

New Micronutrient Fertilisers for Alkaline Soils

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In fulfilment of the requirements for the degree of

Doctor of Philosophy

A thesis submitted to

Soil and Land Systems

School of Earth and Environmental Sciences

The University of Adelaide

Australia

February, 2007

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Abstract

Trace element deficiencies represent an ongoing limitation to agricultural productivity in Southern Australia and in many regions of the world. Trace element deficiencies are commonly encountered on alkaline and calcareous soils due to their high metal adsorption and fixation capacities. Chelating agents, such as EDTA, have been used to reduce fertiliser fixation in these soils and increase trace element transport to the rhizosphere. However, EDTA, which is the most commonly used chelating agent, can be relatively ineffective on alkaline soils and may have negative environmental implications due to its long-term persistence.

This study has identified two novel sequestering agents for use on alkaline and calcareous soils. The novel products differ significantly from EDTA in terms of their structure and functionality. For example, rhamnolipid is synthesised by *Pseudomonas* bacteria, is non-toxic, biodegradable and forms a lipophilic complex with cationic metal ions. The other chelating agent, polyethylenimine (PEI) can complex up to 4 times more metal (g Cu(II)/g ligand) than EDTA, which has important implications for chelate application rates and the cost effectiveness of chelate use.

In solution culture experiments, rhamnolipid and PEI facilitated Zn absorption into the root symplast; the kinetic rate of Zn absorption was greater than that of ZnCl₂ alone. On alkaline and calcareous soils the novel products were significantly ($P \leq 0.05$) more effective Zn sources than EDTA or the SO₄²⁻ salt. EDTA increased the concentration of Zn in soil solution. However, this did not translate to increased Zn uptake by canola plants. This was not surprising as EDTA inhibited Zn absorption by roots in the solution culture experiments.

Radioisotope experiments showed that rhamnolipid and PEI increased Zn adsorption to the soils solid phase. However, PEI increased the size of the total Zn labile pool ($P \leq 0.05$) and mobilised Zn from the pool of fixed native soil Zn ($P \leq 0.05$). Rhamnolipid did not significantly ($P \geq 0.05$) increase the total size of the Zn labile pool in either soil, but significantly ($P \leq 0.05$) increased Zn uptake by canola, probably by facilitating root absorption by the formation of lipophilic complexes with Zn.

These results showed that, on alkaline soils, chelates that increased the rate of trace element absorption into the root symplast were significantly more effective than EDTA, which was not readily absorbed by canola roots.

Experiments were also undertaken to explore the effect of chelation on the absorption of foliar applied trace element fertilisers. Perhaps not-unexpectedly, chelation reduced the absorption of foliar applied Zn. The lipophilic chelate, rhamnolipid, quadrupled Zn absorption by enzymatically excised *Citrus sinensis* cuticles but did not significantly ($P > 0.05$) increase Zn absorption by live leaf tissue. Therefore, there was no discernable relationship between the $K_{c/w}$ of fertiliser solutions and Zn permeability.

This body of work has important implications for future fertiliser development, the cost effectiveness of chelate use and the treatment of micronutrient deficiencies on alkaline soils in the world today.

Declaration

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

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

Samuel Peter Stacey

Date 23/2/2007

Acknowledgements

I am very grateful to my two supervisors Prof. Mike McLaughlin and Dr Enzo Lombi. Thankyou for mentoring me, critiquing my work and encouraging my growth as a scientist. I greatly appreciated the time and energy that you both invested in me. I also appreciated the friendship that developed between us.

I am very grateful to Prof. Derrick Oosterhuis for the opportunity to undertake some of my PhD research at the University of Arkansas. Thankyou for looking after me so well and sharing some great US culture with me. My time in the US was fantastic and truly one of life's great experiences (hopefully I will see you in Australia in the not too distant future).

Thankyou to Caroline Johnston for helping with sample analysis, particularly for working really hard to ensure that Zn analysis on the GF-AAS was accurate (very important). I am also grateful to Colin Rivers for making sure I had access to laboratory equipment and for the great fishing stories.

Finally I would like to thank my wife, Naomi, who supported me throughout this journey and who encouraged me to pursue my dreams (even though I was taking a break from full-time employment to do so).

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Chapter 1. Introduction and Literature Review

The cost of micronutrient deficiencies to humankind is enormous. Costs are not limited to lost agricultural production or economic penalties at the farm gate level. They can also have a considerable deleterious effect on human nutrition and health. The World Health Organization ranked Zn deficiency as the 5th leading cause of disease in developing countries (Edejer et al. 2002). The primary cause of micronutrient disorders in humans is low dietary intake of micronutrient-rich foods (Buyckx 1993). Therefore, overcoming deficiencies in agriculture would go a long way towards solving these disorders in humans (FAO 1993).

Micronutrient deficient soils are particularly prevalent in the world's lower rainfall environments such as the Middle East, Northern Africa, south-west USA and Australia where cereals are usually grown as staple crops (Figure 1.1). While one cannot say that all calcareous soils are micronutrient deficient, many are because of their high pH and predominant calcium carbonate contents. In fact, Fageria et al. (2002) claimed that 553 million hectares of the world's ice-free land area suffers from micronutrient deficiencies. Much of this land probably included sodic soils, which are often alkaline but non-calcareous. Thus micronutrient deficiencies are of considerable concern to crop production in many regions of the world (Table 1.1).

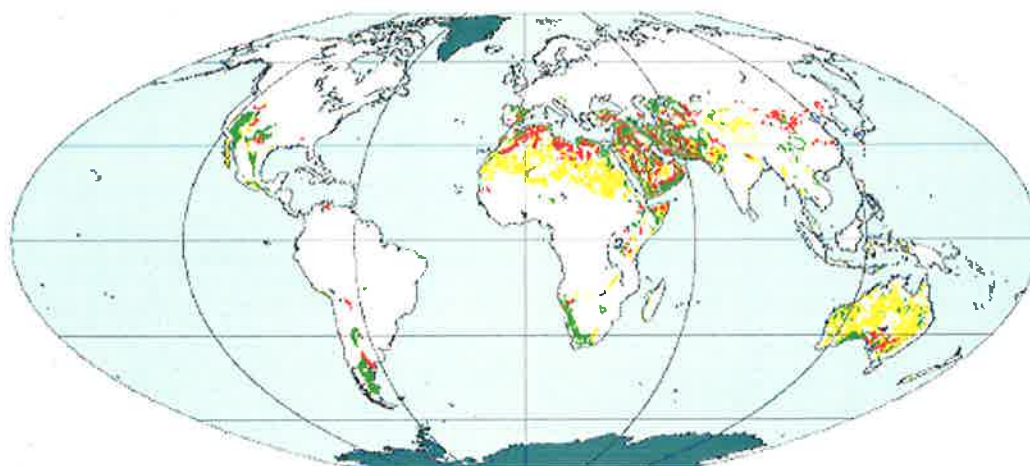


Figure 1.1. World distribution of calcareous soils, ■ dominant, ■ associated, ■ inclusions (from FAO, 2003).

Table 1.1. World-wide distribution of alkaline soils (*000 ha) (from FAO, 2003).

Region	Calcareous Soils	Sodic Soils
Africa	171 237	13 800
Australasia	113 905	38 099
Europe	56 657	7 906
North America	114 720	10 748
North and Central Asia	95 264	30 062
South and Central America	24 318	34 652
South and SE Asia	220 068	0
Total	796 169	135 267

Australia is not immune to these problems. In South Australia 16600 km² of cereal cropping land on the Eyre Peninsula is calcareous, with some topsoils having CaCO₃ contents as high as 70% (Coventry et al. 1998). Vast areas of alkaline soils also exist in the South Australian and Victorian mallee (Coventry et al. 1998), in New South

Wales (Milthorpe 1991; Walker 1991), Western Australia (Brennan 1991) and Queensland (Duncan 1967), and in these regions micronutrient deficiencies are commonplace. Therefore, in South Australia, 83% of Mallee farmers and 89% of Eyre Peninsula farmers widely use Zn-enriched fertilisers (Coventry et al. 1998).

PLANT UPTAKE OF MICRONUTRIENTS

The micronutrients that are essential for the growth and development of field crops are boron (B), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni) and molybdenum (Mo) (Fageria et al. 2002). Their roles in plants are many and varied as they are required for the biosynthesis of proteins and enzymes (Zn, Fe, Mn, Cu, Ni, Mo), the activation of enzymes (Mn, Zn), carbohydrate synthesis (Cu) and metabolism (Zn), chlorophyll content and photosynthetic activity (Fe, Mn, Cu, Zn), lignin biosynthesis (Mn, Cu), lipid biosynthesis (Mn), membrane integrity (Zn, B) and as a structural component of ribosomes (Zn) (Marschner 1995). Deficiencies can severely restrict root development (Mn, Fe, B), vegetative growth (Mn, Cu, B) and preclude flowering, grain and fruit formation (Cu, B) (Marschner 1995). However, mild Zn deficiency may increase root and stele diameters, possibly as a Zn acquisition strategy (Rosolem et al. 2005). The most common micronutrient deficiency found in the cereal crops of southern Australia is Zn, though Mn and Cu deficiencies are regionally important. Therefore this study will primarily focus on overcoming the deficiencies of these three elements.

Plant absorption of Mn and Zn ions is regulated by ion transport across root-cell membranes. The Casparian strip blocks passive diffusion of ions into the stele (Figure 1.2). Therefore ions moving through the apoplastic route must be transported across a cell membrane before absorption into the stele is permitted.

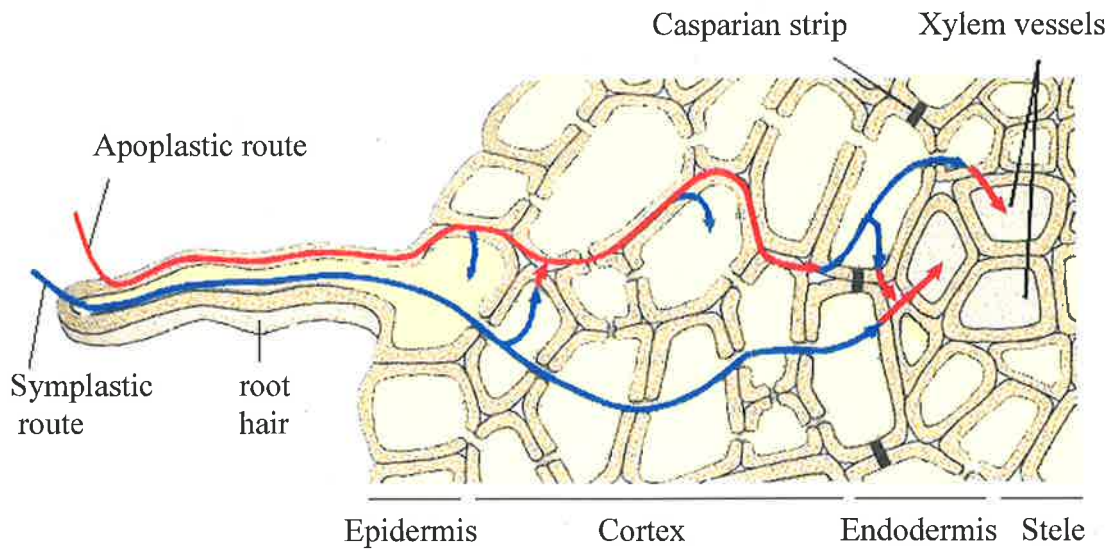


Figure 1.2. Absorption of minerals by roots via apoplastic and symplastic routes (from Campbell, 1993).

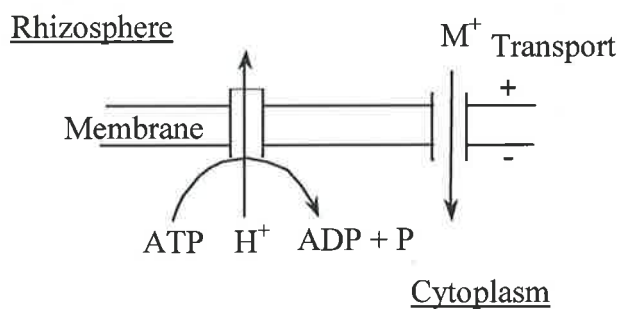


Figure 1.3. Metal transport across root membranes.

