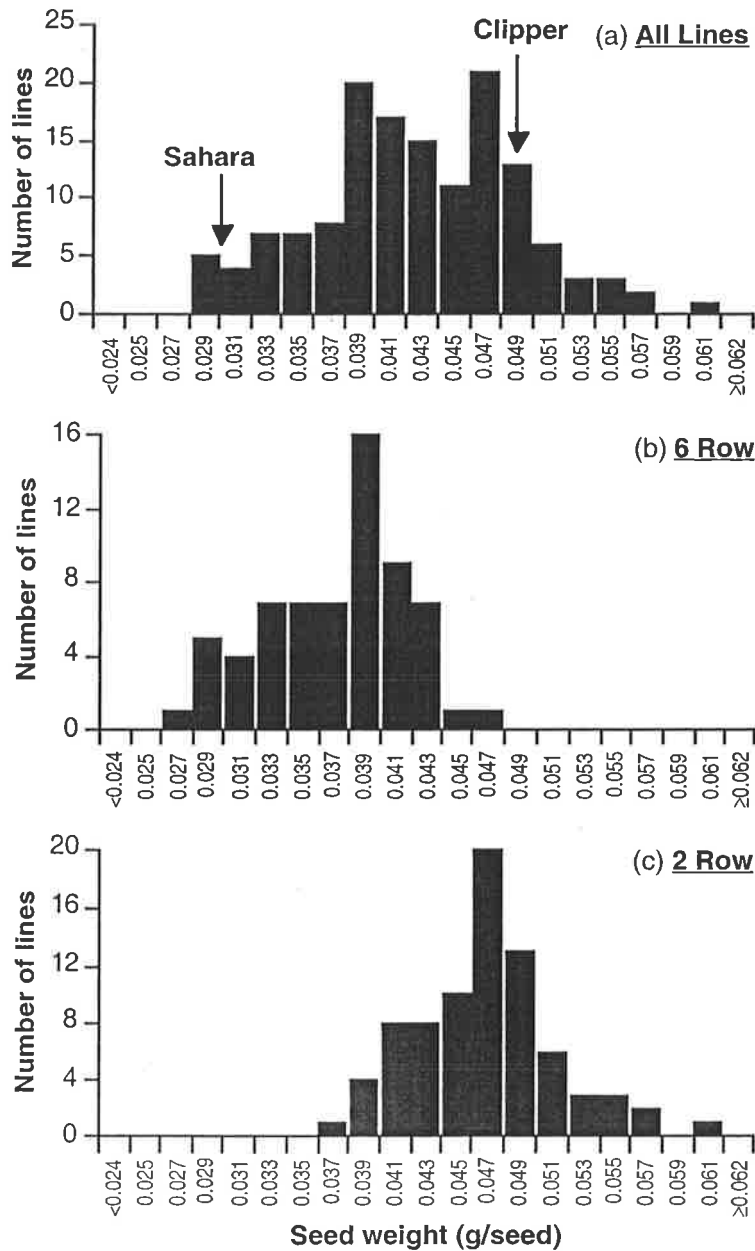
Figures 7.3a, b and c

Frequency distribution of seed weight expressed in 150 doubled haploid barley lines and their parents Clipper and Sahara. Figure 7.3a shows the total population, 7.3b and 7.3c show subgroups based on head row type.

Mapping

Analysis of seed Zn content identified a region in the centre of chromosome 2H and one of less significance on the short arm of chromosome 2 (2HS) associated with this trait (Figure 7.4). The region of major association coincided with the morphological marker for head row type (hex-v). Analysis of seed weight identified a strong association with the same marker (Figure 7.5). Analysis of seed Zn concentration identified association to the minor region on 2HS mapped for seed Zn content (Figure 7.6).

151
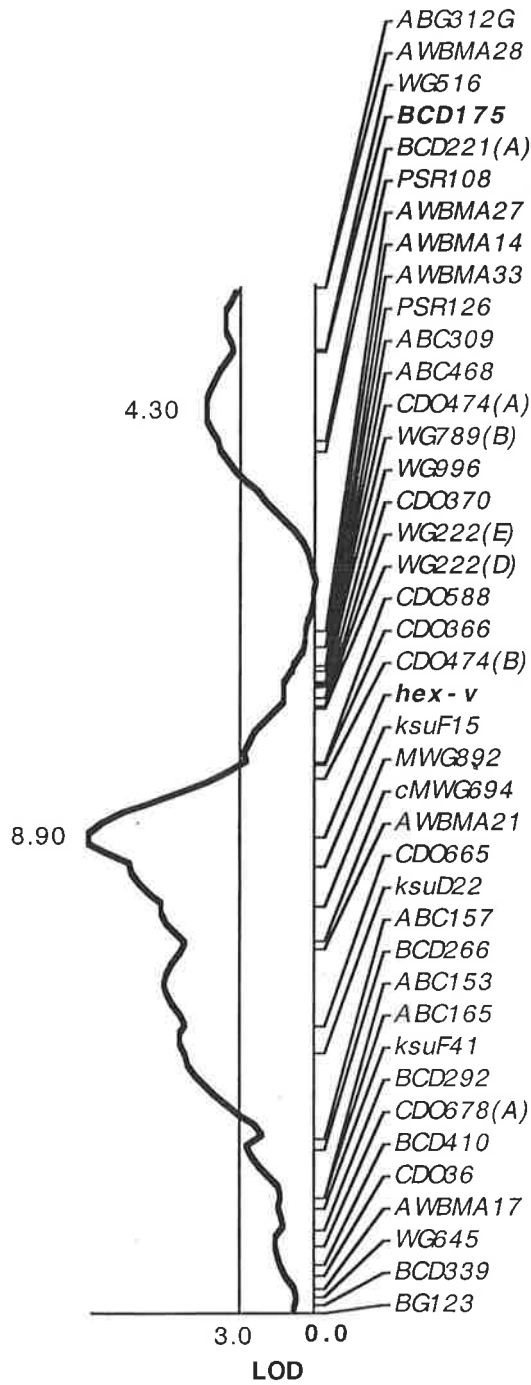
Figure 7.4

Location on chromosome 2H of two regions associated with zinc content per seed expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP markers most significantly associated with the trait are presented in bold type.
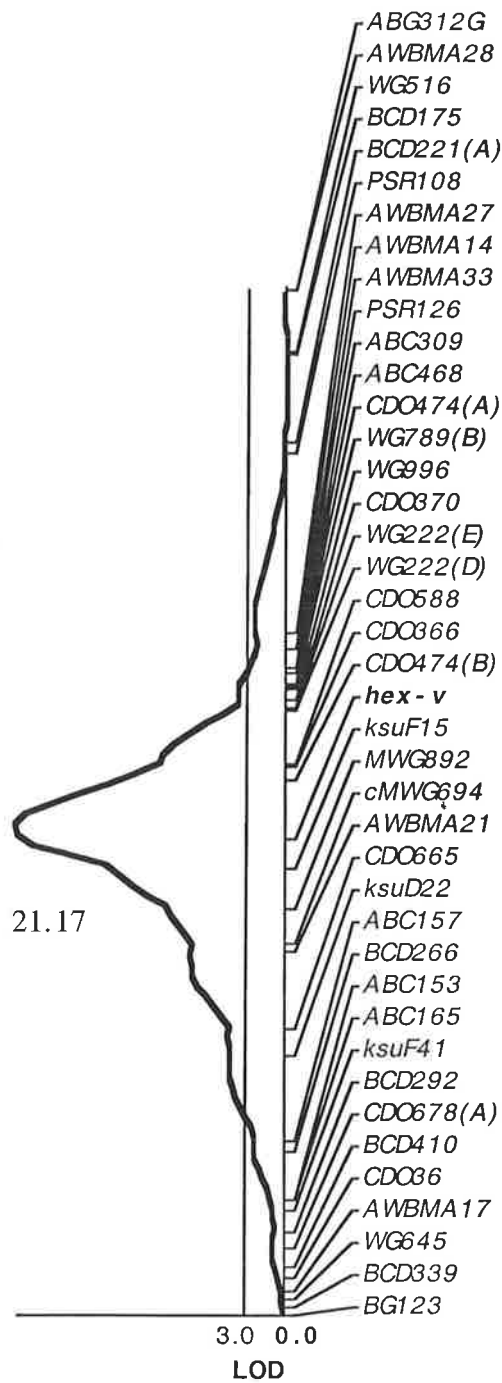
Figure 7.5

Location on chromosome 2H of a region associated with seed size expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

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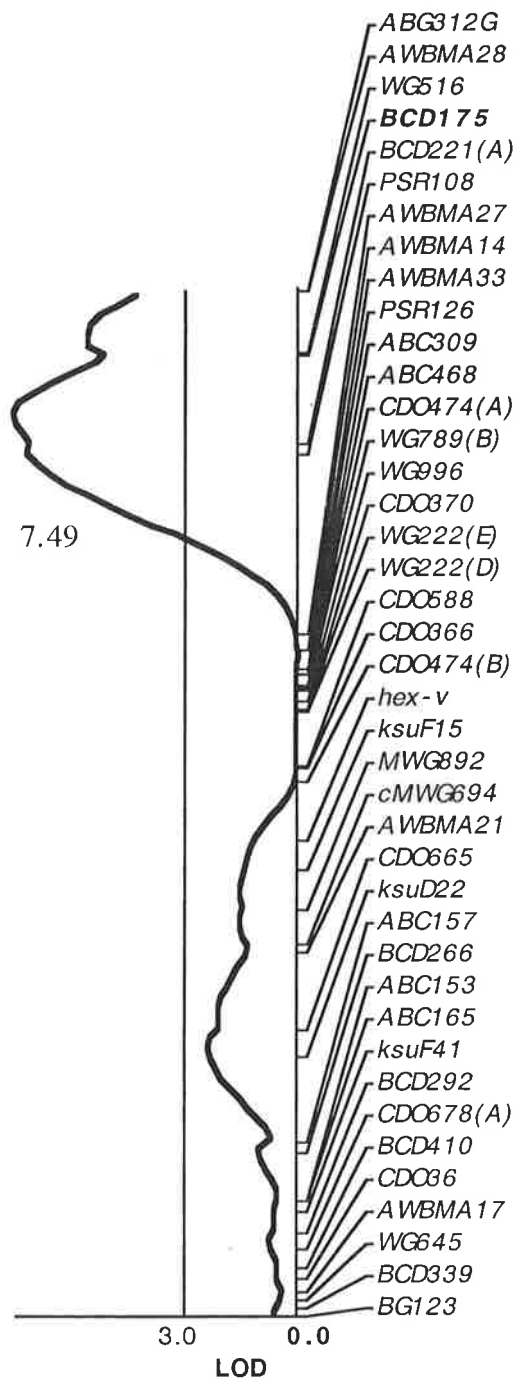
Figure 7.6

Location on chromosome 2H of a region associated with seed zinc concentration expressed in 150 doubled haploid barley lines derived from the cross Clipper x Sahara. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

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As a consequence of these results it was decided to analyse the two groups separately in order to remove the overriding effect of head type. Marker analysis of the 2 row lines identified a significant association with a region on the short arm of chromosome 2H for both seed Zn concentration and content (Figures 7.7a,b). Similar analysis of the 6 row lines identified the same region on the short arm of chromosome 2H as being associated with seed Zn concentration and to a lesser extent with seed Zn content (Figures 7.8a,b).