Membrane transport of metal ions is usually powered by ATP hydrolysis within the cytoplasm (Hughes and Williams 1988; Palmgren and Harper 1999). ATP-dependent proton pumps expel H^+ ions across the root membrane and metal uptake is driven by the resulting electrochemical gradient, which exists between the root cytoplasm and the rhizosphere solution (Figure 1.3). Reid (2001) and Grusak et al. (1999) have suggested that metal transporters are channel proteins that do not differentiate between divalent metal ions. Thus carrier proteins that transport Fe^{2+} may also transport Mn^{2+} and Zn^{2+} ions. Clarkson (1988) also states that Mn^{2+} absorption across root membranes is not tightly controlled, even though Hughes and Williams (1988) explain that its transport into organelles such as vacuoles and chloroplasts is highly regulated. Recent studies have uncovered a broad range of genes for membrane transport proteins, therefore the exact nature of Mn and Zn transport across root membranes and other plant organelles may differ between plant species (Palmgren and Harper 1999; Ramesh et al. 2003; Ghandilyan et al. 2006). Competitive inhibition of Zn^{2+} absorption by Cu^{2+} and Co^{2+} suggests that these metals compete for the same non-specific absorption sites in wheat (Chaudhry and Loneragan 1972a). However Mn^{2+} and Fe^{2+} did not appear to inhibit Zn^{2+} absorption (Chaudhry and Loneragan 1972a), which suggested that Mn^{2+} and Zn^{2+} may in fact be transported by different carrier proteins, or alternatively the absorption sites may have a higher affinity for Zn^{2+} ions. Ca^{2+} , Mg^{2+} and Zn^{2+} are known to depress Mn^{2+} absorption, and Zn^{2+} absorption may be inhibited by Ca^{2+} , K^+ , Mg^{2+} and NH_4^+ , though non-competitively (Chaudhry and Loneragan 1972b; c; Marschner 1988). Thus Zn and Mn deficiencies may be partially induced by high Ca^{2+} concentrations on calcareous soils.

The bioavailability of metals in aquatic systems has been linked to the activity of free metal ions in solution (Sunda et al. 1978). The Free Ion Activity Model (FIAM) has also been used to describe plant absorption of micronutrients in soil, generally indicating that intact plant roots do not absorb micronutrients that are complexed with organic or inorganic ligands (Parker and Pedler 1997). Parker and Pedler (1997) suggest that metals (M) complexed with organic or inorganic ligands (L) are absorbed by plant roots following ligand exchange at the root surface (X), according to the formula:



Indeed, synthetic chelating agents are probably too large to be absorbed by intact plant roots and are possibly only absorbed by passive diffusion into damaged roots or at breaks in the endodermis where lateral roots bud (Collins et al. 2002; McLaughlin et al. 1997). Additionally, the overall negative charge of many synthetic chelates (i.e. EDTA⁴⁻, DTPA⁵⁻) may cause an electrostatic repulsion of the chelated metal away from anionic root surfaces. Chen et al. (2004) showed that arbuscular mycorrhizal associations increased Zn²⁺ uptake by maize, but did not increase the absorption of ZnEDTA²⁻ complexes.

A few studies have suggested exceptions to the FIAM. Smolders and McLaughlin (1996) proposed that CdCl₂²⁻ⁿ species might be absorbed by Swiss chard. They found that Cd uptake increased with increasing CdCl₂²⁻ⁿ activities in solution despite constant Cd²⁺ activities. In another example, membrane transport sites in the roots of strategy II plants are thought to preferentially absorb intact Fe(III)-phytosiderophores (Marschner 1995; Schaaf et al. 2004).

Plant reaction to Fe deficiency has been categorised by two responses; Strategy I (dicots and non-graminaceous monocots) and Strategy II (graminaceous sp.) plants. These responses may also influence plant absorption of Mn^{2+} and Zn^{2+} ions. When Fe^{2+} deficient, the roots of strategy I plants excrete protons and phenolic compounds that acidify the rhizosphere and reduce Fe^{3+} to Fe^{2+} (Marschner 1995), thus solubilising the Fe. Strategy II plants excrete chelating amino acids and phenolic compounds called phytosiderophores, which form highly stable complexes with Fe^{3+} and can enhance the dissolution of sparingly soluble compounds of other trace elements e.g. MnO (Marschner 1988; 1995). Some authors prefer the more general term 'phytometallophore' because the organic compounds may also chelate Mn and Zn (McLaughlin 2002). The formation of stable chelates enhances micronutrient transport to and through the crop rhizosphere, probably by increasing the total concentration of metal ions in the soil solution. Strategy II root membranes contain highly specific transport sites for the organic Fe(III)-phytometallophores, however the transport sites generally have a lower affinity for phytometallophore complexes with Zn^{2+} and Mn^{2+} ions (Marschner 1995).

Trace element fertilisers can also be applied to crop foliage. The leaf cuticle is the primary barrier for fertiliser absorption by leaves. By dissolving cuticle waxes in chloroform, Baur et al (1999) found that the absorption of organic solutes increased by 28-fold to 759-fold, depending on the species of plant. The leaf cuticle is primarily hydrophobic, being comprised of high molecular weight biopolymers such as cutins and suberins, and hydrophobic C_{14} - C_{72} epicuticular waxes (Holloway 1993). Recent physiological studies have identified polar aqueous pores, which may facilitate the absorption of charged ions into leaf epidermal cells (Schonherr 2000). Aqueous pores

exhibit strong size selectivity for soluble ions. By measuring size selectivity, Popp et al. (2005) calculated that cuticles of *Hedera helix* L. had a mean pore radius of 0.3 nm with a standard deviation of 0.02 nm. The size selectivity for diffusion of hydrophilic compounds suggests that chelating agents would hinder trace element diffusion through narrow aqueous pores (Schonherr et al. 2005).

An alternative lipophilic absorption pathway exists for lipophilic compounds (Schonherr and Riederer 1989). The lipophilic pathway may also exhibit some size selectivity (Popp et al. 2005). There is some contention within the scientific literature whether the rate of absorption is greater through the hydrophilic or lipophilic absorption pathway. Popp et al (2005) and Schonherr and Schreiber (2004) argued that hydrophilic absorption pathway was more rapid than the lipophilic pathway, whereas de Ruiter et al (1993) showed that the absorption of uncharged (lipophilic) 2,4-D into the leaves of pea and black nightshade was 1.3 and 2.4 times faster than that of the hydrophilic 2,4-D salt. However, highly lipophilic compounds may remain within the cuticle rather than diffuse into the epidermal leaf tissue (Liu 2004). Polar trace element ions are generally absorbed through leaf cuticles via the hydrophilic pathway (Schonherr 2000) and would need to be chelated by a lipophilic ligand in order to diffuse through the hydrophobic matrix.

FATE OF ZN AND MN IN ALKALINE SOILS

Adsorption/desorption and fixation reactions largely determine the phytoavailability of micronutrients in soils (Fotovat et al. 1997). The term 'adsorption' describes

electrostatic associations between ions and the exposed surfaces of clay lattices and organic matter. Adsorbed ions are exchangeable and thus may equilibrate with ions dissolved in the soil solution or be displaced (exchanged) by cations of a higher valency, or lower hydrated radius, generally following the Irving-Williams order (Irving and Williams 1948). 'Fixation' is generally used to describe ion immobilisation by precipitation or strong chemisorption (Reddy and Perkins 1974; Sidhu et al. 1977). Fixed ions do not exchange readily with ions in the soil solution, although some fixation reactions may be very slowly reversible.

The magnitudes of soil adsorption/desorption reactions are highly pH dependent. With increasing soil pH, variable charge sorption sites are progressively deprotonated, which increases the net negative surface charge of soil particles and organic matter (Bowden et al. 1977). At high pH the soil solution contains fewer competing protons (Sposito 1989), so the adsorption of micronutrients to clay minerals and organic matter increases significantly (McLaughlin et al. 1998). The number of adsorption sites depends on the surface area of the clay particles and the degree of isomorphic substitution, often following the order vermiculites > smectites > illite > kaolinite (Alloway 1995). Both Mn and Zn are electrostatically attracted to surface exchange sites on clay particles (non-specific adsorption) or may be chemisorbed (fixed) by hydrous oxides and high surface area aluminosilicates (Clark and McBride 1984; McLaughlin et al. 1998; Norvell 1988).

Humic compounds, present in soil organic matter, have an enormous capacity to complex metal cations due to their abundant anionic hydroxyl, phenoxyl and carboxyl groups (Alloway 1995; Fageria et al. 2002; Helmke and Naidu 1996). Over time, the

biodegradation of organic matter by the soil microbial biomass may release complexed metals into the plant-available pool (Stacey et al. 2001). While some humic compounds can reduce the solubility of micronutrient cations, low molecular weight fulvic acids may increase their solubility through the formation of soluble chelates (Geering and Hodgson 1969; Kiekens 1995; Stevenson and Fitch 1986). Nevertheless the importance of fulvic acids as chelates for Zn and Mn is limited by their relatively low concentrations in alkaline soils (Dang et al. 1996; Norvell 1988).

High soil pH favours the precipitation of Fe and Mn oxides (Figure 1.4), commonly in the forms ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$), goethite (FeOOH), birnessite ($\gamma\text{-MnO}_{1.8}$) and pyrolusite ($\beta\text{-MnO}_2$) (Alloway 1995; Norvell 1988). Iron and Mn oxides can specifically adsorb (fix) micronutrient cations on their major external surfaces and within the microstructure of the oxides (McKenzie 1972; Tiller 1996), and while they are believed to represent a significant sink for micronutrient cations (Alloway 1995; McKenzie 1972; Norvell 1988; Shuman 1991), their adsorption/fixation capacity has been difficult to accurately quantify (Tiller 1996).

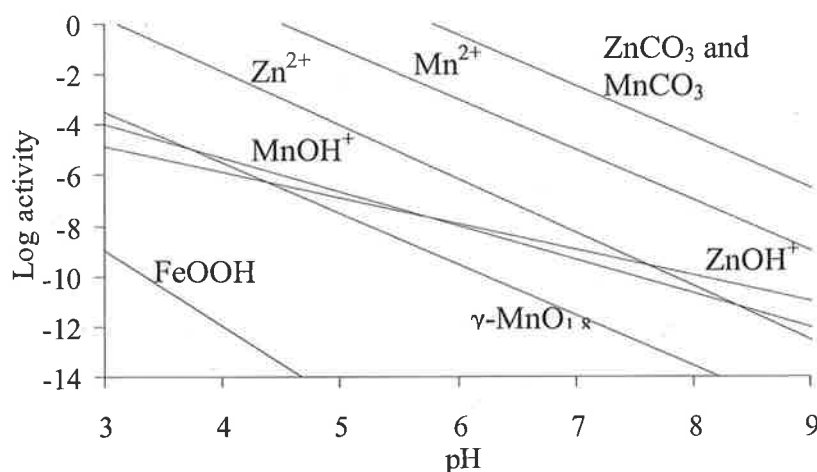


Figure 1.4. The activities of Zn, Mn and Fe species as a function of solution pH (from Lindsay, 1979).

The most readily available Zn and Mn is that found in soluble or exchangeable forms. Using GEOCHEM, Dang et al. (1996) calculated that the predominant forms of solution Zn in alkaline Vertisols were Zn^{2+} , $ZnHCO_3^+$ and $ZnCO_3$. In alkaline soils greater than pH 7.5 $ZnOH^+$ may be important, as might $Zn(OH)_2^0$ above pH 8.5 (Fotovat and Naidu 1997), and in calcareous soils the solution equilibrium probably shifts in favour of $ZnHCO_3^+$ and $ZnCO_3$ species (Figure 1.5) (Dang et al. 1996). For Mn, alkaline soil solutions are predominantly comprised of $MnCO_3$, $MnHCO_3$ and Mn^{2+} , again favouring the CO_3 and HCO_3 species in calcareous soils (Figure 1.4) (Norvell 1988).

Micronutrient deficiencies may also occur in acidic soils, however they are normally the result of deficient parent material, inadequate fertilisation, or leaching from highly weathered soils in high rainfall environments (Marschner 1995). The exception is Mo, which normally occurs in aqueous solution as MoO_4^{2-} , and thus its net negative charge makes it more prone to adsorption and fixation at low pH (Marschner 1995).

MICRONUTRIENT FERTILISERS AND THEIR APPLICATION

The choice of mineral fertiliser and its method of application can profoundly affect the nutrient status of the growing crop. Micronutrient application rates are normally low which can result in poor distribution in the crop root-zone (Mortvedt 1991a). Poor distribution inhibits crop uptake (Mortvedt 1994), so the use of micronutrient-coated fertiliser granules (e.g. ZnO coated DAP, MAP and urea) is a popular way to improve nutrient distribution and increase the probability of root interception. Zinc-coated

DAP is by far the most popular coated granule used on the calcareous soils of the Eyre Peninsula (Figure 1.5).

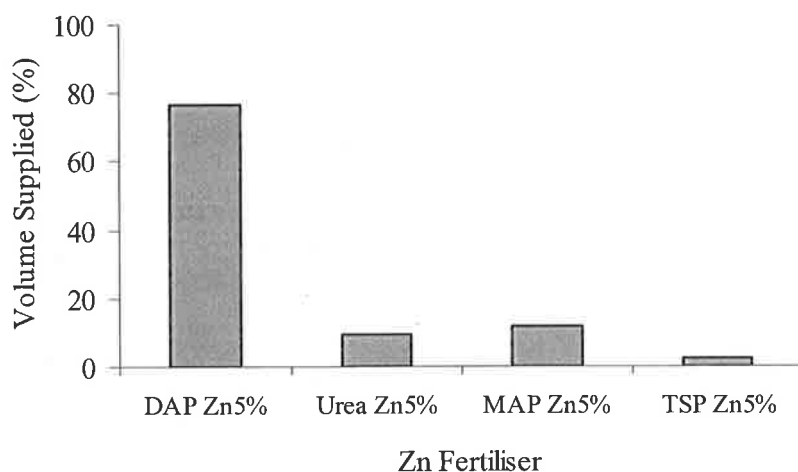


Figure 1.5. ZnO coated fertiliser granules delivered to Port Lincoln during 2002 (unpublished data, Hi-Fert).

Coated granular fertilisers are typically applied in narrow subsurface bands during planting using either combine drill or air-seeder implements (Cook et al. 1989) (Pers Comm. Peter Flavel, Hi-Fert, 8/4/03). Banding is generally considered to be more effective than broadcast applications. In fact, Wild (1993), Bailey and Grant (1990), Norvell (1988) and Raun et al. (1987) reasoned that banding reduces fertiliser exposure to high energy adsorption/fixation sites in the soil (due to the curvilinear relationship between adsorbed and solution P). However, this effect seems unlikely in Australia because fertiliser rates are low (110 kg DAP/ha) and the average distance between individual granules within the fertiliser band is approximately 1-1.5cm, depending on the crops row spacing. Therefore, in southern Australian broadacre agriculture, a continuous fertiliser band probably rarely exists. Sleight et al. (1984)

argued that yield increases from banding were mainly caused by concentrating the fertiliser close to the root zone of the emerging seedling, which improved the probability of interception of P-enriched soil by the seedling roots and boosted early crop vigour (Raun et al. 1987). Indeed, this would explain why banding has been effective on the Eyre Peninsula and Mallee soils. Other authors argued that fertiliser bands were too narrow and should be increased on calcareous soils to improve the probability of root interception (Brown and Krantz 1966; Mortvedt and Giordano 1967; Soper et al. 1989). Again, in Australian broadacre production systems the distance between individual granules is probably sufficient, and increasing the width of fertiliser bands would be neither practical nor necessary.

Numerous studies have suggested that micronutrient-coated granules are relatively inefficient fertilisers on alkaline soils. Mortvedt (1991a) discussed how granular Zn, PO_4 and NH_4 can coprecipitate as ZnNH_4PO_4 and $\text{Zn}_3(\text{PO}_4)_2$ following their application to soil. Likewise, $\text{MnNH}_4\text{PO}_4 \cdot \text{H}_2\text{O}$ precipitates have been found in granule residues when MnSO_4 was incorporated with DAP or MAP and applied to alkaline soil (Norvell 1988). The precipitates $\text{Mn}(\text{NH}_4)_2\text{H}_4(\text{P}_2\text{O}_7)_2 \cdot 2\text{H}_2\text{O}$, $\text{Mn}(\text{NH}_4)_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ and $\text{Mn}_3(\text{NH}_4)_2(\text{P}_2\text{O}_7)_2 \cdot 2\text{H}_2\text{O}$ can form when MnSO_4 is granulated in combination with pyrophosphate P, thereby reducing both Mn^{2+} and PO_4 absorption by the crop (Giordano and Mortvedt 1969; Mortvedt and Giordano 1970; Norvell 1988). Furthermore, the formation of precipitates during manufacture and/or the influx of soluble cations from the soil solution may further reduce the effectiveness of granular fertilisers with respect to P (Lombi et al. 2003). In Lombi et al. (2003) the influx of soluble cations (Ca and Fe) into fertiliser granules was caused by the strong osmotic gradient between the soil solution and fertiliser granule

(Hettiarachchi et al. 2006), which may also be antagonistic to the diffusion of nutrients out of the granule and towards the rhizosphere (Lombi et al. 2003; Mortvedt 1991a).

Some farmers prefer to apply micronutrients in foliar sprays (Duncan 1967; Reuter et al. 1988). Foliar sprays are generally inexpensive and their rapid absorption by the crop can create a striking visual impact (Brennan 1991; Duncan 1967; MacNaedhe and Flemming 1988). In addition, foliar applications of micronutrients have been used to increase the nutritional value of food and fodder crops when soil availability was insufficient to provide adequate dietary intake (Belak et al. 1970). Thus, foliar application provides an important and rapid way to increase micronutrient concentrations in food and fodder crops.

However foliar sprays do not provide the unequivocal answer to micronutrient deficiencies on alkaline soils because they hold little residual value and crops often require subsequent applications in any given year (Reuter et al. 1988). Rising oil prices also make this option increasingly expensive. Furthermore foliar sprays do not solve severe deficiencies, such as those encountered on highly alkaline soils (Sharma and Katyal 1986). Their value must also be weighed against granular fertilisers, which may hold significant residual value for up to five years (Boawn 1974; Duncan 1967; Reuter et al. 1988). In field studies, banded micronutrients have achieved higher yields than those applied by foliar spray, particularly on severely deficient soils (Brennan 1991; Sharma and Katyal 1986). Foliar sprays can be made inefficient by poor penetration rates in leaves with thick cuticles, wash-off by rain, rapid drying of

solutions and runoff from hydrophobic surfaces (Marschner 1995). Whilst beneficial, foliar fertilisation should supplement, not replace, soil micronutrient applications.

Fluid fertilisers, an alternative to granular and foliar sprays, have received widespread adoption in the United States of America because they provide farmers with the flexibility to mix fertiliser preparations according to their own requirements. In Australia, adoption of fluid fertilisers has been greatest in Western Australia with the introduction of urea-ammonium nitrate solutions. Micronutrient concentrations in granular fertilisers are fixed which means that application rates are strongly dependent on the application rates of P and N (Mortvedt 1991a). Until recently it was thought that there was no significant difference in efficiency between granular and fluid fertilisers (Lombi et al. 2002). However, recent studies on Australian calcareous soils have shown considerable yield increases in wheat with fluid P fertilisers (Bertrand et al. 2001; Hancock 2002; Holloway et al. 2001b; 2002a; b). Fewer studies have compared micronutrient fertilisers in fluid and granular forms, though Holloway et al. (2001a) and Mortvedt and Giordano (1967) showed that there can be improvements in the efficiency of Zn and Mn fertilisers when applied as fluids. Holloway et al. (2001a) demonstrated an increase in grain yield (wheat) of 11% with fluid Zn compared with granular products. Furthermore, the published data of Holloway et al. (2002a) showed that the Zn concentration in grains increased by approximately 40% when Zn was applied in fluid form without P fertiliser.

The reason for yield increases with fluid fertilisers is a contentious issue amongst scientists and industry personnel alike. Frischke et al. (2003) argued that yield increases were due to improvements in nutrient placement/distribution effects in soil,

when fertilisers were applied in the fluid form. However, distribution alone probably does not fully account for the yield increases achieved with fluid fertilisers because fluid application volumes are very low (60-120 L/ha) and at higher fluid volumes, when nutrient distribution was improved, yield responses were variable (Frischke 2002). Furthermore the distribution of granular fertilisers, when banded, appeared to be reasonable (Frischke 2002). Frischke et al. (2003) suggested that yield increases measured with crushed MAP indicated a significant yield advantage through improved fertiliser distribution in the soil. However, other effects might have confounded their results because crushing the fertiliser granules would have altered more than just nutrient placement in the soil. For example, the increase in granule surface area may have exposed and released some of the residual P that would normally be retained within intact granules (Lombi et al. 2003). Furthermore, crushing would have reduced the osmotic gradient between the granule and soil solution, which may have enhanced the rate of P diffusion from crushed granules. It is more probable that the incomplete dissolution of fertiliser granules, the precipitation of micronutrients with PO_4 and NH_4 , and the impediment to nutrient diffusion caused by the strong osmotic gradient accounted for the poor performance of fertiliser granules when compared against fluid fertilisers (Lombi et al. 2003; Mortvedt 1991a; Norvell 1988).

COMPATIBILITY OF MICRONUTRIENTS AND PHOSPHATES IN FLUID FERTILISERS

The precipitation of micronutrients from fluid fertiliser solutions is a major limitation to their use in fluid blends (Holloway et al. 2002a). Polyphosphate solutions, which are valuable macronutrient sources, may sequester small volumes of micronutrient cations and retain them within solution (Grzmill and Bogumil 2002; Hignett 1985; Lindsay et al. 1962; Mortvedt and Giordano 1967). Micronutrient cations are complexed by the negatively charged binding sites on the polyphosphate molecules (Figure 1.6). Thus the hydrolysis of polyphosphate to orthophosphate reduces the solutions carrying capacity for micronutrient cations (Mortvedt and Giordano 1967; Mortvedt and Osborn 1977).

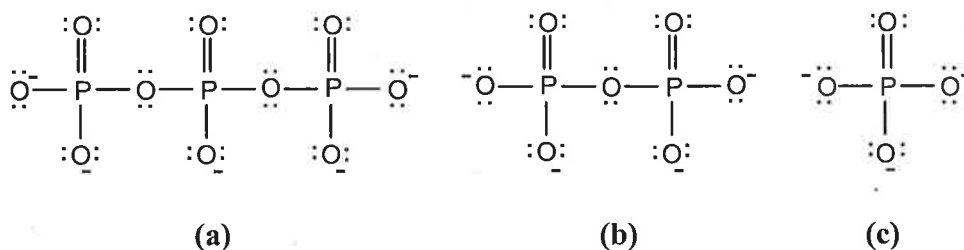


Figure 1.6. Chemical structures of (a) tripolyphosphate (b) pyrophosphate and (c) orthophosphate.

The metal complexing-capacity of polyphosphates is relatively low (Table 1.2). For example less than 0.2% Mn can be dissolved in commercial ammonium polyphosphate (APP) (Giordano and Mortvedt 1969). Therefore it is difficult to dissolve useful amounts of micronutrients in fluid phosphate fertilisers (Mortvedt and Giordano 1967). The insoluble precipitates $\text{Mn}(\text{NH}_4)_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ and

$Zn_3(NH_4)_2(P_2O_7)_2 \cdot 2H_2O$ may form within the fertiliser tank in the presence of excess Mn and Zn, which can cause blockages in the distribution nozzles (Hossner and Blanchar 1970; Yadav and D'Souza 1992).

Table 1.2. Solubility of ZnO in polyphosphate solutions (from Mortvedt, 1991a).

Polyphosphate content (% of total P)	Fertiliser Solution pH		
	5.0	6.0	7.0
	-----	% Zn	-----
40	0.6	1.3	1.6
50	0.7	1.6	1.9
60	0.7	1.7	2.0
70	0.7	1.7	1.6
80	0.7	1.4	2.6

Higher concentrations of micronutrient cations can be applied either via a separate solution, with acidified phosphates (to increase the solubility of the Zn salt, not to increase the carrying capacity of APP), or in suspension fertilisers where an addition of 1-3% attapulgate clay keeps the fertiliser mixture in a relatively stable suspension (Holloway et al. 2002a; b; Mortvedt and Giordano 1967). Mortvedt and Giordano (1967) found that the attapulgate clay did not retard the plant-availability of the micronutrient cations, and field trial results from South Australia appeared to confirm their deductions (Holloway et al. 2002b). Chelation by EDTA, DTPA or other chelating agents can vastly increase the solubility of micronutrients in phosphate fertiliser solutions (Wallace 1983). However, chelating agents have only been used sparingly in the broadacre cropping systems of southern Australia due to their high cost.

The scientific literature has questioned whether polyphosphates themselves have a “chelating effect” on micronutrient cations in soil. When polyphosphates sequester micronutrients they retain their linear structure rather than form a ring or claw arrangement around the micronutrient (Mortvedt 1991b), nevertheless the practical implications of these complexes might be similar. Experimental investigations have had mixed results. Mortvedt and Giordano (1967) measured a fourfold increase in Zn uptake by corn when Zn oxide was mixed with a 50% polyphosphate solution prior to application. Holloway et al. (2002a) also found that Zn uptake increased in wheat when Zn was mixed with APP prior to application (Figure 1.7). Likewise, Giordano (1971) and Djinadou (1995) detected an increase in extractable Zn and Cu following the addition of polyphosphates to soil. Numerous other studies have found conflicting results. For example, Giordano et al. (1971) only found high levels of extractable Zn with very high rates of tripolyphosphate. Even then, the effect only lasted one day, after which there was no discernable increase in extractable Zn. The rapid hydrolysis of polyphosphate to orthophosphate negated the sequestering properties of the phosphate solution (Giordano et al. 1971). Similar conclusions were reached by Mortvedt and Osborn (1977) although both of these studies were carried out on American soils. The rate of polyphosphate hydrolysis might be slower on Eyre Peninsula soils because they are drier, have lower OM contents and therefore probably have a less active microbial biomass. Although polyphosphates are known to sequester micronutrients their usefulness as producers of a “chelating effect” in soil is probably limited by their rapid hydrolysis in soil and poor carrying capacity of micronutrient cations (relative to EDTA).