7.3.2 Experiment 7B

7.3.2.1 ICPAES measurements (anthesis)

<u>Stem zinc concentration and content</u>

Spatially adjusted values for stem Zn concentration at anthesis ranged from 33 mg/kg to 109 mg/kg with Clipper having a concentration of 52 mg/kg and Sahara 57 mg/kg (Figure 7.9a). Spatially adjusted values for stem Zn content ranged from 16.4 µg/pot to 98.7 µg/pot (Figure 7.9b). Clipper had a stem Zn content of 40.4 µg/pot and Sahara 46.9 µg/pot. The differences observed here between Clipper and Sahara for both Zn concentration and Zn content were far less than those for whole shoots measured at 4 weeks growth in a similar bioassay (see Chapter 5, Figures 5.5a and b).
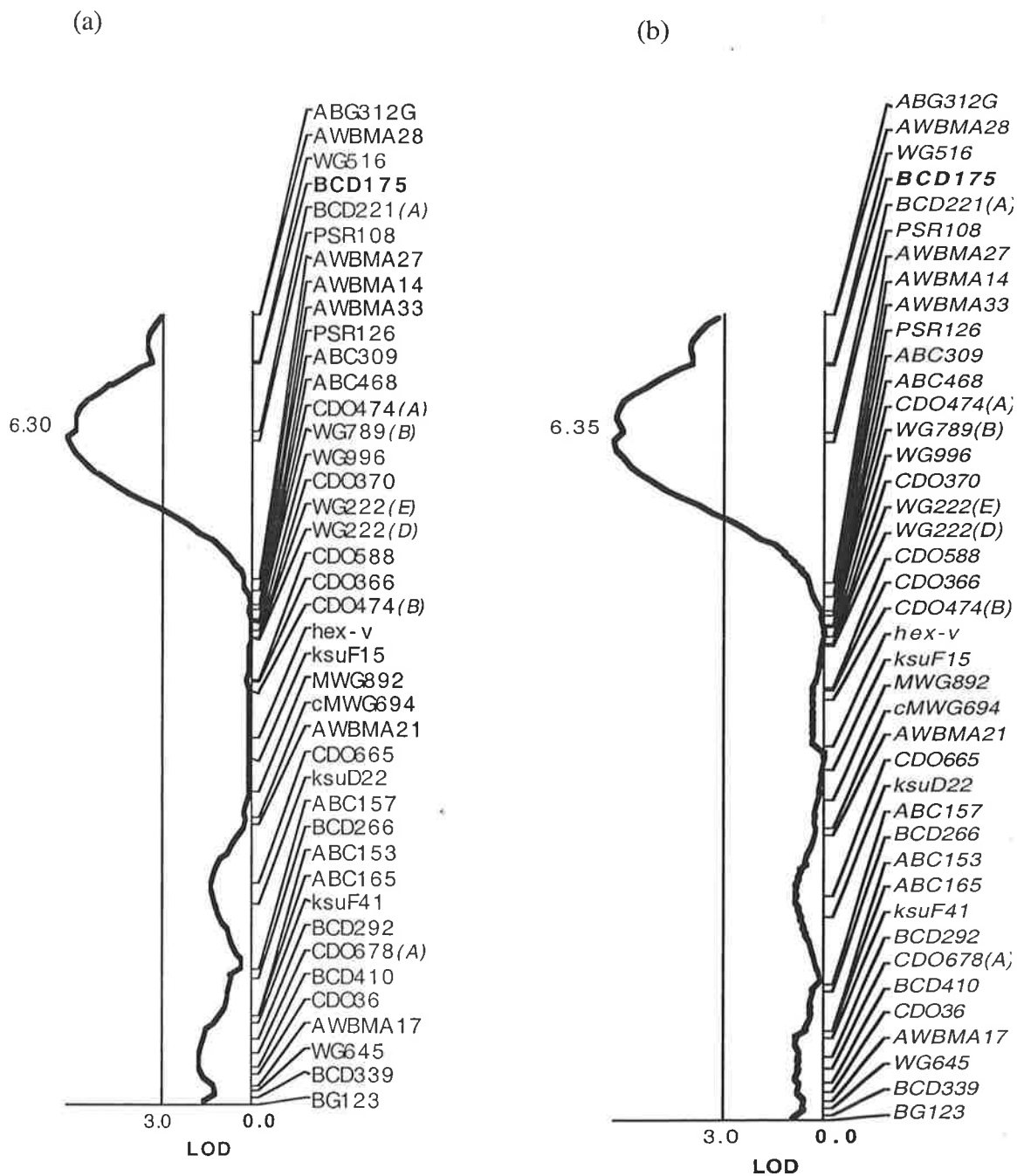
Figures 7.7a and b

Location of a region on chromosome 2H associated with (a) seed zinc content and (b) seed zinc concentration expressed in 78 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represented 2-row types only. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

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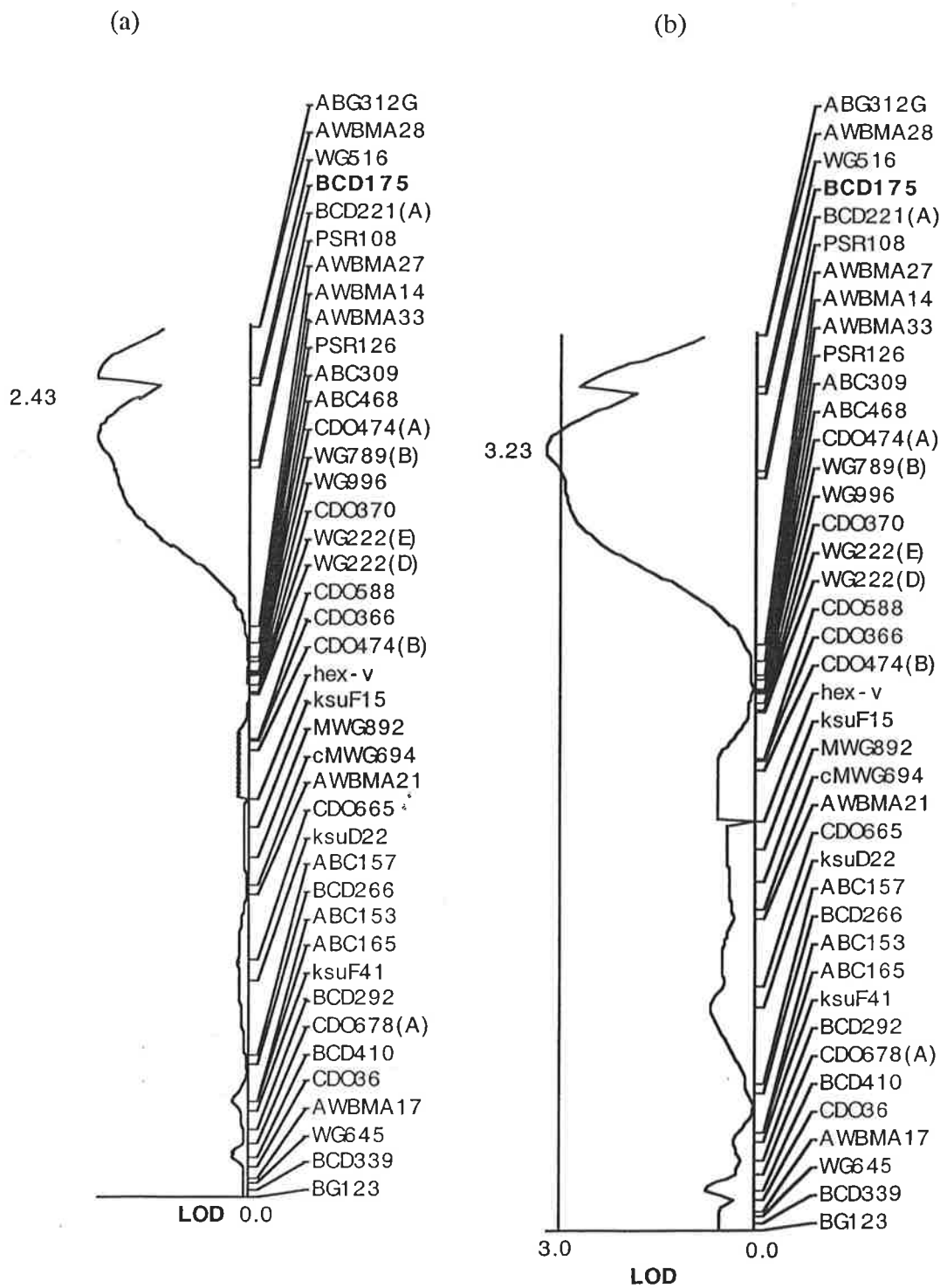
Figures 7.8a and b

Location of a region on chromosome 2H associated with (a) seed zinc content and (b) seed zinc concentration expressed in 64 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represented 6-rowed types only. The short arms are towards the tops of the chromosomes. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.
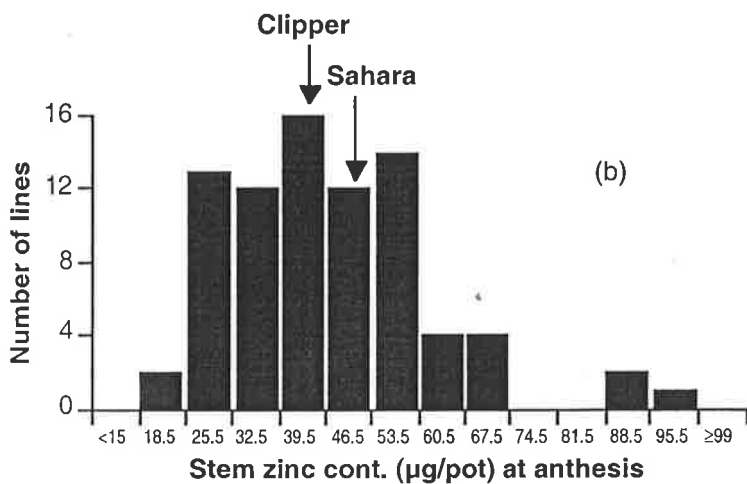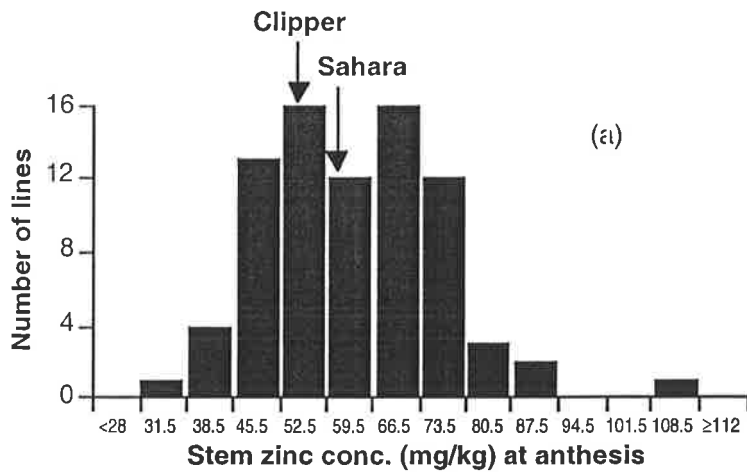
Figures 7.9a and b

Frequency distribution of (a) stem zinc concentration and (b) stem zinc content at anthesis for 78 doubled haploid barley lines and their parents Clipper and Sahara. These lines represented 2-row types only.

Zinc concentration and content of developing head

Spatially adjusted values for Zn concentration of the developing head ranged from 27 mg/kg to 62 mg/kg with Clipper having a concentration of 40 mg/kg and Sahara 35 mg/kg. Zinc contents of the developing head ranged from 3.1 µg/pot to 17.9 µg/pot with Clipper having a content of 10.5 µg/pot and Sahara 7.4 µg/pot (Figures 7.10a and b).
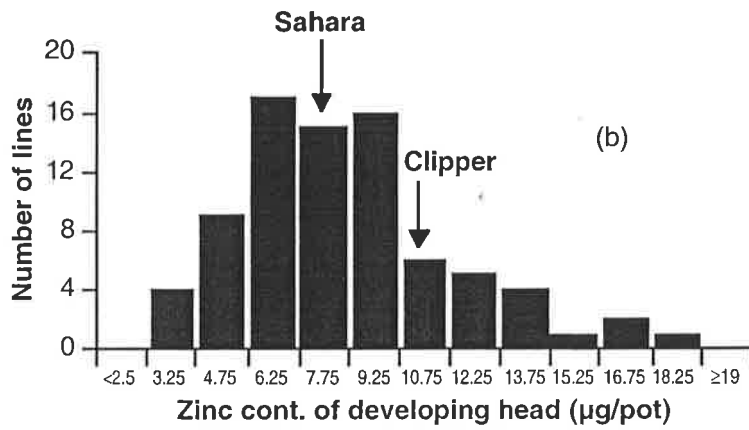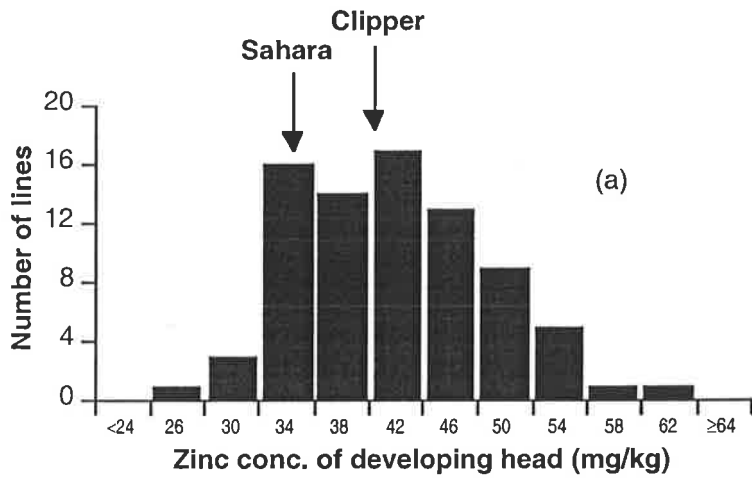
Figures 7.10a and b

Frequency distribution of (a) zinc concentration and (b) zinc content of the developing head of 78 doubled haploid barley lines and their parents Clipper and Sahara sampled at anthesis. These lines represent 2-row types only.