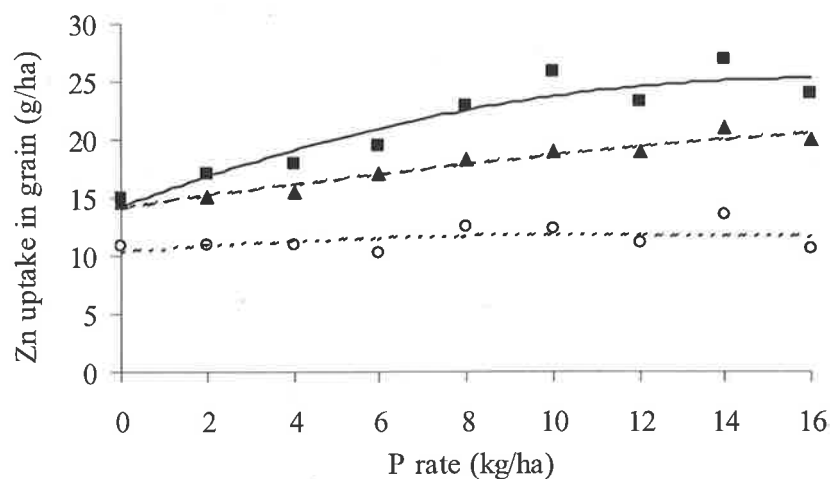


Figure 1.7. Grain Zn content of Krichauff wheat with increasing P rate when Zn was applied in granules (○), as a separate fluid to P (▲) or as a fluid with P (■) (from Holloway et al., 2002a).

In calcareous soils, mycorrhizae play an important role in the supply of Zn and Cu to plants. Kothari et al. (1991) found that Vesicular-arbuscular mycorrhizae supplied between 15% and 45% of Zn to maize plants, depending on the mycorrhizae used and the concentrations of P and micronutrients supplied to the plants. Mycorrhizae also promoted a 2-3 fold increase in root Cu concentrations (Kothari et al. 1991). High concentrations of phosphatic fertilisers (including polyphosphates) are known to suppress mycorrhizal activity in the rhizosphere (Louis and Lim 1988), which could inadvertently reduce trace element acquisition by crop plants.

SYNTHETIC CHELATES IN AGRICULTURE

Synthetic chelates have been used to enhance plant uptake of trace elements for approximately 50 years (MacNaoidhe and Flemming 1988; Murphy and Walsh 1972; Norvell 1972; Takkar and Walker 1993). The term ‘chelate’ describes the formation of a ring structure when a metal ion complexes with two or more functional groups on the ligand and derives from the Greek word meaning “claw” (Wallace 1962a; b). The chelate ring formed between EDTA and a tetracoordinate metal ion is shown (Figure 1.8). While carboxylate groups are the key metal-complexing functional groups of EDTA and DTPA, N, S, P, As and Se may also donate electrons to the chelated ion where they are present (Howard and Wilson 1993).

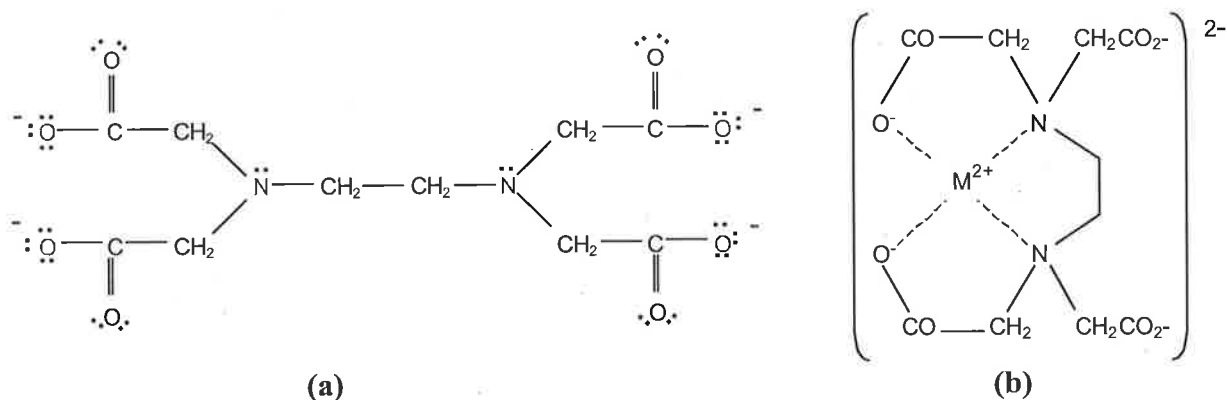
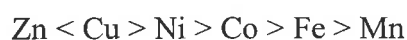


Figure 1.8. Structure of (a) EDTA with potential binding sites in red and (b) EDTA complex with a tetracoordinate metal ion (from Howard and Wilson, 1993 and McQuarrie and Rock, 1991).

The formation of the ring structure is integral to the stability of the resulting complex. Mellor (1964) argued that metal chelates were inherently more stable than closely related non-chelated complexes, and furthermore, that ring number was positively

correlated with the overall stability of the complex (Table 1.3). For example, EDTA and DTPA have six and eight metal-complexing sites respectively, and as a result can generate high stability constants when they chelate metals (Mortvedt 1991b). The stability of metal chelates is also dependent on the species of central metal ion (Table 1.3), and for bivalent ions follows the order (Irving and Williams 1948; Mellor 1964):



Within agricultural production systems we are chiefly interested in the chelates ability to increase crop yield. Chelation reduces the cationic characteristics of the metal, therefore reducing its electrostatic attraction to adsorption sites in the soil solid phase (McLaughlin et al. 1998; Wallace 1962a). This means that chelation may protect metals from fixation reactions in soils and furthermore retain them within the soil solution (Wallace 1983). The reduction in the metals cationic characteristics can also enhance the rate of micronutrient transport to the crop root system (Treeby et al. 1989; Wallace 1983). Though, transport to crop roots does not necessarily imply that the chelated metal will be readily absorbed. In solution culture, chelating agents have substantially reduced trace element absorption by plants (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995). The importance of root exclusion to the overall effectiveness of chelated fertilisers in soil has not been adequately defined. Neither does chelation guarantee that *all* complexed metal will persist in the soils solution phase. Chelating agents can themselves be subject to adsorption, the severity of which depends on the ligand, the chelated metal and the soil's pH (Norvell and Lindsay 1969; Wallace et al. 1955; Wallace and Wallace 1983b).

Mortvedt and Gilkes (1993) estimated that soil applied ZnEDTA^{2-} could be 2-5 times more effective than non-chelated Zn fertilisers. Despite their value, metal chelates have not received widespread adoption because their high cost has restricted use to high value crops (Mortvedt et al. 1992). At current fertiliser prices, ZnEDTA^{2-} costs approximately \$56 /kg Zn, whereas Zn sulphate heptahydrate is substantially cheaper, at only \$4.50 /kg Zn (R. Holloway, pers. comm. 30/04/03). Actual fertiliser costs are grower specific, with discounts given for bulk purchase. A horizontal cost comparison showed that ZnEDTA^{2-} would need to be 12.5 times more effective than Zn sulphate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) to be cost effective on cereal cropping soils (Figure 1.9).

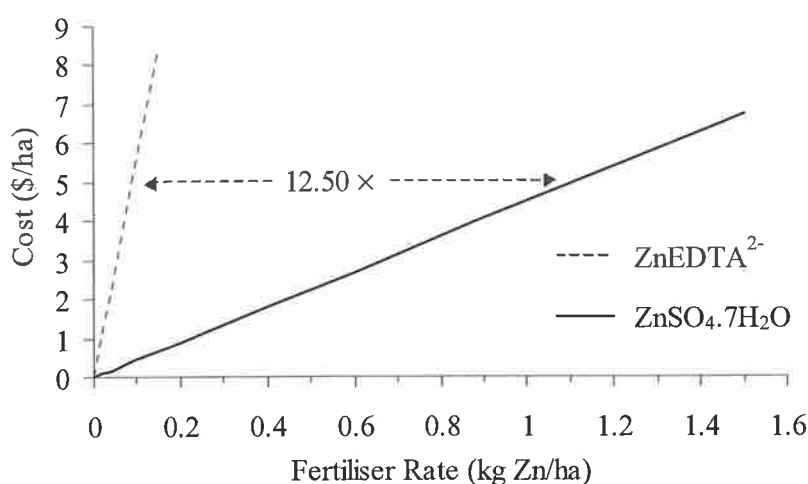


Figure 1.9. Cost comparison between ZnEDTA^{2-} and ZnSO_4 .

Norvell (1972) produced stability diagrams for ZnEDTA^{2-} to estimate the chelates effectiveness as a function of solution pH. His calculations suggested that only 10% of Zn was likely to be found as ZnEDTA^{2-} above pH 7.5 (Figure 1.10). Other authors argued that EDTA should not be used to chelate Zn, Mn or Cu on alkaline soils, rather DTPA because of the instability of EDTA in high pH soils (Wallace 1983; Wallace and Wallace 1983a). Nevertheless manufacturers have traditionally favoured EDTA

because it is less expensive than DTPA (Wallace and Wallace 1983a). Clearly the use of conventional chelating agents cannot be justified in broadacre agriculture because of their poor cost to benefit ratio.

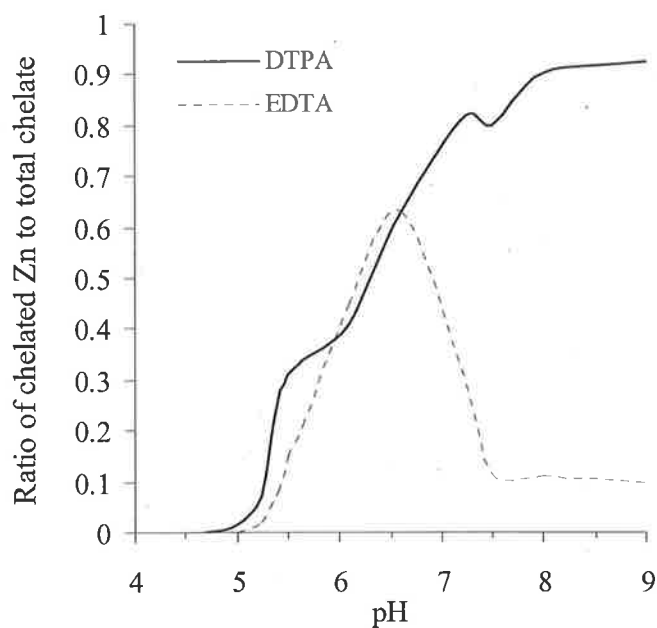


Figure 1.10. The effect of pH on the stability of Zn-chelates in soil solution (from Norvell, 1972).

CHELATE STABILITY CONSTANTS

Stable metal-ligand complexes are those that form readily and once formed, remain in their complexed state. The empirical measure of stability, the stability, equilibrium or formation constant, has for some time provided the rationale to predict complex formation and the activity of the resulting complex in the soil environment (Laurie and Manthey 1994; Mortvedt 1991b; Norvell 1972).

Stability constants (K_n) are calculated from known activities of metal (M), ligand (L) and the metal ligand complex (ML) using the formula (Laurie and Manthey 1994):

$$K_n = \frac{(ML_n)}{(M)(L)^n} \quad [2]$$

A large stability constant implies the complex is likely to form and persist following its application to soil. Published stability constants are often presented as logarithms (base 10), and termed the log K value (Table 1.3). It is worth noting that first constants (Table 1.3) provide information about the affinity of complex formation when 1:1 metal: ligand complexes predominate.

Table 1.3. First constants (log₁₀ K) for selected chelating agents at 25°, ionic strength 0.1M (from Martell and Smith, 1974; 1976).

Ion	EDTA	DTPA	P ₂ O ₇ H ₄	P ₃ O ₁₀ H ₅
Ca ²⁺	10.61	10.75	5.4	5.2
Mg ²⁺	8.83	9.34	5.45	5.76
Cu ²⁺	18.7	21.38	7.6	8.3
Fe ³⁺	25.0	28.0	NA	NA
Mn ²⁺	13.81	15.51	NA	7.15
Zn ²⁺	16.44	18.29	NA	7.5

NA, not available.

Lindsay and Norvell (1969) combined stability constants from numerous equilibrium reactions to determine the log K of final reaction products. In doing so they were able to account for proton activity and amend stability constants based on alterations to solution pH. Although they could not directly predict the influence of unknown

cations solubilising from the soil solid phase they suggested that the ratio of stability constants between two competing cations could be used to predict the importance of such competition (Lindsay and Norvell 1969). This computation would require one to know of the presence of competing cations in the soils solid phase. The procedure described by Lindsay and Norvell (1969) showed that the ratio of metal-chelate to total chelate could be calculated as a function of pH. This is a powerful tool for predicting the formation and persistence of metal-chelates within aqueous solutions.