7.3.2.2 ICPAES measurements (maturity)

Zinc concentration and content of main stem

Zinc concentration in stems ranged from 19 mg/kg to 63 mg/kg with Clipper having a concentration of 41 mg/kg and Sahara 27 mg/kg (Figure 7.11a). This result stands in marked contrast to Experiment 5B where Clipper had a shoot Zn concentration of 38 mg/kg, while Sahara had a shoot Zn concentration of 66 mg/kg after 28 days growth.

Correspondingly, results for Zn content of the main stem show a similar turnaround with Clipper having a content of 24.9 μg/pot and Sahara 19.4 μg/pot while the range for the doubled haploid lines was 6.3 μg/pot to 46.4 μg/pot (Figure 7.11b).
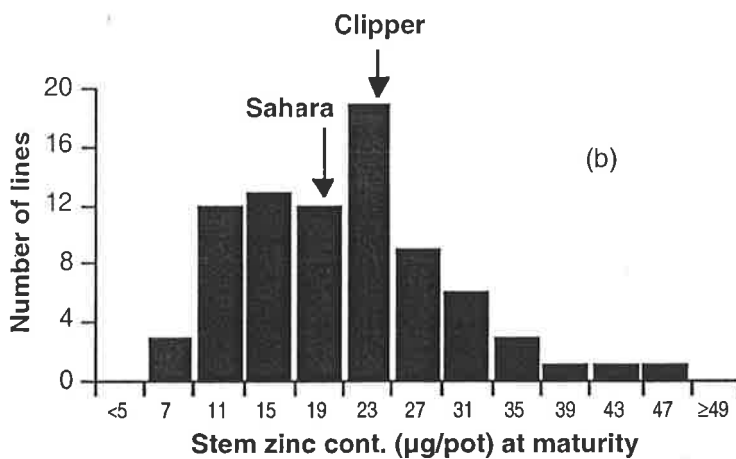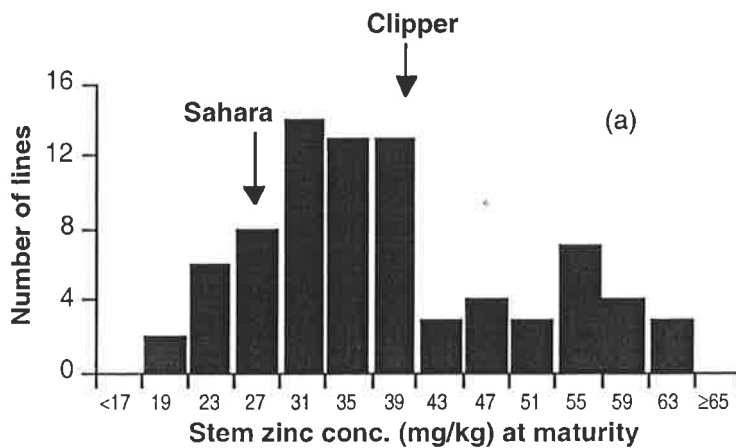
Figures 7.11a and b

Frequency distribution of (a) zinc concentration and (b) zinc content of the main stem of 78 doubled haploid barley lines and their parents Clipper and Sahara sampled at maturity. These lines represent 2-row types only.

Seed zinc concentration and content

Seed Zn concentrations ranged from 43 mg/kg to 101 mg/kg with Clipper having a concentration of 69 mg/kg and Sahara 54 mg/kg (Figure 7.12a). Seed Zn content ranged from 1.6 to 4.8 µg/seed with Clipper having a content of 3.6 µg/seed and Sahara 1.9 µg/seed (Figure 7.12b). Figure 7.12c shows the distribution for seed weight which is still wide-ranging despite the fact that only 2-row doubled haploids were included in this experiment.
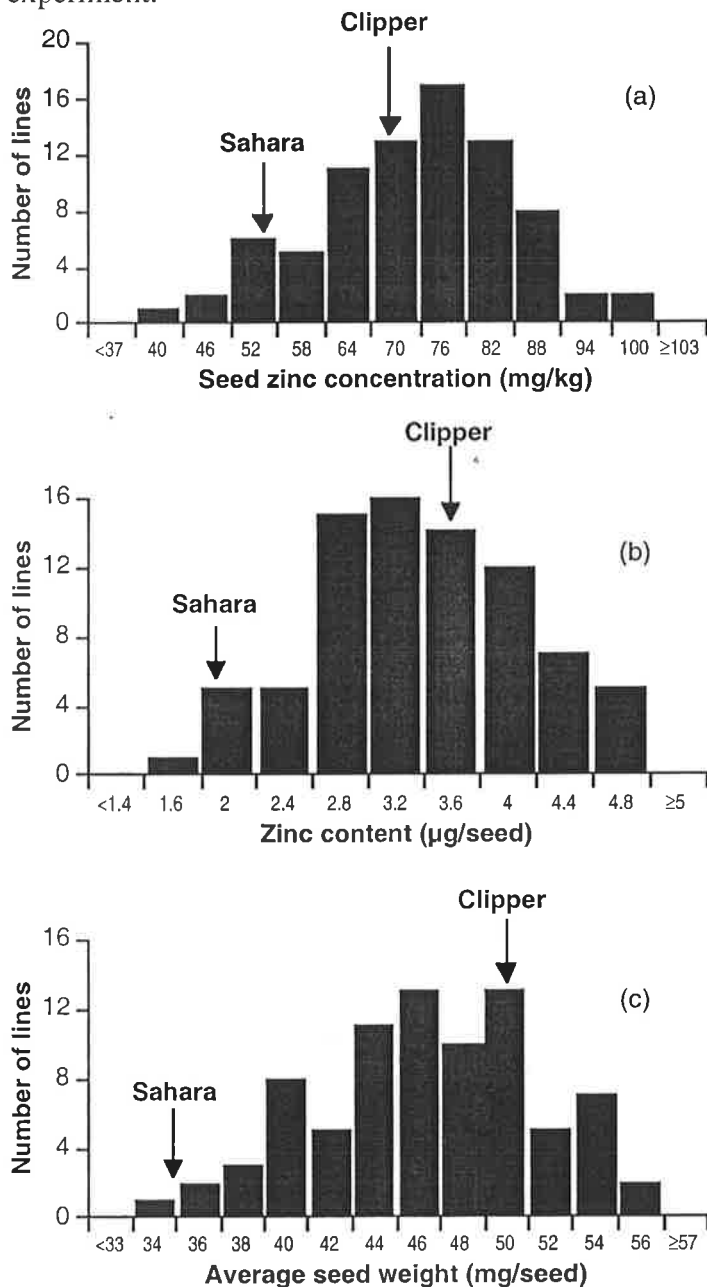
Figures 7.12a, b and c

Frequency distributions for (a) seed zinc concentration (b) zinc content/seed and (c) average seed weight for 78 doubled haploid barley lines and their parents Clipper and Sahara. These lines represent 2-row types only.

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Chaff zinc concentration and content

Zinc concentrations for chaff ranged from 14 mg/kg to 155 mg/kg with Clipper having a Zn concentration of 66 mg/kg and Sahara 25 mg/kg (Figure 7.13a). Chaff Zn content ranged from 2.0 μg/pot to 29.1 μg/pot with Clipper having a content of 11.0 μg/pot and Sahara 4.7 μg/pot (Figure 7.13b).
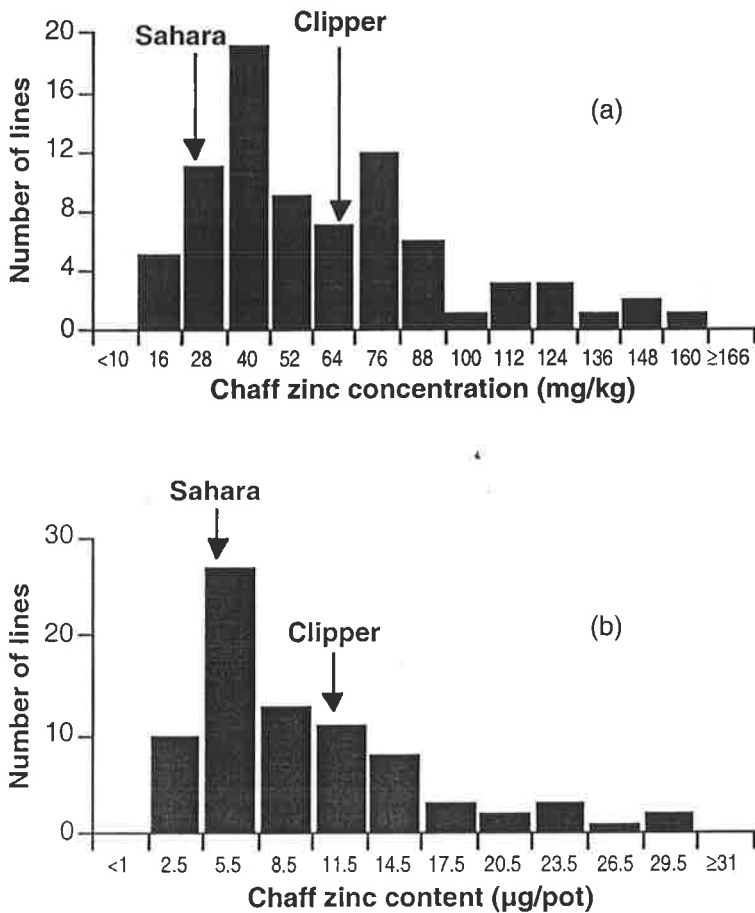
Figures 7.13a and b

Frequency distribution of (a) chaff zinc concentration and (b) chaff zinc content of 78 doubled haploid barley lines and their parents Clipper and Sahara. These lines represent 2-row types only.

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Entire head zinc content

In order to examine the possibility that Zn loading mechanisms in the stem result in a net movement of Zn to reproductive tissues, the total amount of Zn per head (i.e. seed and chaff) was calculated. Values for the individual lines ranged from 32.0 μg/pot to 93.4 μg/pot with Clipper having a content of 68.8 μg/pot and Sahara 80.5 μg/pot (Figure 7.14a). In a further attempt to separate processes involved in loading and unloading of Zn into seeds, the total seed Zn content of a head was calculated as a percentage of the total head Zn content. This value showed a large variation (54 - 94%) amongst the doubled haploid lines (Figure 7.14b). It was hoped that mapping of this value might identify putative loci, independent of seed Zn loci, that indicate a separate mechanism for unloading of Zn into grains.
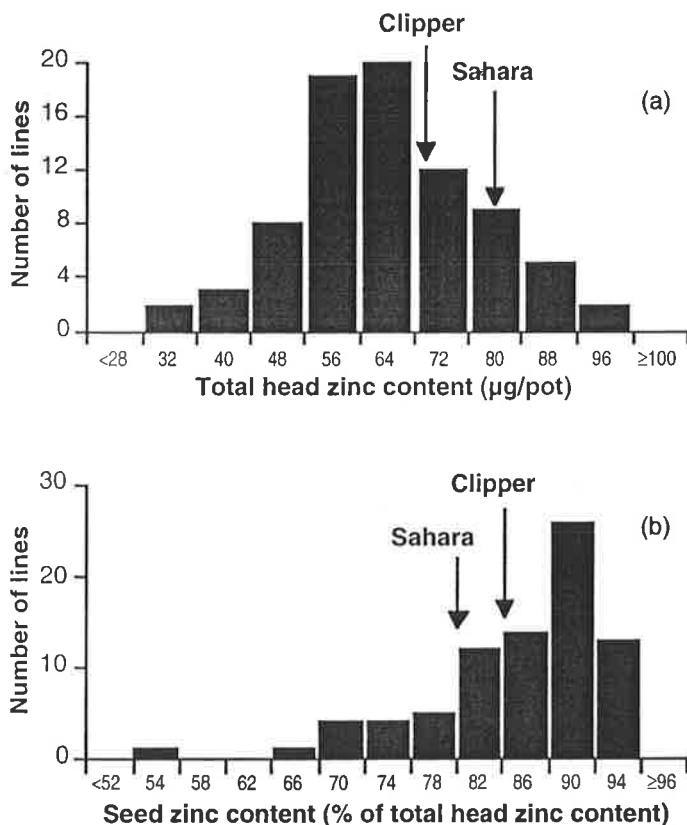
Figures 7.14a and b

Frequency distributions for (a) total head zinc content and (b) seed zinc content as a proportion of total head zinc content expressed in 78 doubled haploid barley lines and their parents Clipper and Sahara. These lines represent 2-row types only.

7.3.2.3 Mapping (anthesis)

Vegetative zinc concentration and content

Marker analysis identified a region on the long arm of chromosome 4H associated with vegetative Zn concentration at anthesis (Figure 7.15a). Analysis of vegetative Zn content at anthesis, however, identified a region of association on the short arm of chromosome 2H (Figure 7.15b) corresponding with the region mapped for seed Zn concentration and content (Experiment 7A). There was also weak association with the region on the long arm of chromosome 2H (again associated with seed Zn accumulation in Experiment 7A).

Zinc concentration and content of developing head

Analysis of the Zn concentration and content of the developing head showed no significant association with any of the markers across the genome.

7.3.2.4 Mapping (maturity)

Zinc concentration and content of main stem

The results for the main stem at maturity were similar to those obtained for the vegetative tissue at anthesis, with Zn concentration showing an association with the same region on chromosome 4H (Figure 7.16a). Zinc content was associated with a similar region on the short arm of chromosome 2H (Figure 7.16b) as for anthesis but the association with the long arm was no longer present.

Seed zinc concentration and content

Analysis of seed Zn concentration yielded significant association with 3 RFLPs, one on the short arm of chromosome 2H (BCD175), one on the long arm of 2H (ksuD22) (Figure 7.17a) and one of unknown location (BCD225B). The regions on 2H were the same as those mapped for vegetative Zn content at anthesis. Mapping of seed Zn content showed association in the same 2 regions on 2H as Zn concentration (Figure 7.17b) and also the region of unknown location.

(a)

(b)

5.38

GERMIN
AWBMA30
WG876
BCD808 *(B)*
BCD265 *(A)*
Mne1A
WG232
CDO795
CDO358
PSR163
AWBMA29
PSR141 *(A)*
WG464
BlueAleurone
ABC152 *(B)*
CDO541
ABC152 *(A)*
Mne2
ksuG10
WG719
BSP
WG114
CDO669
CDO1312
CDO63
ksuE2
BCD1130
ABC305
HVM67

3.0   0.0

LOD

4H

6.28

2.95

ABG312G
AWBMA28
WG516
BCD175
BCD221(A)
PSR108
AWBMA27
AWBMA14
AWBMA33
PSR126
ABC309
ABC468
CDO474(A)
WG789(B)
WG996
CDO370
WG222(E)
WG222(D)
CDO588
CDO366
CDO474(B)
hex-v
ksuF15
MWG892
cMWG694
AWBMA21
CDO665
ksuD22
ABC157
BCD266
ABC153
ABC165
ksuF41
BCD292
CDO678(A)
BCD410
CDO36
AWBMA17
WG645
BCD339
BG123

3.0   0.0

LOD

2H

Figures 7.15a and b

Location of chromosomal regions associated with (a) vegetative zinc concentration and (b) vegetative zinc content at anthesis expressed in 78 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represent 2-row types only. The short arms are towards the tops of the chromosomes. The LOD scores are shown on the x-axis. The RFLP marker most significantly associated with the trait is presented in bold type.

(a)

GERMIN
AWBMA30
WG876
BCD808(B)
BCD265(A)
Mne1A
WG232
CDO795
CDO358
PSR163
AWBMA29
PSR141(A)
WG464
BlueAleurone
ABC152(B)
CDO541
ABC152(A)
Mne2
ksuG10
WG719
BSP
WG114
CDO669
CDO1312
CDO63
ksuE2
BCD1130
ABC305
HVM67

7.96

3.0   0.0
LOD

4H

(b)

4.69

ABG312G
AWBMA28
WG516
BCD175
BCD221(A)
PSR108
AWBMA27
AWBMA14
AWBMA33
PSR126
ABC309
ABC468
CDO474(A)
WG789(B)
WG996
CDO370
WG222(E)
WG222(D)
CDO588
CDO366
CDO474(B)
hex-v
ksuF15
MWG892
cMWG694
AWBMA21
CDO665
ksuD22
ABC157
BCD266
ABC153
ABC165
ksuF41
BCD292
CDO678(A)
BCD410
CDO36
AWBMA17
WG645
BCD339
BG123

3.0   0.0
LOD

2H
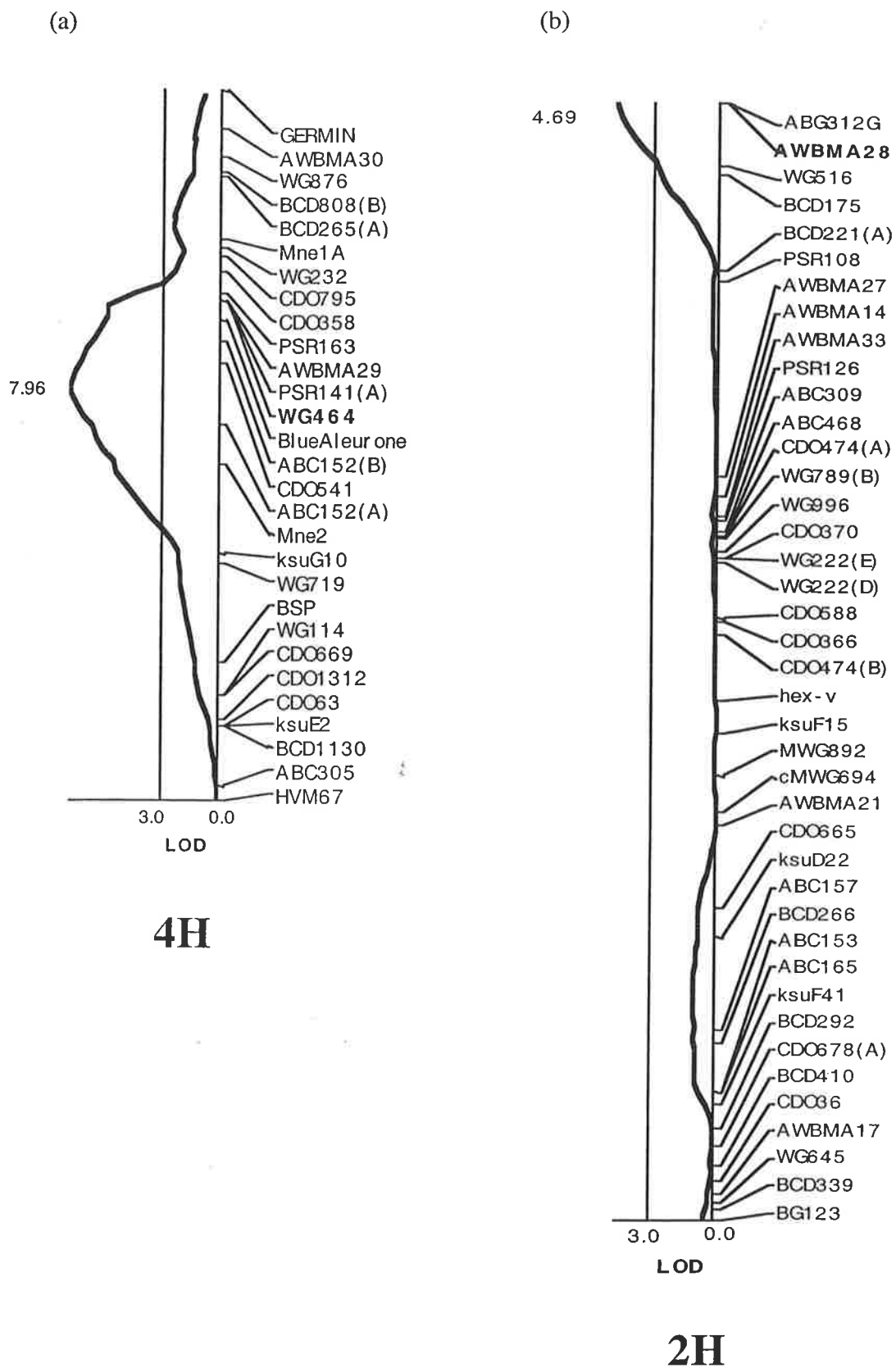
Figure 7.16

Location of chromosomal regions associated with (a) zinc concentration and (b) zinc content of the main culm at maturity, expressed in 78 doubled haploid lines derived from the cross Clipper x Sahara. These lines represented 2-rowed types only. Short arms are towards tops of chromosomes. LOD scores are shown on the x-axis. The RFLP marker most significantly associated with the trait is shown in bold type.

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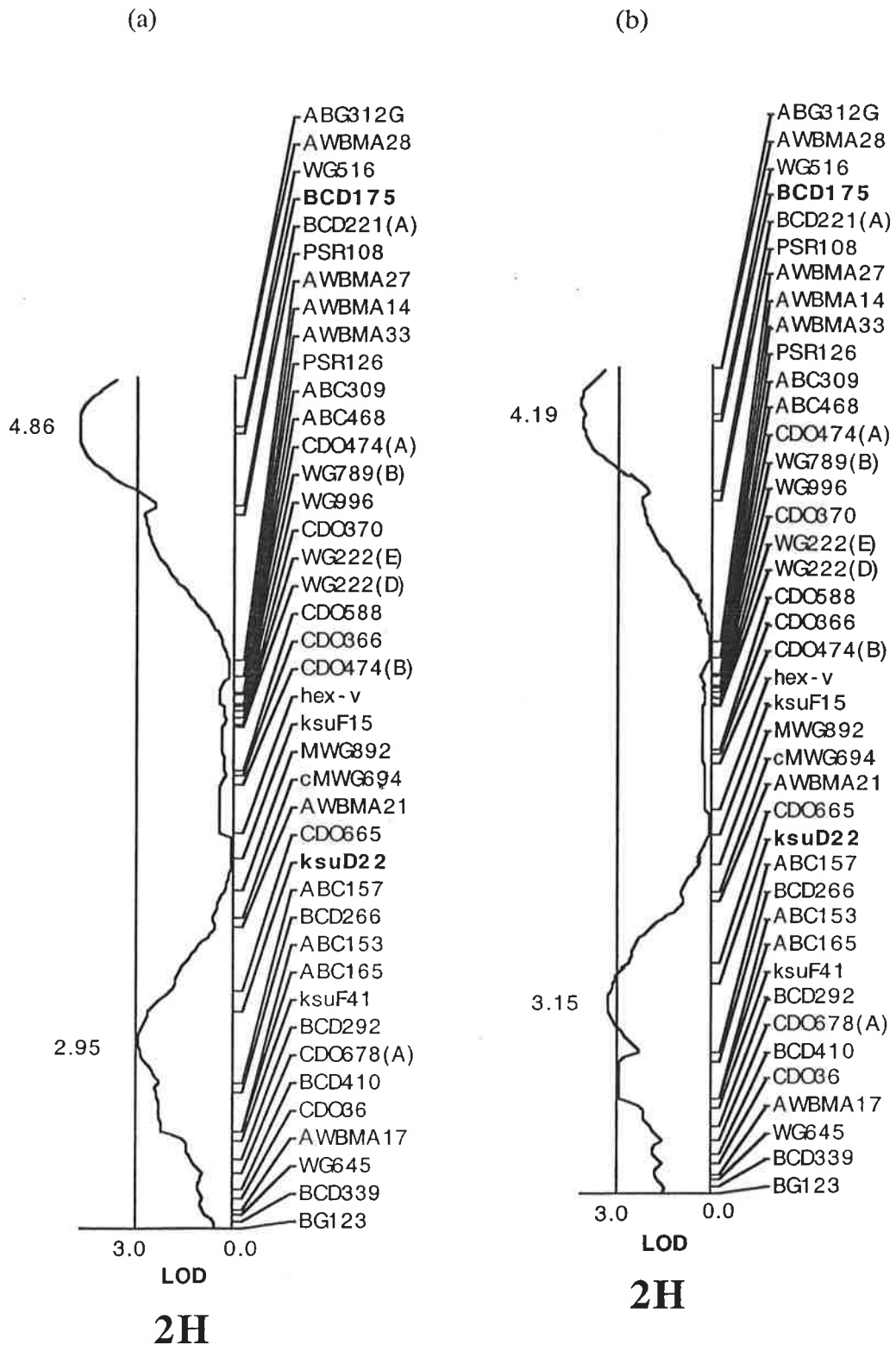
Figures 7.17a and b

Co-location of regions on chromosome 2H associated with (a) seed zinc concentration and (b) seed zinc content expressed in 78 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represent 2-row types only. The short arm is towards the tops of the chromosomes. The LOD scores are shown on the x-axis. The RFLP marker most significantly associated with the trait is shown in bold type.