In recent years computer equilibrium models, such as GEOCHEM-PC have been developed to rapidly perform these computations (Parker et al. 1995). GEOCHEM-PC combines known stability constants to simultaneously predict equilibrium reaction products for numerous cations and anions with regard to ionic strength, soil pH and redox potential (Mattigod and Zachara 1996).

ALTERNATIVE SEQUESTERING AGENTS FOR TRACE ELEMENT IONS

1) Chelating Polymers

For over 25 years synthetic polymers have been used on agricultural soils to remedy poor physical, hydrological and chemical soil properties. Insoluble, hydrophilic polymer gels such as high-molecular-weight polyamides, polyacrylates, and polyacrylamides have been applied to increase the water holding capacity of soils and improve water use efficiency and crop yields (Durai et al. 1996; Varennes et al. 1999). Their usefulness results from their ability to absorb water to between 40-500 times their own weight, depending on their structural makeup (Johnson 1984). Some

authors believe hydrophilic gels do not promote water conservation because the gels do not significantly affect evapotranspiration (Letey et al. 1992). Nevertheless, water conservation is inevitable if, in the presence of hydrophilic polymer, the soil can store more rainfall. Polymer products have been effective in the treatment of sodic soils by improving soil aggregation and water infiltration (Ben-Hur and Keren 1997; Mitchell 1986; Wallace et al. 1986). They have also been used to reduce soil erosion by wind and water (El-Asswad et al. 1986; Wallace and Wallace 1986).

Polymers may also affect the solubility of micronutrients in soils. Negatively charged carboxylate groups, a structural component of many synthetic polymers, have the ability to sequester micro- and macronutrient cations in soil through the formation of coordinate bonds (Falatah and Al-omran 1995; Lindim et al. 2001; Mortvedt et al. 1992; Varennes and Torres 1998; 1999; Wilson and Crisp 1977). Carboxylate groups are also the functional groups of conventional chelating agents, such as EDTA and DTPA (Figure 1.8). Synthetic polymers can form true chelate rings with micronutrient ions (Tomida et al. 2001); the stability of the resulting complexes appears to be polymer specific (Falatah et al. 1996; Mortvedt et al. 1992). The relationship between polymer application and micronutrient solubility is a paradox between the polymers sequestering properties, solubility, resistance to degradation and influence on soil pH and electrical conductivity, both of which may rise sharply following polymer addition soil (Falatah and Al-omran 1995; Falatah et al. 1996). For example, Lindim et al (2001) and Varennes and Torres (1998; 1999) found that insoluble polyacrylate gels were useful for short-term remediation of metal-contaminated soils. Copper and Cd ions were complexed by the insoluble polymers, thus facilitating their removal from the soil solution. Other authors have measured increases in the solubility of

micronutrients, sometimes despite significant increases in soil pH (Falatah and Al-omran 1995; Falatah et al. 1996; Mortvedt et al. 1992). Water-soluble polymers would probably function more like conventional chelating agents than the cross-linked, insoluble gels used in previous studies. However, the potential use of chelating polymers in trace element fertilisers has not been investigated in the scientific literature.

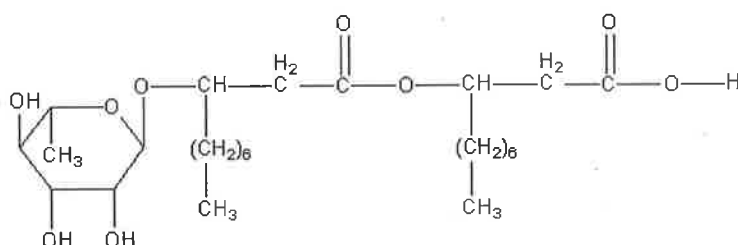
One water-soluble polymer, polyethylenimine (PEI), is of interest in this study because it has previously been used to chelate Pb, retain the metal in solution and facilitate its removal from the soil (Rampley and Ogden 1998). The PEI polymer is based on the reoccurring $[-\text{CH}_2-\text{CH}_2-\text{NH}-]^n$ monomer and can be synthesised in either highly branched or linear forms. Amine groups in the polymer backbone associate with water through hydrogen bonding, rendering the complexes water-soluble (Glass 1998). The very high charge density of PEI, 16-20 meq/g (BASF, 1996), reflective of the large number of amine groups, suggests that the polymer may have a very high complexing capacity for metal ions. However, prior to this study the author could not find any data in the scientific literature to either support or reject this hypothesis.

As already discussed, EDTA complexes divalent metals with a 1:1 molar ratio (Norvell 1991). If, for example, each mole of the chelating ligand complexed two moles of metal, the chelate application rate (moles/ha) could potentially be halved. Therefore, polymers with high complexing capacities for trace element ions could potentially be used at lower rates, which could ultimately reduce the cost of chelated fertilisers.

2) Biosurfactants

Biosurfactants are metabolites of various microorganisms that possess surfactant properties. A very large number of biosurfactants have been described, some that have carboxylate functional groups and possess the ability to complex metals. Rhamnolipid is one such biosurfactant, produced by *Pseudomonas* bacteria (Arino et al. 1996; Gunther et al. 2005). Six structural forms of rhamnolipid have been described, two of which are produced commercially, denoted R1 and R2 (Figure 1.11).

R1: L-rhamnosyl- β - hydroxydecanoyl- β -hydroxydecanoate



R2: L-rhamnosyl-L-rhamnosyl- β - hydroxydecanoyl- β -hydroxydecanoate

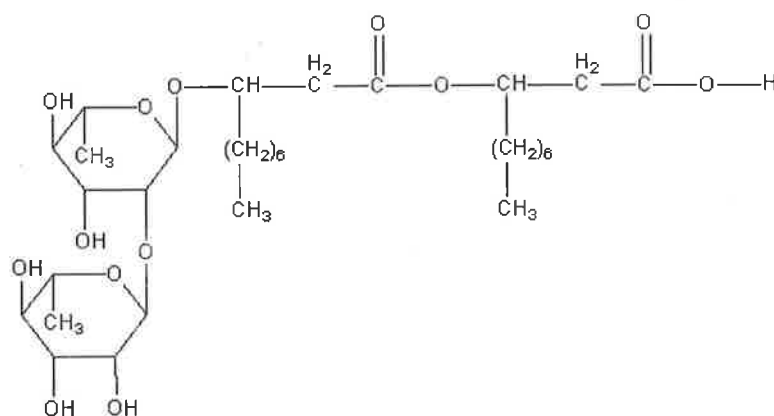


Figure 1.11. Structures of R1 and R2 rhamnolipids (from Sim et al., 1997).

Rhamnolipids complex a wide range of metal ions (Table 1.4) and have been used to complex and remove heavy metals from contaminated soils (Frazer 2000; Tan et al. 1994; Torrens et al. 1998). Stability constants of metal-rhamnolipid complexes are considerably lower than those with EDTA and DTPA (Table 1.2), which suggests that rhamnolipid complexes may not persist for long periods following their addition to soil.

One biosurfactant, known as surfactin, forms lipophilic complexes with trace element ions (Mulligan et al. 1999). The spontaneous formation of micelles above the critical micelle concentration (CMC) may account for the lipophilic properties of surfactin (Mulligan et al. 1999). Rhamnolipids also form micelles above their CMC (Champion et al. 1995), though it is not known whether trace element-rhamnolipid complexes are lipophilic. The formation of lipophilic complexes seems likely due to the presence of two 7-carbon alkyl chains (likely hydrophobic) on both R1 and R2 structures (Figure 1.11). The presence of these non-polar alkyl groups, plus the polar carboxyl group probably explains micelle formation, as the alkyl chains would probably not hydrogen bond with water. Rhamnolipid has a much lower molecular weight than surfactin; the M_w of R1 = 504 amu, whereas the M_w of surfactin is 1036 amu (Mulligan et al. 1999). Therefore, for plant absorption studies, trace-element rhamnolipid complexes are arguably of greater interest.

Lipophilic trace element complexes could potentially be absorbed by plants more readily than conventional chelates. Conventional products, such as EDTA, significantly reduce the rate of trace element absorption by plant roots (Halvorson and

Lindsay 1977; Malzer and Barber 1976; Marschner 1995), which may reduce the overall effectiveness of chelated fertilisers in soils.

Table 1.4. Conditional average stability constants of rhamnolipid with various cations (from Ochoa-Loza et al., 2001).

Metal ion	Log K \pm 1 S.E.	Complex stoichiometry rhamnolipid/ cation
Al ³⁺	10.30 \pm 0.73	2.48
Cu ²⁺	9.27 \pm 0.66	2.31
Cd ²⁺	6.89 \pm 0.25	1.91
Zn ²⁺	5.62 \pm 0.21	1.58
Fe ³⁺	5.16 \pm 0.71	1.22
Ca ²⁺	4.10 \pm 0.64	1.32
Mn ²⁺	2.85 \pm 0.45	0.9
Mg ²⁺	2.66 \pm 0.32	0.84
K ⁺	0.96 \pm 0.12	0.57

ECOTOXICOLOGICAL CONSIDERATIONS

Numerous ecotoxicological studies have detected chelating agents, especially EDTA, in waterways across the Northern Hemisphere (Eklund et al. 2002; Geschke and Zehringer 1997; Nakashima and Yagi 1991; Pietsch et al. 1995). EDTA may mobilise toxic heavy metals from river sediments and stimulate the eutrophication of waterways, which may have detrimental effects on aquatic organisms and/or the

quality of drinking water for human consumption (Geschke and Zehringer 1997; Nakashima and Yagi 1991; Pietsch et al. 1995; Yu et al. 1996). The long-term persistence of chelating agents is of particular concern. For example Eklund et al. (2002) found no measurable degradation of EDTA over a period of 5.5 months and the continued presence of chelating agents in industrial effluents and wastewater treatment plants may maintain or increase their presence in the environment (Eklund et al. 2002; Geschke and Zehringer 1997). Tiedje (1975) showed that microorganisms present in three Michigan soils could biodegrade EDTA, albeit slowly. In suboptimal conditions, i.e. below 30°C and in the absence of an organic (0.4% glucose, glycine, acetate and peptone) amendment, 1.6% and 6.7% of EDTA was degraded over a four-week period in Spinks and Conover soils respectively.

An alternative chelating agent, nitrilotriacetic acid (NTA), is more readily biodegradable than EDTA but is believed to be carcinogenic and has been banned from use in the USA (Iqbal et al. 2003; Tiedje and Mason 1974).

Unlike EDTA, rhamnolipids are microbially synthesised, biodegradable in soil and have very low toxicity (Banat et al. 2000). PEI is a cationic polymer, even at the high pH values typical of alkaline soils (von Harpe et al. 2000), which could potentially increase polymer adsorption to the soil solid phase and reduce trace element leaching, compared with EDTA. However, strong adsorption to the soil could potentially reduce the effectiveness of this chelate, as trace element delivery to the rhizosphere would diminish. Low molecular weight PEI has relatively low toxicity (Fischer et al. 1999). One benefit of using water-soluble polymers is that functional groups can be

readily modified, which can improve polymer biodegradation and reduce the risk of monomer toxicity (Forrest et al. 2003; Kim et al. 2005).

SUMMARY

Micronutrient deficiencies are a serious problem affecting crop production and human health. The World Health Organisation ranked Zn deficiency as the 5th leading cause of disease in developing countries. Therefore, there is a significant need to improve Zn nutrition in agricultural crops and Zn concentrations in harvested foodstuffs.

Micronutrient deficiencies are prevalent throughout the world's lower rainfall environments where cereals are often grown as staple crops. Their deficiencies are often associated with, but not limited to, alkaline calcareous and sodic soils due to complex soil adsorption and fixation reactions that are primarily the result of elevated soil pH. Granular Mn and Zn fertilisers have been found to perform poorly on alkaline soils when compared with fluid applications, however precipitation with fluid PO_4 and NH_4 severely limits the addition of Zn and Mn in fluid blends. Precipitation can be reduced by using chelated micronutrients however the exorbitant cost of EDTA and DTPA prohibits their use in all but very small concentrations.

Chelate cost is strongly dependent on both the required application rate and the efficiency with which the chelated fertiliser increases trace element absorption by plants. Synthetic water-soluble polymers may provide a less expensive alternative to conventional chelating agents. Unlike conventional chelating agents, each polymer

strand may chelate large numbers of cations, which could ultimately reduce the required chelate application rate. Moreover, there may be opportunities to improve the performance of chelated fertilisers by overcoming a key limitation of the conventional products; that is, root exclusion of the chelated fertiliser. One way of overcoming exclusion may be to use a novel lipophilic sequestering agent that can be absorbed more readily into the root symplast.

Given the magnitude of micronutrient deficiencies throughout the World and their concern for crop production, it is vital that we explore all avenues that may help to resolve these problems. Cost effective chelating agents would go a long way towards preventing micronutrient deficiencies and may significantly increase the profitability of farming on alkaline soils.