The frequency distributions of seed Zn concentration and content (Figures 7.12a and b) suggested transgressive segregation with respect to these characters as some of the doubled haploid lines were better than the best parent (Clipper) and some were worse than the worst parent (Sahara). This was confirmed on study of the allelic configuration at each of the 3 markers with Clipper alleles confirming increased Zn concentration and content at the 2HL locus and unmapped locus and the Sahara allele associated with increased Zn at the 2HS locus. The presence of the Sahara allele at this locus conferred an average 20.2% increase in Zn concentration and a 26.3% increase in Zn content. Clipper alleles at the 2HL locus and locus of unknown location increased Zn concentration by an average of 16.5% and 15.9% respectively. The average increase in Zn content at these two loci due to the Clipper alleles was 24.6% and 22.2%. Figure 7.18 shows the average Zn concentrations, contents and seed weights of allelic forms at the 3 loci as well as the averages for the maximum and minimum allelic combinations.

Chaff zinc concentration and content

Analysis of chaff Zn concentration and content showed no association with any markers across the genome.

Entire head zinc content

Analysis of the entire head Zn content showed association with the same 3 markers as for seed Zn concentration and content. The two mapped markers on chromosome 2H are shown in Figure 7.19.

Seed zinc as % of total head zinc

Analysis of this variable showed an association with an area on the long arm of chromosome 5H (Figure 7.20).
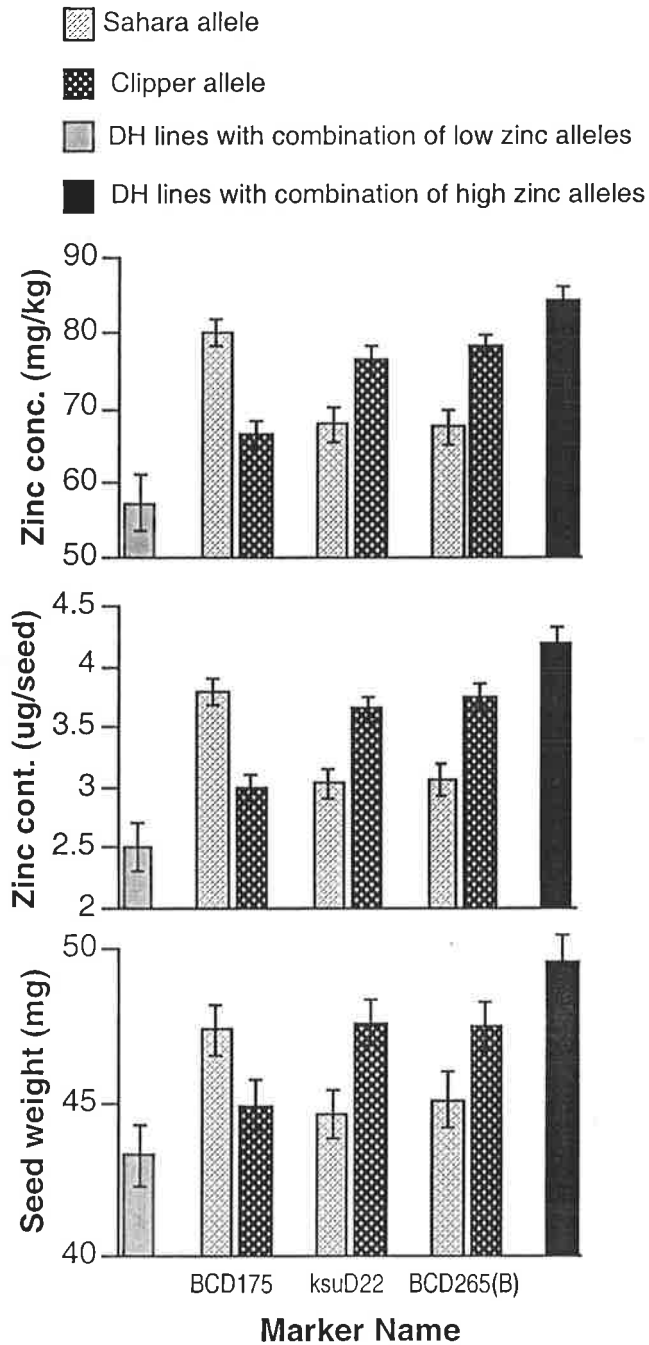
Figures 7.18a, b and c

(a) seed zinc concentration (b) seed zinc content and (c) seed weight expressed in 78 doubled haploid barley lines with respect to their allelic configuration at 3 RFLP markers. Hatched areas show average values for the 3 traits at each marker (shown on the x-axis). Solid areas show average values of maximum and minimum allelic combinations. (Vertical bars represent standard errors).
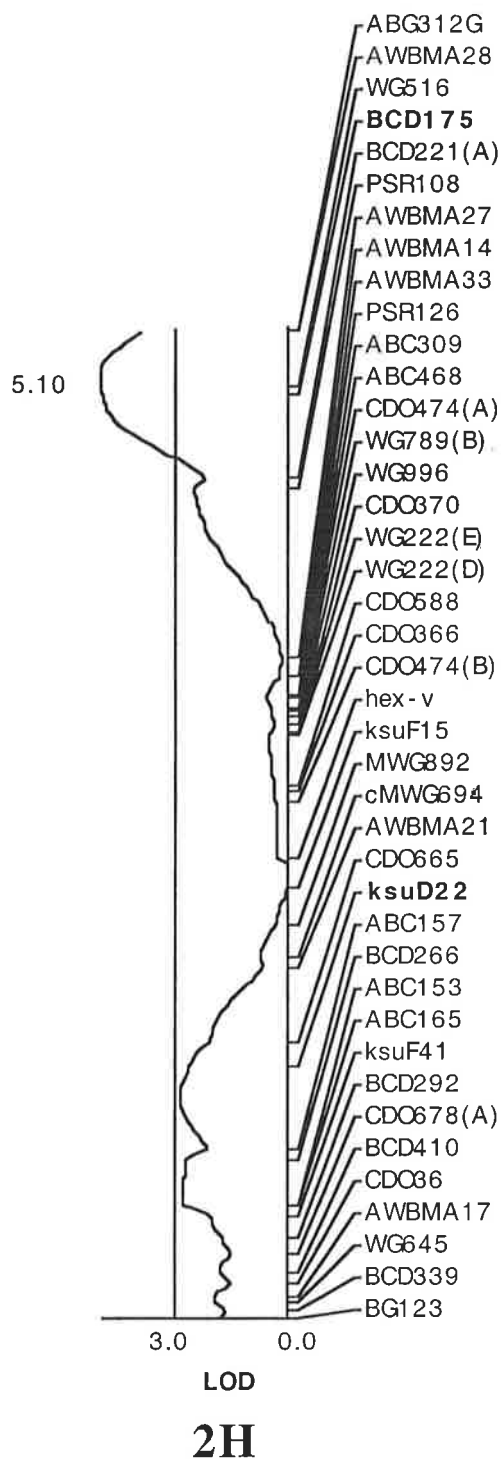
Figure 7.19

Location of chromosomal regions associated with total head zinc content expressed in 78 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represent 2-row types only. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is shown in bold type.
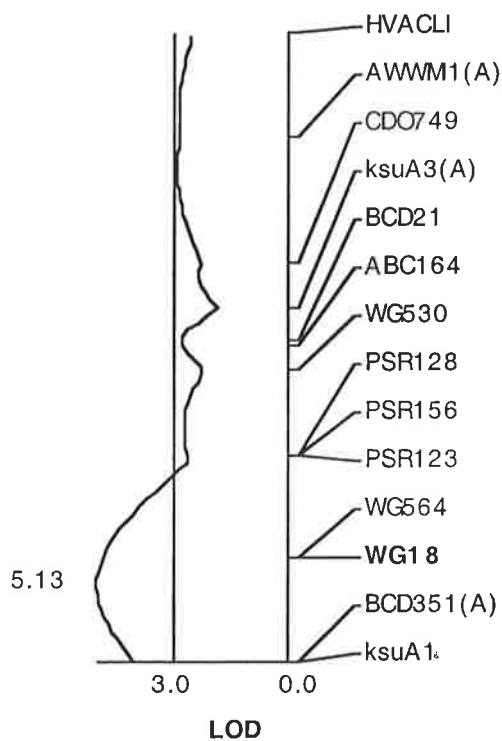
5H

Figure 7.20

Location of chromosomal region associated with seed zinc content as a percentage of total head zinc content, expressed in 78 doubled haploid barley lines derived from the cross Clipper x Sahara. These lines represent 2-row types only. The short arm is towards the top of the chromosome. The LOD score is shown on the x-axis. The RFLP marker most significantly associated with the trait is shown in bold type.

7.4 Discussion

The experiments described in this chapter have identified five chromosomal regions associated with Zn accumulation in barley grain and three chromosomal regions associated with Zn accumulation in vegetative tissues (Table 7.1).

Table 7.1

Detail of zinc traits mapped in Chapter 7, showing tissue type and growth stage, chromosomal location and most significantly associated RFLP marker.

Tissue	Trait	Chromosome	RFLP
Seed	Zn unloading	5H	WG18
	Zn concentration	2HL, 2HS, ?H	ksuD22, BCD175, BCD265B
	Zn content	2H, 2HL, 2HS, ?H	hex-v ksuD22, BCD175, BCD265B
Stem (at maturity)	Zn concentration	4HL	WG464
	Zn content	2HS	AWBMA28
Stem (at anthesis)	Zn concentration	4HL	WG464
	Zn content	2HL, 2HS	ksuD22, BCD175

The nature of the central region of chromosome 2H (mapped in Experiment 7A) appears to be quite different from the other 3 regions mapped in Experiment 7B. The presence of the Clipper allele in this region is associated with higher levels of seed Zn content but not seed Zn concentration. This is explained by the morphological marker for head type (hex-v), which is coincident in this region. Clipper alleles at this locus confer the two row character which results in a larger average seed weight. By contrast, the 3 QTLs mapped in Experiment 7B are associated with higher levels of both Zn concentration and content. Interestingly, these increases correspond with an increase in seed size but are not related to seed number per head. From a breeding perspective, this is a positive result since incorporation of these QTLs for the purpose of increasing seed Zn concentration or content would not consequently reduce yield. Furthermore, larger seed size is generally

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associated with higher protein content which has been shown to be correlated with seed viability and seedling vigour in other species (Ries, 1971; Sawan *et al.*, 1989; Mian and Nafziger, 1992). It remains unclear whether the genetic seed size determines how much Zn enters the seed or, conversely, the amount of incoming Zn affects seed size. Regardless of this, identification of these 3 QTLs potentially enables significant increase in seed Zn through conventional breeding methods. In a similar manner to seed size, increased seed Zn content has been shown to increase seedling vigour and final yield of wheat and barley, particularly when grown on Zn deficient soils (Rengel and Graham, 1995a,b; Yilmaz *et al.*, 1997; Genc *et al.*, 2000).

Wolswinkel (1999) described a need for a better understanding of the factors involved in micronutrient transport into cereal grains, including the route of transport from vegetative to reproductive tissues and the timing and rate of remobilisation from vegetative plant parts. Results of Experiment 7B support what is already known about these processes. The correlation of the 2HL and 2HS regions for vegetative Zn content at anthesis and seed Zn content suggests a possible role for these loci in the loading of Zn into the phloem. Although not perfectly understood, it is generally accepted that nutrients move into developing seeds via the phloem. Photosynthetically active but senescing structures such as leaves serve as sources of nutrients while developing fruits act as sinks with 'sink strength' defined as the potential capacity of sink tissues to accumulate assimilates (Wareing and Patrick, 1975; Patrick, 1981; Wolswinkel, 1984; Ho *et al.*, 1989). The ease of transport varies between nutrients with Zn considered to be intermediate in its mobility. Such long distance transport of Zn via the phloem is dependent on phloem loading and unloading in the source and sink regions. Transfer of nutrients from xylem to phloem occurs all along the pathway from roots to shoots via transfer cells (Pate and Gunning, 1972) with intense transfer occurring at the nodes of stems (Pate and Jeschke, 1995). This being the case, it seems reasonable to assume that differences in vegetative Zn content at anthesis are related to differences in ability to transport Zn from roots to shoots and/or load it into the phloem, ready for further transport to the developing seed. The co-segregation of seed Zn accumulation with vegetative Zn accumulation at anthesis at the 2HS and 2HL regions lends support to this theory. This is

also in agreement with the generally accepted mass flow theory of long distance transport (Munch, 1930) which postulates that total contents of solutes are the determining factor of sink content rather than solute concentrations. The non-correlation of vegetative Zn concentrations with seed Zn content is therefore in accordance with Munch's theory.

At maturity, stem Zn content mapped to a region on the short arm of chromosome 2H that was close to that mapped for seed Zn accumulation but not at the 2HL region. It would appear that, by this time, whatever mechanism(s) are controlled by the 2HL region are no longer operational. The fact that the 2HS region is still having an effect indicates that it might be responsible not only for phloem loading but also for remobilisation of Zn from root to shoot.

The QTL of unknown location did not co-segregate with vegetative Zn accumulation, suggesting it is involved with processes other than phloem loading. These could include phloem unloading into the extracellular space (apoplast) and/or subsequent efflux from there into the developing seed. As there is no direct vascular connection between the maternal plant and filial tissues (Wolswinkel, 1992), the accumulation of nutrients in seed is dependent on unloading from the phloem to the apoplast separating the two generations, and, uptake from the apoplast to the tissues of the embryo or endosperm (Jenner, 1980; Thorne, 1985; Patrick, 1990). Alternatively, this QTL could still be involved in phloem loading of Zn but occurrence of this event would need to have started after anthesis and concluded before maturity. The QTL mapped on chromosome 5H (Figure 7.20) however, is likely to be associated with uptake from the apoplast into the seed tissues since it relates to the amount of Zn in the entire head that subsequently enters the seed.

Although Sahara transports a greater net amount of Zn to the head than Clipper, it could be considered to be less efficient since the sink strength should be much stronger due to a larger number of seeds per head. This inefficiency manifests itself as a reduced Zn content per seed and is a general result for the 6 row doubled haploid lines in comparison to the 2 row lines. In considering the 2 row group alone, seed number is not correlated

with seed Zn accumulation so it seems that differences in total head Zn content are related to the ability of different lines to load Zn into the phloem and transport it to the developing head. With regard to vegetative Zn efficiency, it is obvious that significant changes have occurred between the parents over time. The reversal in ranking of Sahara and Clipper with respect to Zn accumulation between the 4 week harvest (Chapter 5) and harvests at anthesis and maturity casts serious doubt over the validity of an early bioassay (such as that used in Chapter 3) being related to final yield, particularly when comparing varieties derived from widely varying germplasm.

It is clear that vegetative Zn accumulation in the early stage of growth is controlled by mechanisms separate from those relating to seed Zn accumulation. Correlation has been found between vegetative Zn content at anthesis and seed Zn content and although evidence is not conclusive, a role in phloem loading has been proposed for two of the 3 QTLs mapped. It should be remembered that all results were obtained under Zn sufficiency, a condition in which Zn can be readily transported from vegetative tissues to reproductive tissues (Welch, 1986). Under Zn deficiency, however, there is little transport to developing seeds. With this in mind, further research is needed regarding the effect of Zn deficiency on the 3 QTLs discussed. Information is needed as to whether increased seed Zn creates a 'sink strength' that cannot be supplied under Zn deficiency, resulting in yield decline. One must be careful in that selecting for increased seed Zn, one does not inadvertently breed varieties that are Zn inefficient.

Chapter 8

General Discussion

Zinc is the most widespread micronutrient deficiency in the world, limiting grain yield in the major cereals wheat, rice and maize (Takkar and Walker, 1993). Alleviation of zinc deficiency through fertiliser application is costly and its effectiveness is limited by topsoil drying, subsoil constraints and disease interactions (Graham and Rengel, 1993). An alternative and more sustainable approach is to breed cultivars that are zinc efficient, ie. have an ability to grow and yield well in soils too deficient in zinc for a standard cultivar (Graham, 1984). A combination of these strategies often results in highest yields (Rengel, 1999).

Zinc deficiency also results in poor quality of seed in terms of reduced zinc concentration (mg Zn/kg) and zinc content (μg Zn/seed). These two parameters, although interrelated, are of separate significance. Zinc concentration is important for human and animal health since food grains are bought and consumed by weight whereas zinc content is more important for the seedling vigour of the subsequent plant generation, particularly if grown on zinc deficient soils (Rengel and Graham, 1995a,b; Genc *et al.*, 2000). The ability to increase both zinc concentration and zinc content, therefore, has important implications not only for alleviation of zinc deficiency in people reliant on these grains as the major component of their diet but also for improved yield on zinc deficient soils. Identification of the genes controlling these agronomic factors followed by their incorporation into adapted varieties should provide a sustainable solution to the global problem of zinc deficiency.

Prior to this project, zinc concentrations in the barley varieties Clipper and Sahara (Pallotta, unpublished) gathered from a manganese efficiency study showed that Sahara

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had greater shoot zinc concentration than Clipper after 4 weeks growth in both manganese deficient and manganese sufficient conditions. The availability of a doubled haploid population derived from these two parents, together with an RFLP linkage map showed promise as tools by which to map zinc efficiency and/or accumulation.

In the past, screening for tolerance to zinc deficiency has relied on field experiments (Cakmak *et al.*, 1997; Yilmaz *et al.*, 1996) but spatial variation in soil zinc availability (Kubota and Alloway, 1972; Nable and Webb, 1993), together with seasonal variations in climate, have made analysis difficult. In addition, field sites usually cannot be closely monitored due to accessibility, and high maintenance costs are involved. Development of molecular markers, followed by their implementation in a breeding program presents a cost effective alternative to large scale field trials. Successful breeding of zinc efficient cultivars in this way would rely on correlation of yield results to a suitable bioassay (preferably at the seedling stage) that allows large numbers of plants to be screened in a reasonable time. Such an assay has been used by many researchers on various crops: wheat (Graham *et al.*, 1992; Rengel and Graham, 1995a) canola (Grewal *et al.*, 1997) and chickpea (Khan *et al.*, 1998).

The assay used in chapter 3 was adapted from Rengel and Graham (1995a) but proved to require extensive refinement to make it a suitable test for barley. This refinement is documented throughout chapter 3 and highlighted the difficulties associated with accurately measuring zinc efficiency owing to the effects of other nutrients, in this case high levels of boron and molybdenum. Further difficulties due to genotype-specific response curves were identified in both chapter 3 and 4 suggesting that multi level screening was necessary. This supports the opinions of some authors who stressed the importance of screening over a full range of stress (Boken, 1966; Blum, 1988; Paull, 1990); however this clearly becomes unmanageable with large numbers of genotypes such as are needed for mapping purposes.

A further difficulty was the range of starting seed zinc contents of the doubled haploid lines. Results from Chapter 7 indicated that the doubled haploid seed increased by

approximately four-fold from the lowest to the highest for zinc concentration and almost six-fold for zinc content. It was decided that such a wide range of seed zinc content would confound results (Rengel and Graham, 1995a; Genc *et al.*, 2000) and possibly lead to an incorrect identification of genomic regions associated with zinc efficiency.

Beyond these problems, there was serious concern regarding the correlation of vegetative zinc efficiency with grain yield efficiency (Chapter 4). Although these observations were based on a visual score of relative growth at the vegetative stage, the non-correlation was strong enough to suggest that the two measures of efficiency (vegetative and grain yield) are controlled by independent mechanisms. This notion is partly supported by the identification of different genomic regions controlling zinc accumulation at different growth stages (Chapters 6 and 7).

Little is known about the genetics of zinc accumulation in plants but it is thought that two sets of genes are responsible, those associated with uptake from the soil and those involved in transport around the plant (Graham *et al.*, 1999). This, indeed, appears to be the case with the present study identifying 5 separate chromosomal regions associated with differential zinc accumulation in shoots and grain of barley. Figure 8.1 depicts a model barley plant showing the putative sites of influence of the particular chromosomal regions identified in this study.

The region on the short arm of chromosome 4H (mapped in Chapter 5) is associated with increased vegetative shoot zinc concentration and content due to the Sahara allele. This correlation was no longer present at anthesis or maturity (Chapter 7) suggesting that this region was associated with zinc uptake from the soil and/or transport to the developing shoot during the vegetative phase. Possible mechanisms by which this could occur include: (i) root induced changes in rhizosphere pH resulting from release of zinc-chelating phytosiderophores (Cakmak *et al.*, 1994; Rengel *et al.*, 1998) (ii) an increased maximum uptake rate (I_{max}) resulting in increased net zinc accumulation (Cakmak *et al.*, 1996; Rengel and Graham, 1996; Rengel and Wheal, 1997) or (iii) a greater ability to transport zinc from root to shoots. The solution culture experiment

(Chapter 6) suggested that the third mechanism is unlikely since, under zinc sufficiency, Clipper was the more efficient translocator. This result should be treated with caution, however, as Clipper was also the higher accumulator of shoot zinc in this experiment.
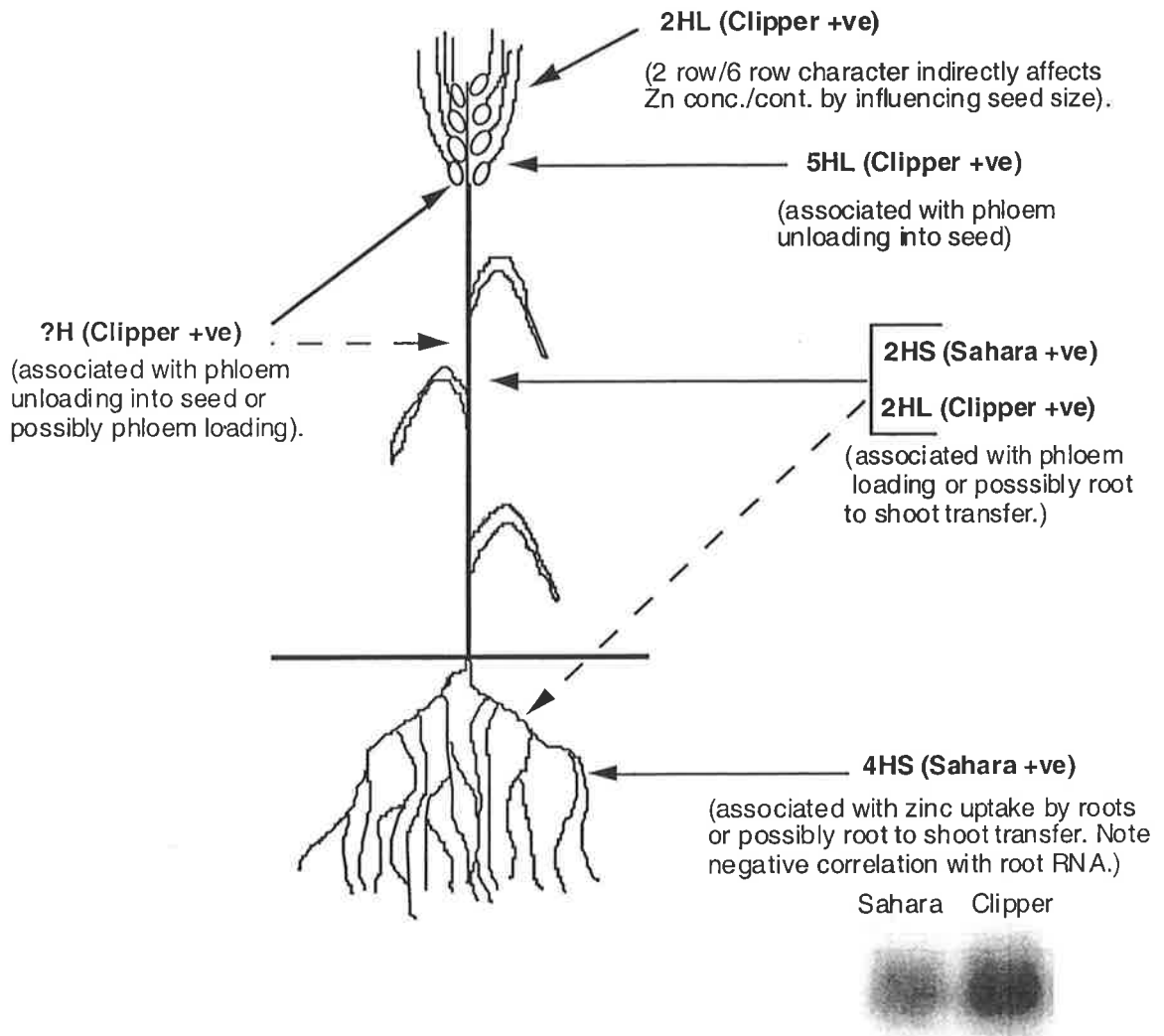
Figure 8.1