AIMS AND OBJECTIVES

There were two main objectives of this study. Firstly to determine whether water-soluble polymers, such as PEI, would be more effective than conventional chelates, such as EDTA, when applied to alkaline and calcareous soils. Analyses will primarily compare the fate of the chelated fertilisers in alkaline and calcareous soils and the efficacy with which the chelates increase trace element absorption by plants. In addition, this study will compare the metal complexing-capacities of PEI and EDTA to determine whether polymers could be used to significantly reduce chelate application rates.

Secondly, this study will determine whether metal-rhamnolipid complexes are lipophilic and, if so, examine the effect of lipophilicity on the absorption of chelated fertilisers through plant roots and shoots. This study will also explore the fate of trace element-rhamnolipid complexes following their addition to alkaline and calcareous soils.

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Chapter 2. Significant chemical properties of EDTA, PEI and rhamnolipid

INTRODUCTION

The value of a chelating agent to crop nutrition depends on its ability to form stable complexes with metal ions and its fate following application to soil or to plant foliage (Norvell 1972). Trace element adsorption and fixation in soil may depend on the polarity of the chelate, which is influenced by its acid dissociation constants (pKa) and the pH of the soil solution. Furthermore, the fate of the chelate may be influenced by its lipophilic properties; lipophilicity may increase adsorption to soil organic matter (Negre et al. 2001), or perhaps absorption across the roots lipid bi-layer membrane into the root symplast (Briggs et al. 1982; Iwasaki and Takahashi 1989)

In the literature review (Chapter 1), I hypothesised that lipophilic chelates would be absorbed readily by plant roots and leaf cuticles. The lipophilic properties of a chelate may be affected by its pKa, as dissociation of H⁺ ions with fluctuations in pH would alter the chelate's polarity (Negre et al. 2001).

The formation of stable metal-chelates depends on the stoichiometry of complex formation, chelate orientation and the type of electron-donating functional groups present on the chelate molecule (Mellor 1964). In addition, the atomic properties of metal ions play a critical role in the stability of metal-chelate complexes (Irving and Williams 1948). In the literature review (Chapter 1), I discussed how the polymer PEI might possess a high complexing capacity (CC) for metal ions due to its extremely high concentration of nitrogen functional groups. The use of chelates with high CC's

could ultimately lead to a reduction in chelate application rates in agricultural production.

The aim of this work was to measure the pK_a of each ligand, the octanol/water partition coefficients ($K_{o/w}$) of each metal-ligand complex and the CC and stability constants of Cu²⁺ with PEI, rhamnolipid and EDTA. Results from these experiments have been discussed in relation to the likelihood of complex formation, the potential fate of these fertilisers in soils and possible absorption pathways in plants.

MATERIALS AND METHODS

The Jeneil Biosurfactant Company kindly supplied a 25% rhamnolipid solution that contained equal proportions of R1 (504amu) and R2 (650amu) rhamnolipids. BASF Germany kindly supplied a highly branched 50% polyethylenimine (PEI) solution with an average molecular weight of 800amu. Sub-samples of both products were digested in concentrated HNO₃ and analysed by inductively-coupled plasma optical emission spectroscopy (ICP-OES; SpectroFlame Modula, Spectro) to determine the concentrations of contaminant ions. Both products contained negligible Cu, Mn, phosphorus (P) and Zn and were used without further purification.

Octanol/water partition coefficients

Octanol/water partition coefficients ($K_{o/w}$) were determined using the shake-flask method. A series of 20ml solutions were prepared, containing 1.5mM ZnSO₄·7H₂O, CuSO₄·5H₂O and MnSO₄·4H₂O chelated with 20mM rhamnolipid, PEI or EDTA. High chelate rates were used to ensure that most of the metal was in the complexed state. Two millilitres of *n*-Octanol was added to each vial before they were shaken

end-over-end for 24 hours. Following shaking, 3ml of solution was removed from the lower 2cm of the water phase and digested in concentrated HNO₃ before analysis for total Zn, Mn and Cu by ICP-OES. The concentration of each metal partitioned in the octanol phase was determined by mass balance. Each treatment was replicated in triplicate. The partition coefficient ($K_{o/w}$) was calculated according to equation [1].

$$K_{o/w} = \frac{C_o}{C_w} \quad [1]$$

where C_o and C_w referred to the concentration of Zn in the *n*-octanol and water phase respectively (Chiou et al. 1977).

A further study measured the effect of rhamnolipid concentration on the $K_{o/w}$ of Cu, Zn and Mn ions. For each metal solution, 20ml of 1mM ZnSO₄·7H₂O, CuSO₄·5H₂O or MnSO₄·4H₂O, was mixed with rhamnolipid biosurfactant in 50ml polyethylene tubes. Final rhamnolipid concentrations were (mM) 0, 0.1, 0.24, 0.5, 1, 1.5, 2, and 2.5. Each solution was buffered at pH 6.0 with 2mM KMES (2-morpholinoethanesulphonic acid, 50% as potassium salt). Trace element $K_{o/w}$'s were determined using the method described above.

pKa

The pKa of rhamnolipid was measured by titrating a 2mM rhamnolipid solution, pH 4.95, with 20mM NaOH. The Rhamnolipid solution was stirred continuously and the pH was measured after each addition of NaOH. The titration was continued until the pH of the rhamnolipid solution reached a constant value. The titration curve was plotted and the pKa determined from the pH when half of the acid groups were neutralised by NaOH, according to the Henderson-Hasselbach equation:

$$pKa = pH - \log_{10} \frac{[A^-]}{[HA]} \quad [2]$$

Branched PEI is an alkaline solution, pH 11.05. Therefore, pKa's were determined according to the methods of Choosakoonkriang et al. (2003) and von Harpe et al. (2000). Initially the pH buffering capacity of PEI was measured by potentiometric titration with HCl. A 6.25mM solution of PEI was titrated with 0.1M HCl. The pH of the PEI solution was recorded at each titration step until a constant pH was obtained.

The buffering capacity (β) was calculated as the reciprocal of the titration slope (Choosakoonkriang et al. 2003):

$$\beta \text{ (mol/L)} = \frac{d[HCl]}{dpH} \quad [3]$$

The pKa of PEI was equal to the pH at the highest recorded buffering capacity.

Complexing capacity and stoichiometry of complex formation

The Cu(II) complexing capacity of each ligand was measured with the Ion Selective Electrode (ISE) titration method used by (Kaschl et al. 2002). Free copper activity in the titrate was measured using a Cu(II) ion selective electrode (Orion 9629).

Calibration of the ISE was performed in a solution containing 1mM CuSO₄, 84mM KNO₃ and 4.5mM ethylenediamine (EN). All reagents were made using MilliQ water. The Cu(II) ISE was polished according to the manufacturers instructions prior to each titration. Solution pH was altered by incremental addition of 0.1M KOH and the activity of Cu(II) in solution was calculated using GEOCHEM-PC with each pH change (Oliver et al. 2005). The calibration curves consistently followed the relationship ($R^2 = 0.99$, Figure 2.1):

$$\text{mV} = 237.72 - 27.47 \log(\text{Cu(II)}) \quad [4]$$

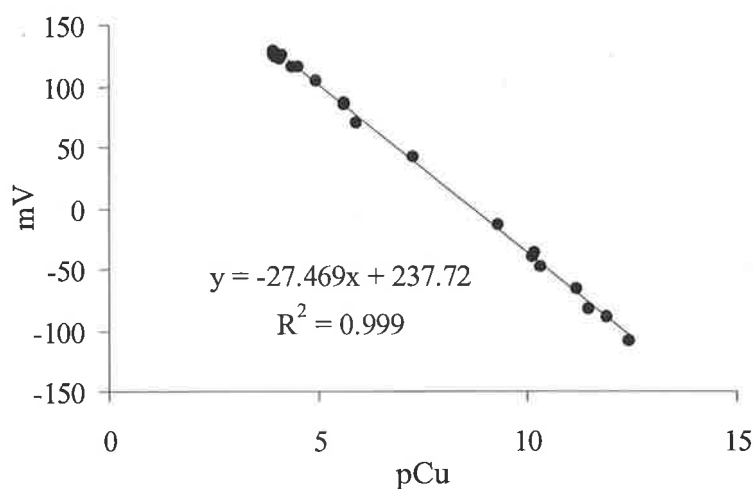


Figure 2.1. Calibration curve of the Cu(II) Ion Selective Electrode.

A weighed sample of each experimental ligand was mixed into a salt-buffered solution containing 95mM KNO_3 and 5mM EN. The solution was stirred continuously with a magnetic stirrer bar and the pH altered to 5.8 using 0.1M KOH or 0.1M HNO_3 . Measured volumes of 10mM CuSO_4 were titrated, and incremental additions of 0.1M KOH were used to maintain constant pH. The mV output from the Cu(II) probe was recorded when a stable reading was achieved (~5 minutes). The activity of free Cu^{2+} was calculated from the calibration curve (Figure 2.1). Each titration was replicated in triplicate.

The free Cu^{2+} activity was plotted against the ratio of total Cu^{2+} and ligand in the titration vessel. A linear regression was established when the maximum binding

capacity of the chelating ligand was exceeded (Kaschl et al. 2002). The x -axis intercept of the linear regression indicated the ligands maximum binding capacity.

The formation constant (θ) was defined by:

$$\theta = \frac{\text{sites bound}}{MBA} \quad [5]$$

Where MBA was the maximum binding capacity of the polymer. Hence, $\theta = 1$ at maximum binding capacity.

Scatchard plots were graphed (θ/M versus θ), where M was the activity of free Cu^{2+} ions, from which incremental values of the conditional average stability constant (K_i) were derived (Stevenson 1994). Conditional average stability constants (pK_i) were graphed against the fraction of binding sites occupied by Cu^{2+} (θ).

Stability constants were measured in a solution buffered with 95mM KNO_3 and 5mM EN. Adjustment for infinite dilution was performed using the Davies equation:

$$\log(\gamma_{\pm}) = -A |Z_m||Z_n| \left[\frac{\sqrt{\mu}}{1 + \sqrt{\mu}} - 0.2\mu \right] \quad [6]$$

Where μ = ionic strength, Z_m & Z_n = ion charges, γ = activity coefficient at $\mu=0$, A = constant unique to the solvent & temperature ($A = 0.512$ for water at 25°C).

PEI stability constants were not adjusted for infinite dilution because the exact polarity of the polymer was unknown.

RESULTS

n-Octanol/water partition coefficients ($K_{o/w}$) are commonly used to determine whether molecules can partition into hydrophobic (lipid-soluble) phases. Polar solutes, such as trace element ions, partition within the water phase. Neutral, lipid soluble molecules may partition within the hydrophobic octanol phase according to their $K_{o/w}$. Most conventional chelate complexes (i.e. ZnEDTA²⁻) are polar and therefore partition exclusively within the water phase.

As SO₄²⁻ salts, the metals Zn, Cu and Mn were not absorbed into the hydrophobic octanol phase (Figure 2.2). Likewise, the metals were non-lipophilic when complexed by EDTA and PEI. These results were expected due to the polarity of the metals and their chelate complexes. Zinc, Cu and Mn complexes with rhamnolipid were lipophilic and readily absorbed into the octanol phase (Figure 2.2). The concentration of Zn, Cu and Mn absorbed into the octanol phase was strongly dependent on the concentration of rhamnolipid that was used to complex the metal ($P \leq 0.05$) (Figure 2.3). These results indicate that either the metal-rhamnolipid complexes were predominantly non-polar or the formation of micelles facilitated their absorption into the hydrophobic layer. The measured $K_{o/w}$ differed between the two experiments due to the higher rate of Rhamnolipid in experiment 1 (Figure 2.2) and the use of 2mM KMES in experiment 2 (Figure 2.3) to buffer the solutions at pH 6.0.

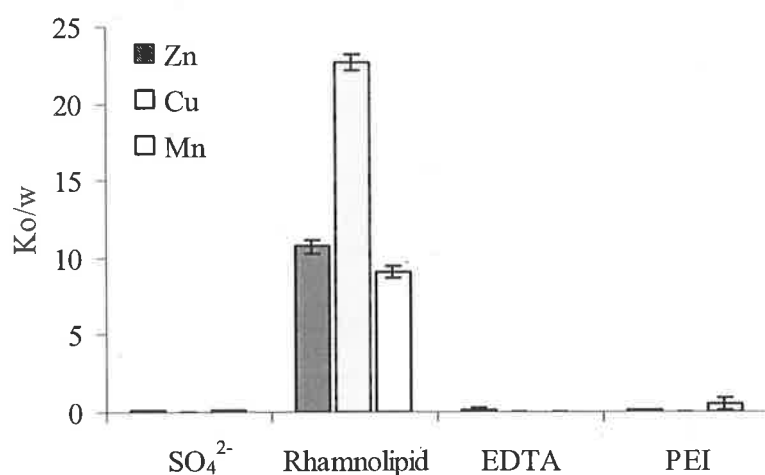


Figure 2.2. The effect of ligand species on the octanol/water partition coefficients of Cu, Mn and Zn (± 1 S.E.).

The rhamnolipid R1 and R2 mixture had an overall pK_a of 5.9 between pH 4.95 and pH 12.0 (Figure 2.4). The dissociating H⁺ ions were probably derived from the single carboxyl group on each molecule, as dissociation of H⁺ ions from the rhamnosyl groups probably occurs above pH 11 (Ozdemir et al. 2004). The single carboxylate group was believed to be involved in the formation of metal complexes. However, in order to be lipophilic the overall complex would need to be uncharged. For divalent cations, ML⁺ species would not be lipophilic. Therefore, probably either ML₂⁰ species formed or one of the hydroxyl groups may have ionised (von Wiren et al. 2000), resulting in the formation of ML⁰ species.

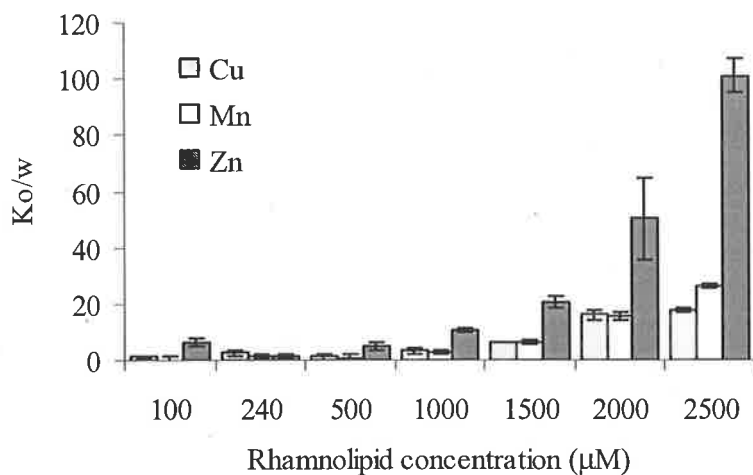


Figure 2.3. Octanol/water partition coefficients for Cu, Mn and Zn with varying rhamnolipid concentrations (± 1 S.E.).

Interestingly, only one pKa was measured, which indicated that the pKa's of R1 and R2 rhamnolipids were very similar. The pKa was only slightly different to the value published by Ishigami et al. (1993) for rhamnolipid B of (pKa 5.6). rhamnolipid B was structurally different from the R1 and R2 forms used in this study; it contained three hydrophobic carbon chains compared to only two on R1 and R2 rhamnolipids.

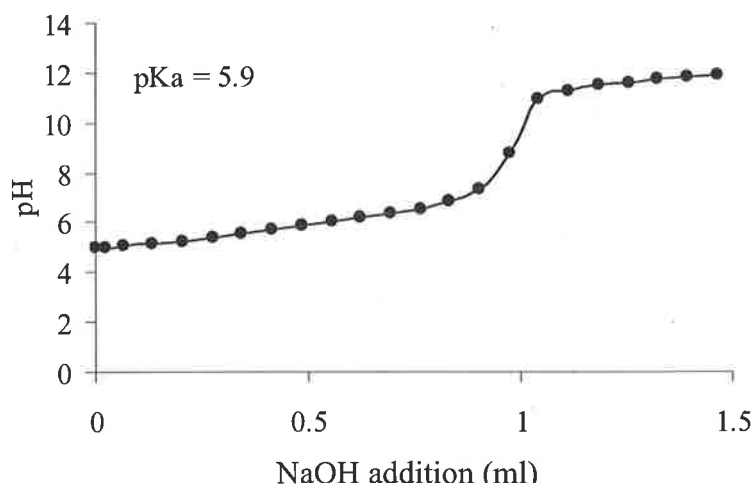


Figure 2.4. The Rhamnolipid titration curve and pKa.

The pKa of PEI was at pH 9.7 (Figure 2.5). This pKa was within the ranges published by Choosakoonkriang et al. (2003) and von Harpe et al. (2000) for PEI. Therefore, below pH 9.7, the PEI used in this study was predominantly found in the protonated, cationic form (von Harpe et al. 2000).

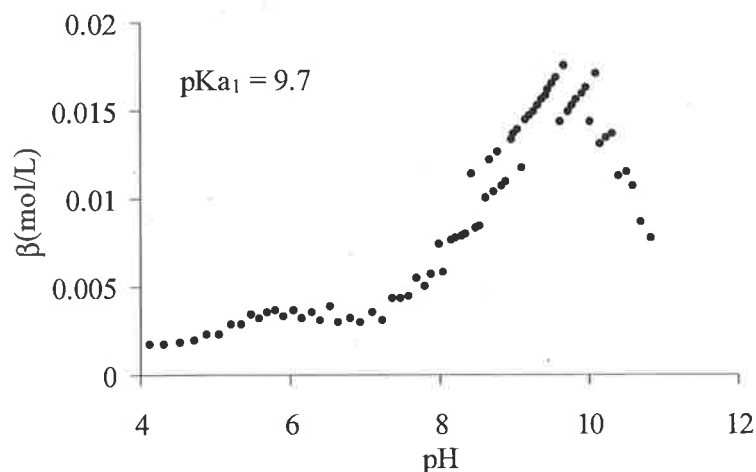


Figure 2.5. The pKa and pH buffering capacity of PEI.

The measured CC of EDTA, 0.37g Cu(II)/g EDTA, equated to a 2:1 molar complexing ratio of Cu:EDTA (Figure 2.6). According to the literature, EDTA complexes divalent metal ions with a 1:1 molar ratio (Norvell 1991), which suggests that the ISE overestimated Cu complex formation. The EDTA structure has four carboxyl groups. Therefore the CuEDTA^{2-} complex is anionic. With a molar excess of Cu^{2+} ions, there may have been electrostatic adsorption between Cu^{2+} and CuEDTA^{2-} , which was measured as complex formation by the ISE. The conditional average stability constant, $\log K = 12.7$ at the maximum CC (Table 2.1), was considerably lower than the highest recorded average conditional $\log K$ of 17.0, which was also lower than published $\log K$ values for EDTA (Martell and Smith 1974). Stability constants, measured by the ISE, were average conditional constants (or incremental

stability constants) that probably included a combination of both coordinate bonding and electrostatic interactions. The Scatchard method was developed from the continuous distribution model, which produces conceptually different values to those published by Martell and Smith (1974), measured using the Bjerrum method (Kaschl et al. 2002; Logan et al. 1997).

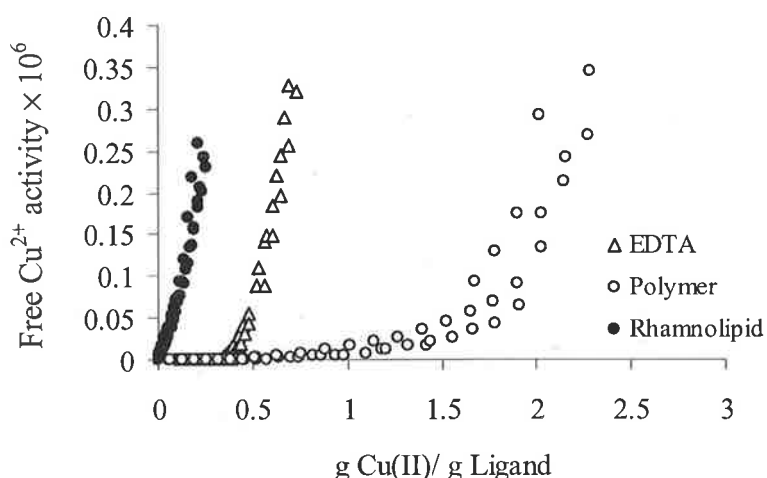


Figure 2.6. Cu(II) complexing capacities of EDTA, rhamnolipid and PEI.

The CC of PEI was approximately 1.5 g Cu(II)/g PEI, more than four fold higher than that for EDTA on a ligand weight basis (Figure 2.6). The CC showed that each mole of PEI complexed approximately 18.9 moles of Cu²⁺. However, as with EDTA, the ISE may have overestimated the number of Cu-PEI complexes. The conditional average stability constant for PEI was $\log K = 13.3$, not adjusted for infinite dilution (Table 2.1).

The rhamnolipid had a very low Cu CC of 0.05 g Cu(II)/g Rhamnolipid (Figure 2.6). The reason for the low CC was a combination of the unfavourable stoichiometry (1:2 molar ratio of Cu: rhamnolipid) of the complex and the relatively high molecular

weight of the ligand; R1 M.W. = 504, R2 M.W. = 650. Moreover, the Cu-rhamnolipid stability constant ($\log K = 9.4$) was much lower than those for Cu and EDTA or PEI (Table 2.1). The measured $\log K$ was very similar to the previously published value for rhamnolipids (Ochoa-Loza et al. 2001).

Table 2.1. Upper and lower conditional average stability constants ($\log K$) for each ligand, adjusted to zero ionic strength using the Davies equation.

Ligand	Highest recorded conditional average $\log K$	$\log K$ at CC
EDTA	17.0	12.7
Rhamnolipid	9.4	8.2
PEI	13.3*	9.0*

**Conditional average stability constants, not adjusted to zero ionic strength because the polarity of PEI was not accurately known.*

DISCUSSION

The stability of metal-ligand complexes governs the likelihood of complex formation and their persistence in the soil environment. Stability also affects the plants ability to absorb the metal ion. For example, the Free Ion Activity Model (FIAM) states that only free metal ion is favoured for absorption across root membranes (Parker and Pedler 1997). Therefore, chelates that form highly stable complexes may compete against the plant root for the free metal ion.

In this study, EDTA formed the most stable complexes with Cu(II), followed by PEI, which also formed very stable complexes. Rhamnolipid formed the least stable complexes with Cu(II) (Table 2.1). The stability of Cu-rhamnolipid complexes was significantly greater than published values for Cu-oxalate and Cu-citrate; $\log K = 5.6$

and $\log K = 6.7$ respectively (Norvell 1972). Oxalate and citrate are known to increase Cu and Zn solubility in soils through complex formation (Romer and Keller 2001). Therefore, Cu-rhamnolipid complexes could persist, at least for a short time, following their addition to soil. However, in highly calcareous soils, a large molar excess of Ca^{2+} in the soil solution may reduce the persistence of trace element-rhamnolipid complexes due to cation exchange. The importance of Ca^{2+} substitution was unknown at this stage of the experimental work, but was explored in more detail in Chapter 3.

Conventional chelating agents, such as EDTA, may reduce metal absorption of trace ions in nutrient solutions because of the very high stability of metal-EDTA complexes and membrane exclusion of the EDTA ligand (Marschner 1995). EDTA may still improve Zn uptake from soils by increasing the concentration of Zn in the soil solution and by increasing Zn transport to the rhizosphere. However, the overall effectiveness of the chelated fertiliser would still be limited by competition between the root and EDTA for the metal ion. Therefore, the relative effectiveness of EDTA, compared with PEI and rhamnolipid (which have lower stability constants and are potentially more 'plant available'), has been explored in soil and solution culture experiments in Chapters 3 and 4.

Plants may absorb organic lipophilic molecules more readily than anionic ligands (Briggs et al. 1982; Iwasaki and Takahashi 1989). This study showed that rhamnolipid formed lipophilic complexes with Cu, Zn and Mn ions. I hypothesised that the lipophilic trace-element-rhamnolipid complexes would be absorbed more readily than

complexes with EDTA, which are excluded from absorption by the root membrane (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995).

The pKa of PEI was 9.7. Therefore, PEI will be predominantly found in the protonated, cationic form in the soils and nutrient solutions used in this study (von Harpe et al. 2000). Thus, PEI differs from EDTA, which forms anionic complexes with trace element ions. Electrostatic repulsion between EDTA and anionic soil particles helps to retain the chelated metal in the soil solution phase (Wallace 1962). Therefore, I hypothesise that PEI will be adsorbed to soil particles, initially by electrostatic attraction, thereby reducing the concentration of trace element ions in the soil solution phase. Metal-rhamnolipid complexes could potentially be adsorbed by soil organic matter due to their lipophilic properties (Negre et al. 2001), which would also reduce the concentration of trace element ions in the soil solution.

According to conventional wisdom, a reduction in the solution concentration of an ion should reduce its absorption by plants, hence the importance of chelate technology (Wallace 1983). If these assumptions hold true, PEI and rhamnolipid would probably reduce plant absorption of trace element ions. However, if lipophilic compounds are readily absorbed through root membranes, the rhamnolipid might increase trace element absorption by plants. These contradicting hypotheses have been tested in Chapters 3 and 4.

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Chapter 3. Effectiveness of polyethyleneimine (PEI), rhamnolipid and EDTA as trace element chelates on alkaline soils

INTRODUCTION

Trace element deficiencies represent an ongoing limitation to agricultural productivity in southern Australia and in many regions of the world. Deficiencies are commonly encountered on alkaline and calcareous soils due to their high metal sorption and fixation capacities. The southern Australian cereal belt is dominated by alkaline and calcareous soils, some with CO₃ contents of over 70% (Coventry et al. 1998). Responses to Zn, Mn and Cu are commonly reported in field crops grown on these soils (Brennan and Bolland 2003; Holloway 1996; Reuter et al. 1988).