Diagrammatic representation of a barley plant showing the sites of influence of chromosomal regions mapped in Chapters 5 and 7. The allele associated with increased zinc accumulation is shown in brackets after the chromosomal location.

With respect to efficiency, Chapter 3 showed Clipper to be more zinc efficient than Sahara. Even though Sahara accumulated as much or greater zinc than Clipper under zinc sufficiency, under zinc limiting conditions its growth was decreased at a greater rate. This suggests that Clipper has greater utilisation efficiency (Sattelmacher *et al.*, 1994).

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Alternatively, Sahara, rather than having a greater capacity to extract zinc from soil, has a higher internal requirement for zinc than Clipper which is met under sufficiency conditions but is reflected as a greater decline in shoot growth when grown under zinc deficiency. If indeed there are differing mechanisms for zinc efficiency, then transgressive segregation should occur within the doubled haploids resulting in some lines that have all mechanisms, some that have none and some that fall between these two extremes.

Whether the differential zinc efficiency measured between the two parents is related to the 4HS locus remains unknown. Similar growth responses from a Sahara-like doubled haploid (DH51) and a Clipper-like one (DH125) in Chapter 3 give some extra weight to the notion that 4HS and zinc efficiency are related but the evidence is certainly not conclusive.

Further studies showed Clipper to be more effective than Sahara at loading zinc into the seed. Three putative QTLs were identified as being associated with seed zinc concentration and content as well as seed weight. For two of the QTLs, the presence of the Clipper allele was associated with increased seed zinc while for the third QTL it was the Sahara allele that was responsible for higher seed zinc concentration and content.

There is little known about the genetics of micronutrient transport and loading other than the pioneering work by Ripperger and Schreiber (1982) who identified a major gene coding for nicotiamine in tomato. This iron-transporting amine was expressed in the shoot, appearing to facilitate iron transport in phloem. Nicotianamine is ubiquitous in higher plants (Prochàzka and Scholz, 1984; Ripperger and Schreiber, 1982) and is likely to be capable of facilitating movement of all the heavy metals (Welch, 1995). The regions 2HL and 2HS which were associated with seed zinc concentration and content were also associated with vegetative zinc content at anthesis. It is possible that these regions are related to the synthesis of nicotianamine, thereby enhancing the capacity for zinc translocation. The 2HS region was associated with increased zinc due to the Sahara allele and 2HL due to the Clipper allele. Whatever the mechanism, it appears that these two loci are similar in their effect on zinc loading into seed and are the main regions responsible

for this process in their respective parents. The fact that they have an additive effect in combination suggests that they encode two separate functions and that in barley, the capacity for seed zinc loading has not evolved to saturation point.

The third QTL (?H) identified as being responsible for increased seed concentration and content due to the Clipper allele was quite different in nature to the regions at 2HS and 2HL. It was not associated with vegetative zinc content at anthesis suggesting it was related to processes other than phloem loading, possibly phloem unloading or zinc efflux into the seed. The fourth genomic region on chromosome 5HL was calculated from the total seed zinc as a percentage of total head zinc. Such a measurement calculates how much zinc is loaded into the seed once it has been transported to the head. It seems reasonable therefore to assume that this locus is involved with uptake of zinc from the apoplast into the embryo or endosperm of the developing seed.

This study has located several genomic regions associated with differential zinc accumulation in barley. The three regions relating to seed zinc have important consequences for the alleviation of zinc deficiency in humans and animals. Although barley is not used as a major food source by humans, genomic similarity between cereals, together with advances in DNA technology, theoretically enable the development of molecular markers to identify a particular trait in a number of species (Schachtman and Barker, 1999). This method could be used to increase speed and efficiency of breeding for zinc-dense grain of major cereals. Alternatively, isolation of the genes responsible for high seed zinc density in barley, followed by transformation into wheat or rice would achieve the same objective of providing grains of increased zinc concentration and content, thereby providing a sustainable solution to the problem of human zinc deficiency. Implementation of these methods in a breeding program could equally be applied to the other micronutrients, thus providing the tools to alleviate a number of globally important nutritional disorders.

Appendix 1

Analysis of variance was conducted using SuperANOVA™. Homogeneity of variance was examined by a plot of the residual values versus the fitted values of the dependent variable.

3.1 Analysis of variance tables for Experiment 3A

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Zn treatment	11	6.88	.63	466.61	.0001
genotype	1	2.82E-5	2.82E-5	.02	.8858
Zn treatment * genotype	11	.03	2.28E-3	1.70	.1344
Residual	24	.03	1.34E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Zn treatment	11	9.38	.85	308.81	.0001
genotype	1	.01	.01	1.88	.1828
Zn treatment * genotype	11	.07	.01	2.44	.0328
Residual	24	.07	2.76E-3		

Dependent: log zinc content

3.2 Analysis of variance tables for Experiment 3B

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Zn treatment	5	7.44	1.49	25.38	.0001
genotype	1	.01	.01	.23	.6361
Zn treatment * genotype	5	.91	.18	3.11	.0165
Residual	48	2.81	.06		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Zn treatment	5	8.90	1.78	51.11	.0001
genotype	1	.03	.03	1.00	.3215
Zn treatment * genotype	5	.16	.03	.93	.4676
Residual	48	1.67	.03		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Zn treatment	5	12.69	2.54	75.55	.0001
genotype	1	.01	.01	.20	.6582
Zn treatment * genotype	5	.51	.10	3.03	.0185
Residual	48	1.61	.03		

Dependent: log zinc content

3.3 Analysis of variance tables for Experiment 3C

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
soil prep.	2	2.60	1.30	22.65	.0001
Zn treatment	1	10.44	10.44	181.99	.0001
B treatment	1	.03	.03	.53	.4737
genotype	2	.22	.11	1.89	.1734
soil prep. * Zn treatment	2	.01	3.42E-3	.06	.9422
soil prep. * B treatment	2	.04	.02	.39	.6813
Zn treatment * B treatment	1	.02	.02	.35	.5591
soil prep. * genotype	2	.04	.02	.39	.6801
Zn treatment * genotype	2	.20	.10	1.70	.2035
B treatment * genotype	2	.01	3.54E-3	.06	.9402
soil prep. * Zn treatment *...	2	.10	.05	.88	.4273
soil prep. * Zn treatment *...	2	.03	.01	.24	.7891
soil prep. * B treatment * ...	2	.09	.04	.78	.4718
Zn treatment * B treatment...	2	.03	.02	.28	.7563
soil prep. * Zn treatment *...	2	.01	.01	.10	.9082
Residual	24	1.38	.06		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
soil prep.	2	1.69E-3	8.44E-4	.29	.7534
Zn treatment	1	7.62	7.62	2589.13	.0001
B treatment	1	7.01E-5	7.01E-5	.02	.8787
genotype	1	6.22E-4	6.22E-4	.21	.6499
soil prep. * Zn treatment	2	.05	.02	8.17	.0020
soil prep. * B treatment	2	.01	2.72E-3	.92	.4109
Zn treatment * B treatment	1	3.49E-3	3.49E-3	1.19	.2870
soil prep. * genotype	2	4.88E-3	2.44E-3	.83	.4488
Zn treatment * genotype	1	.05	.05	16.77	.0004
B treatment * genotype	1	2.39E-3	2.39E-3	.81	.3762
soil prep. * Zn treatment *...	2	1.84E-3	9.22E-4	.31	.7342
soil prep. * Zn treatment *...	2	.03	.02	5.37	.0118
soil prep. * B treatment * ...	2	1.81E-3	9.05E-4	.31	.7382
Zn treatment * B treatment...	1	6.30E-5	6.30E-5	.02	.8850
soil prep. * Zn treatment *...	2	.01	2.64E-3	.90	.4210
Residual	24	.07	2.94E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
soil prep.	2	.12	.06	31.98	.0001
Zn treatment	1	11.10	11.10	5929.93	.0001
B treatment	1	1.64E-3	1.64E-3	.87	.3591
genotype	1	2.60E-3	2.60E-3	1.39	.2503
soil prep. * Zn treatment	2	.07	.04	19.86	.0001
soil prep. * B treatment	2	.01	3.06E-3	1.63	.2161
Zn treatment * B treatment	1	6.48E-4	6.48E-4	.35	.5619
soil prep. * genotype	2	4.69E-3	2.34E-3	1.25	.3039
Zn treatment * genotype	1	.06	.06	29.87	.0001
B treatment * genotype	1	3.09E-3	3.09E-3	1.65	.2112
soil prep. * Zn treatment *...	2	.01	.01	2.75	.0840
soil prep. * Zn treatment *...	2	.02	.01	4.59	.0205
soil prep. * B treatment * ...	2	.01	2.51E-3	1.34	.2806
Zn treatment * B treatment...	1	1.33E-3	1.33E-3	.71	.4074
soil prep. * Zn treatment *...	2	.02	.01	5.85	.0085
Residual	24	.04	1.87E-3		

Dependent: log zinc content

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
soil prep.	2	.05	.02	23.61	.0001
Zn treatment	1	.04	.04	38.44	.0001
B treatment	1	2.43	2.43	2342.32	.0001
genotype	1	1.00	1.00	961.08	.0001
soil prep. * Zn treatment	2	5.43E-4	2.72E-4	.26	.7716
soil prep. * B treatment	2	.02	.01	9.85	.0008
Zn treatment * B treatment	1	.03	.03	24.93	.0001
soil prep. * genotype	2	4.24E-3	2.12E-3	2.04	.1514
Zn treatment * genotype	1	.05	.05	52.24	.0001
B treatment * genotype	1	.05	.05	51.33	.0001
soil prep. * Zn treatment *...	2	4.91E-3	2.45E-3	2.37	.1152
soil prep. * Zn treatment *...	2	.01	4.69E-3	4.53	.0215
soil prep. * B treatment * ...	2	1.92E-3	9.60E-4	.93	.4094
Zn treatment * B treatment...	1	1.88E-6	1.88E-6	1.81E-3	.9664
soil prep. * Zn treatment *...	2	1.62E-3	8.08E-4	.78	.4699
Residual	24	.02	1.04E-3		

Dependent: log boron conc.

3.4 Analysis of variance tables for Experiment 3D

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	3	.58	.19	28.21	.0001
zinc treatment	2	1.34	.67	98.25	.0001
genotype * zinc treatment	6	.26	.04	6.25	.0011
Residual	18	.12	.01		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	3	.02	.01	4.97	.0110
zinc treatment	2	4.05	2.02	1897.29	.0001
genotype * zinc treatment	6	.04	.01	6.21	.0011
Residual	18	.02	1.07E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	3	.06	.02	76.21	.0001
zinc treatment	2	4.67	2.33	8957.84	.0001
genotype * zinc treatment	6	.07	.01	44.60	.0001
Residual	18	4.69E-3	2.61E-4		

Dependent: log zinc content

4.1 Analysis of variance tables for Experiment 4A

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	6	.30	.05	9.04	.0001
zinc treatment	2	6.28	3.14	567.62	.0001
genotype * zinc treatment	12	.21	.02	3.09	.0114
Residual	21	.12	.01		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	6	.07	.01	8.63	.0001
zinc treatment	2	6.33	3.17	2320.88	.0001
genotype * zinc treatment	12	.08	.01	4.75	.0009
Residual	21	.03	1.36E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	6	.10	.02	14.90	.0001
zinc treatment	2	8.11	4.05	3636.68	.0001
genotype * zinc treatment	12	.07	.01	4.91	.0008
Residual	21	.02	1.11E-3		

Dependent: log zinc content

4.2 Analysis of variance tables for Experiment 4B

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	4	1.10	.28	9.91	.0001
zinc treatment	2	4.73	2.37	85.03	.0001
genotype * zinc treatment	8	.47	.06	2.10	.0831
Residual	21	.58	.03		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	4	.11	.03	32.02	.0001
zinc treatment	2	3.90	1.95	2293.73	.0001
genotype * zinc treatment	8	.02	3.05E-3	3.59	.0089
Residual	21	.02	8.50E-4		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	4	.07	.02	7.70	.0006
zinc treatment	2	5.62	2.81	1270.69	.0001
genotype * zinc treatment	8	.05	.01	2.83	.0268
Residual	21	.05	2.21E-3		

Dependent: log zinc content

5.1 Analysis of variance tables for Experiment 5A

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.09	.09	7.08	.0121
Zn tmt.	1	.04	.04	2.76	.1066
Mn tmt.	1	1.60	1.60	121.74	.0001
genotype * Zn tmt.	1	2.35E-4	2.35E-4	.02	.8945
genotype * Mn tmt.	1	.12	.12	8.77	.0057
Zn tmt. * Mn tmt.	1	.02	.02	1.26	.2709
genotype * Zn tmt. * Mn t...	1	.06	.06	4.31	.0459
Residual	32	.42	.01		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	7745.09	7745.09	198.83	.0001
Zn tmt.	1	7473.66	7473.66	191.86	.0001
Mn tmt.	1	1716.10	1716.10	44.06	.0001
genotype * Zn tmt.	1	83.81	83.81	2.15	.1522
genotype * Mn tmt.	1	133.88	133.88	3.44	.0730
Zn tmt. * Mn tmt.	1	32.08	32.08	.82	.3710
genotype * Zn tmt. * Mn t...	1	424.06	424.06	10.89	.0024
Residual	32	1246.50	38.95		

Dependent: zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	8696.31	8696.31	62.23	.0001
Zn tmt.	1	11609.35	11609.35	83.08	.0001
Mn tmt.	1	848.52	848.52	6.07	.0193
genotype * Zn tmt.	1	179.23	179.23	1.28	.2658
genotype * Mn tmt.	1	450.04	450.04	3.22	.0822
Zn tmt. * Mn tmt.	1	173.85	173.85	1.24	.2730
genotype * Zn tmt. * Mn t...	1	257.10	257.10	1.84	.1845
Residual	32	4471.76	139.74		

Dependent: zinc content

6.1 Analysis of variance tables for Experiment 6A

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.03	.03	8.76	.0119
Zn tmt.	1	2.80E-3	2.80E-3	.79	.3930
harvest	2	1.25	.63	175.55	.0001
genotype * Zn tmt.	1	9.75E-4	9.75E-4	.27	.6102
genotype * harvest	2	.01	2.98E-3	.84	.4569
Zn tmt. * harvest	2	.02	.01	3.28	.0731
genotype * Zn tmt. * harvest	2	.01	2.51E-3	.71	.5133
Residual	12	.04	3.56E-3		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.09	.09	100.67	.0001
Zn tmt.	1	1.21E-4	1.21E-4	.13	.7235
harvest	2	.68	.34	369.59	.0001
genotype * Zn tmt.	1	.01	.01	10.11	.0079
genotype * harvest	2	.04	.02	19.92	.0002
Zn tmt. * harvest	2	.01	3.49E-3	3.77	.0536
genotype * Zn tmt. * harvest	2	.01	4.08E-3	4.40	.0368
Residual	12	.01	9.26E-4		

Dependent: root weight

shoots

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.02	.02	13.04	.0036
Zn tmt.	1	1.96	1.96	1524.93	.0001
harvest	2	.47	.24	183.64	.0001
genotype * Zn tmt.	1	.01	.01	11.47	.0054
genotype * harvest	2	.01	3.24E-3	2.52	.1217
Zn tmt. * harvest	2	.07	.03	25.50	.0001
genotype * Zn tmt. * harvest	2	1.91E-4	9.56E-5	.07	.9286
Residual	12	.02	1.28E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	2.83E-4	2.83E-4	.08	.7839
Zn tmt.	1	1.89	1.89	525.06	.0001
harvest	2	.03	.01	3.90	.0494
genotype * Zn tmt.	1	.01	.01	3.16	.1009
genotype * harvest	2	2.13E-3	1.06E-3	.30	.7491
Zn tmt. * harvest	2	.06	.03	8.63	.0048
genotype * Zn tmt. * harvest	2	3.15E-4	1.58E-4	.04	.9572
Residual	12	.04	3.59E-3		

Dependent: log zinc content

roots

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.07	.07	27.01	.0002
Zn tmt.	1	1.45	1.45	571.05	.0001
harvest	2	.43	.21	83.74	.0001
genotype * Zn tmt.	1	.03	.03	10.28	.0075
genotype * harvest	2	1.41E-3	7.06E-4	.28	.7622
Zn tmt. * harvest	2	.04	.02	6.97	.0098
genotype * Zn tmt. * harvest	2	.02	.01	4.54	.0340
Residual	12	.03	2.54E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.01	.01	.89	.3629
Zn tmt.	1	1.51	1.51	261.92	.0001
harvest	2	.24	.12	21.22	.0001
genotype * Zn tmt.	1	.07	.07	12.49	.0041
genotype * harvest	2	.01	3.55E-3	.62	.5554
Zn tmt. * harvest	2	.03	.01	2.46	.1274
genotype * Zn tmt. * harvest	2	4.26E-3	2.13E-3	.37	.6981
Residual	12	.07	.01		

Dependent: log zinc content

6.2 Analysis of variance tables for Experiment 6B

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.12	.12	20.14	.0001
soil type	5	3.80	.76	126.13	.0001
genotype * soil type	5	.10	.02	3.22	.0214
Residual	26	.16	.01		

Dependent: shoot weight

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.20	.20	161.43	.0001
soil type	5	1.31	.26	208.45	.0001
genotype * soil type	5	.05	.01	8.52	.0001
Residual	26	.03	1.26E-3		

Dependent: log zinc conc.

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
genotype	1	.34	.34	114.37	.0001
soil type	5	2.66	.53	177.17	.0001
genotype * soil type	5	.06	.01	4.05	.0075
Residual	26	.08	3.00E-3		

Dependent: log zinc content

Appendix 2

Average elemental concentrations (mg/kg) for Experiments 3A-D. (N.B. Values for K, P and S are x10,000).

Experiment	Zn treatment	Genotype	Fe	Mn	Cu	B	Ca	Mg	Na	K	P	S
3A	0	Clipper	300	253	24	48	9000	5600	250	6	16.5	0.8
	0.05		160	158	23	35	13000	5100	250	3.2	0.8	0.5
	0.1		94	125	18	27	11000	4300	250	2.7	5.5	0.4
	0.3		61	121	18	33	12000	4700	320	2.7	0.6	0.43
	3.2		49	130	21	30	11000	4200	320	2.7	0.5	0.38
	0	Sahara	428	298	22	22	16000	6000	380	5.3	19	1.2
	0.05		215	179	18	18	15000	4400	540	3.1	0.8	0.5
	0.1		136	141	17	14	15000	3900	570	2.8	0.6	0.4
	0.3		60	102	15	17	12000	3800	470	2.2	0.5	0.4
	3.2		52	101	15	21	10000	3400	460	2.3	0.45	0.36
3B	0	Clipper	200	135	20	29	9000	3000	250	6	0.9	0.54
	0.05		150	100	17	25	11000	3000	270	4	0.6	0.36
	0.1		110	105	16	27	11000	3200	300	3.5	0.5	0.38
	0.3		75	90	17	26	11000	3100	300	3.4	0.45	0.35
	3.2		75	130	19	29	9000	3300	380	3.6	0.5	0.37
	0	Sahara	240	170	16	9	9000	3200	500	7	1	0.6
	0.05		125	85	13	8	11000	2500	490	4.2	0.5	0.36
	0.1		85	75	12	9	11000	2300	500	3.6	0.5	0.38
	0.3		58	60	12	10	11000	2100	750	3.4	0.45	0.35
	3.2		52	80	14	11	9000	2300	710	3.5	0.5	0.37

Experiment	Zn treatment	Genotype	Fe	Mn	Cu	B	Ca	Mg	Na	K	P	S
3C	0 (washed)	Clipper	220	95	15	16	15000	2800	175	2.9	0.68	0.39
	1.6		74	65	9	16	14000	2000	140	1.5	0.38	0.3
	0 (unwashed)		182	98	17	17	15000	4200	210	3.6	0.71	0.49
	1.6		82	45	7.5	12.5	12000	2800	270	2	0.35	0.34
	0 (pure)		185	82	12	18	16000	2500	75	1.9	0.56	0.34
	1.6		64	67	8.9	16	13000	2000	120	1.6	0.4	0.30
	0 (washed)	Sahara	140	71	8	6.5	12000	2200	350	2.8	0.65	0.36
	1.6		50	52	6.5	7.3	10000	2600	330	1.7	0.41	0.30
	0 (unwashed)		270	56	12	5	8500	2600	420	3.4	0.6	0.38
	1.6		66	36	7	6.5	9000	2300	630	2	0.36	0.30
	0 (pure)		134	65	9	7.5	10500	2000	180	2.4	0.62	0.36
	1.6		51	46	6.5	7.3	8700	1500	180	1.7	0.42	0.29
3D	0	Clipper	230	78	14	5.8	13000	3300	230	1.2	0.5	0.41
	0.05		141	53	10.2	5.8	11600	2700	230	1	0.34	0.33
	1.6		54	50	10.5	5.3	10600	2500	260	1.3	0.35	0.34
	0	Sahara	160	66	10.8	4.1	11000	2300	740	1.3	0.47	0.4
	0.05		97	42	8	3.8	8000	1600	530	0.8	0.27	0.36
	1.6		40	33	7.6	4.1	6900	1600	650	1.1	0.3	0.28

Appendix 3

Basal fertiliser for field trials (HiFert)

(15:17:0 plus Cu, Co Mn, Mo coated).

Element	%	Rate@175kg/ha
N	14.7	26.00
P	17.2	30.00
S	1.25	2.20
Cu	1.15	2.00
Co	0.14	0.24
Mn	2.9	5.10
Mo	0.14	0.25

Appendix 4

Recycled Soil (R.S.)

1/2 cubic metre of finished with and composted experimental soils (U.C.; J.I. and R.S.) and plants therein (composted for over 2 years) is steam sterilised at $100^{\circ}C$ for 45 mins and cooled after which $1/10 \ m^3$ of Euroturf peatmoss and the following nutrients are mixed in.

Blood Meal	500g
Agricultural Lime	200g
Potassium Sulphate	200g
Super Phosphate	100g

The mix is then sieved through a 1cm grid size sieve.

University of California Mix (U.C.)

400l of coarse washed sand (Golden Grove) is sterilised at $100^{\circ}C$ for 1/2h in a sterilising mixer. One bale of Euroturf peatmoss (300l) is added and mixed for 10s. The temperature of the combined sand/peat mix drops to about $80^{\circ}C$. After about 10mins, more cooling, the following fertilisers are added and mixed with the sand/peat mix for 20s. The short mixing times are to ensure that sufficient air-porosity remains in the finished soil.

Calcium hydroxide	700g
Calcium carbonate	480g
Nitrophoska (15-4-12)	600g

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