Chelating agents reduce metal sorption to the soil solid phase, preventing the loss of trace element ions from the soils labile pool (Wallace 1983). Chelates are thought to benefit crops by increasing trace element supply to roots (Lindsay 1974; Wallace 1983). Most chelated fertilisers sold in Australia are based on EDTA. However, these products are expensive, particularly as EDTA is a relatively ineffective chelator of metal ions in alkaline and calcareous soils (Hill-Cottingham and Lloyd-Jones 1957; Norvell 1972; Norvell and Lindsay 1969). In addition, the long-term use of EDTA may have negative environmental implications due to its persistence in the environment (Geschke and Zehringer 1997; Nakashima and Yagi 1991; Pietsch et al. 1995; Yu et al. 1996). Other chelates, DTPA and NTA are not commonly used due to their high cost, persistence and evidence that suggests NTA may be carcinogenic (Khan and Sultana 2004).

Two alternative products, polyethylenimine (PEI) and rhamnolipid could prove to be more effective and pose less environmental risk than EDTA. In Chapter 2, I showed that PEI had an extremely high complexing capacity for metal ions. If PEI replaced EDTA, chelate application rates might be substantially reduced. I also found that rhamnolipid formed lipophilic complexes with Cu, Mn and Zn. I hypothesised that the lipophilic complexes would be absorbed more readily than EDTA-based fertilisers, which are not readily absorbed by roots (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995).

The objective of this study was to assess the efficacy of Zn-rhamnolipid and Zn-PEI fertilisers on two alkaline soils. Nutrient absorption by canola was evaluated in relation to the chelates effect on Zn partitioning and the size of the labile Zn pools in each soil.

MATERIALS AND METHODS

Soil samples were collected from field sites known to be Zn responsive at Streaky Bay, South Australia and Birchip, Victoria. Topsoils from each location were collected, oven dried, passed through a 2mm sieve and stored in sealed containers until use. Pertinent soil characteristics have been described in Table 3.1.

Plant uptake of chelated Zn

Chelated fertiliser solutions were mixed with 20g of the Birchip and Streaky Bay soils, which was banded between 100g of the unfertilised bulk soil. Total nutrient application equated to ($\mu\text{g/g}$ soil) P 60, N 27, applied as technical grade monoammonium phosphate (TGMAP), and Zn 0.2 as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ($1.2 \mu\text{g Zn/g}$ soil

in the fertiliser band) either as the free metal salt or chelated by EDTA, PEI or rhamnolipid. Fertiliser Zn was labelled with ^{65}Zn to a specific activity of 3.13 kBq/ μg Zn. Chelate rates were based on the concentrations required to complex between 75% and 100% of the Zn in the fertiliser solution. Rates varied depending on the equilibrium constant (log K) and the stoichiometry of the Zn-ligand complexes (Chapter 2). GEOCHEM-PC was used to predict the degree of chelation in the EDTA and rhamnolipid fertiliser solutions. Published rhamnolipid stability constants were used for GEOCHEM-PC modelling (Ochoa-Loza 2001). For PEI, the Cu(II) complexing capacity, was used to estimate the degree of complex formation with Zn (Chapter 2). Chelate application rates were ($\mu\text{M}/\text{kg}$ soil) rhamnolipid 31.25 (~75% of Zn complexed), EDTA 9.18 (~100% of Zn complexed), PEI 1.07 (~100% of Zn complexed). Experimental controls were chelate free ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ only). Each treatment was replicated four times.

Two pre-germinated canola seeds (*Brassica napus* cv. Pinnacle) were transferred to each pot. Streaky bay soil was watered with deionised water every second day to pF 2, measured using sintered glass funnels. The Birchip soil was watered to pF 2.2 due to its higher clay content and swelling properties. The soil surface was covered with polyethylene beads to reduce evaporation. The plants were grown for 21 days in a controlled environment growth chamber (10 h dark at 15°C, 14 h light at 20°C, 41% humidity) before the shoots were harvested, rinsed, dried, weighed and then digested in concentrated HNO_3 . Plant digests were analysed for ^{65}Zn by gamma spectroscopy and for total nutrient contents by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Plant uptake of fertiliser Zn was calculated from the

activity of ^{65}Zn in plant shoots and the known specific activity of the fertiliser Zn added.

Zn adsorption in alkaline and calcareous soils

Five grams of oven dry Birchip and Streaky Bay soils were weighed into 50ml polypropylene vials. Fertiliser solutions, containing $6\mu\text{g}$ of Zn as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and either EDTA, rhamnolipid or PEI, were applied to the soils. EDTA, rhamnolipid and PEI were applied at seven rates: ($\mu\text{M}/\text{g}$ soil) 0.008, 0.018, 0.03, 0.04, 0.05, 0.062 and 0.07. The soil solution phase was made up to 25ml with deionised water to provide a 1:5 soil:water ratio.

The soils and fertiliser solutions were shaken end-over-end for 24 hours. After shaking, vials were centrifuged for 20 minutes at 1400 RCF. Five millilitres of the supernatant liquid was removed, filtered through a $0.2\mu\text{M}$ syringe filter and digested in concentrated HNO_3 before analysis by graphite furnace atomic adsorption spectrometry (GFAAS) for total Zn. The pH values of the supernatant solutions were measured to assess the effect of the chelating agents on supernatant pH.

E_e and E_a Values

One gram of oven dry soil was weighed into polypropylene vials. Zinc ($1.2\mu\text{g}/\text{g}$ soil) was applied as a fluid fertiliser, made from $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and EDTA, rhamnolipid or PEI at ($\mu\text{M}/\text{g}$ soil) 0, 0.018, 0.04 and 0.07. The solution phase was made up to 10ml with deionised water before the soil suspensions were shaken end-over-end for 24 hours to begin equilibrating the fertiliser with the soil. Soil suspensions were then spiked with $6\text{kBq } ^{65}\text{Zn}/\text{g}$ soil. Additional soil-free controls were spiked with ^{65}Zn to

determine the total ^{65}Zn activity applied to the soil suspensions. Vials were returned to the shaker for 24 hours before being centrifuged for 25mins at 1400 RCF. The supernatant liquid was filtered through $0.2\mu\text{M}$ syringe filters before the filtrate was analysed for ^{65}Zn and cold Zn by gamma spectroscopy and ICP-OES or GF-AAS, respectively.

The concentration of surface exchangeable Zn (E_e) was calculated from (Hamon et al. 2002):

$$E_e (\mu\text{g/g soil}) = C_s \times \frac{(R - r_s)}{r_s} \quad [1]$$

Where:

C_s = cold Zn in solution following equilibrium ($\mu\text{g/g soil}$);

R = Total ^{65}Zn activity introduced to the system (Bq);

r_s = ^{65}Zn activity remaining in the solution phase following equilibrium (Bq).

The most potentially bioavailable pool of Zn in the soil (E_a) is the sum of the surface exchangeable and solution Zn pools (Hamon et al. 2002):

$$E_a = E_e + C_s \quad [2]$$

E_e and E_a (%) was calculated as a percentage of the total soil Zn:

$$E \text{ value (\%)} = \frac{E_a}{\text{Total soil Zn}} \times 100 \quad [3]$$

The total elemental composition of each soil was determined by *aqua regia* digestion, then analysed by ICP-OES.

The E_a of native soil Zn was measured in control soils without fertiliser addition. The pool of native soil Zn mobilised by each chelate (E_n) was then calculated:

$$E_n = E_a (\text{fertilised}) - E_a (\text{unfertilised}) - \text{fertiliser Zn added} \quad [4]$$

Finally, the ratio of exchangeable surface Zn (E_e) to solution Zn (C_s) was expressed as an adsorption coefficient (K_d), calculated from:

$$K_d = \frac{E_e}{C_s} \quad [5]$$

Data for shoot nutrient concentrations, E_e and E_a values were analysed by analysis of variance (ANOVA) with a 95% confidence interval. Residual versus fitted value plots were examined for even scatter to satisfy the constant variance assumption of ANOVA. Significance between treatment means was determined using the Least Significant Difference (LSD) test ($P \leq 0.05$).

RESULTS

The Birchip soil was a alkaline Sodosol with a pH_(1:5 soil:water) of 8.8 (Table 3.1). The Streaky Bay soil was highly calcareous, with a CO₃ content of 39% and pH_(1:5 soil:water) = 8.7 (Table 3.1).

Canola plants were grown under Zn deficient conditions. Therefore, on Streaky Bay soil, shoot Zn concentrations were below the published critical tissue concentrations for Zn of 7-8 mg Zn/kg DM (Huang et al. 1995). However, canola grown on rhamnolipid treated soil had a shoot Zn concentration of 7.74 mg Zn/kg DM (Figure 3.1). Canola plants grown on Birchip soil had Zn concentrations on or above the critical Zn concentration; treatment with rhamnolipid and PEI increased shoot Zn concentration above the critical level (Figure 3.1).

Table 3.1. Soil Properties

Description	Birchip Soil	Streaky Bay soil
	Sodic light clay	Calcareous grey sandy loam
pH _(1:5 soil:water)	8.8 ± 0.01	8.7 ± 0.02
CO ₃ (%)	2.8	39
Clay (%)	40	25
(Total mg/g soil)		
Ca	6.8	73.6
Mg	5.27	4.05
Zn	0.030	0.015
Cu	0.011	0.005
Mn	0.26	0.14

PEI and rhamnolipid significantly ($P \leq 0.05$) increased plant absorption of Zn on Birchip soil (Figure 3.1). Rhamnolipid also significantly ($P \leq 0.05$) increased Zn uptake from the highly calcareous Streaky Bay soil. EDTA did not significantly ($P > 0.05$) increase Zn uptake from either soil, compared to the ZnSO₄ control. These results were surprising, because EDTA substantially increased the solution concentrations of Zn in both soils (Figure 3.2, Figure 3.3). PEI and rhamnolipid increased Zn adsorption to the soil solid phase, thereby reducing the concentrations of solution Zn in both soils (Figure 3.2, Figure 3.3). However, they were the most effective fertiliser products (Figure 3.1). There was no significant difference ($P > 0.05$) in shoot biomass production between fertiliser treatments (data not shown).

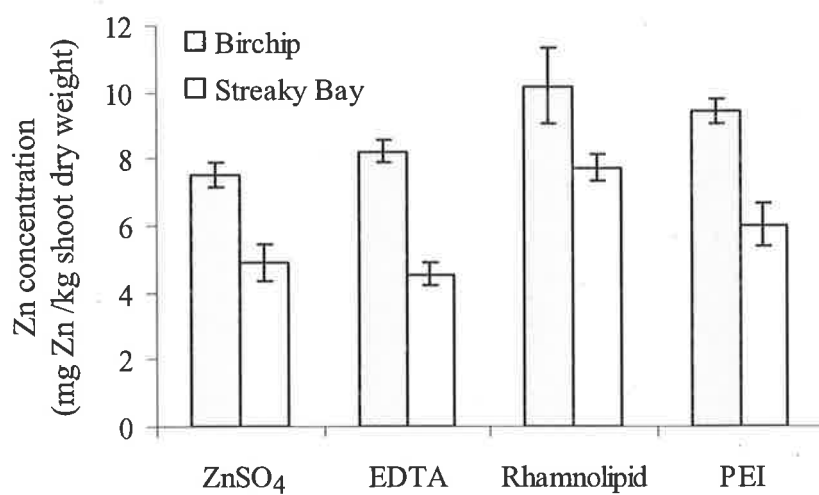


Figure 3.1. Uptake and translocation of Zn to canola shoots (± 1 S.E.).

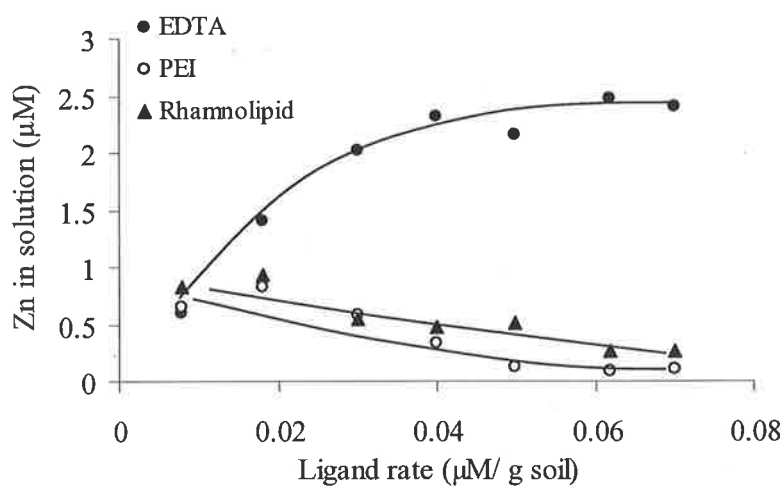


Figure 3.2. Effect of EDTA, PEI and rhamnolipid on solution concentrations of Zn in Birchip soil.

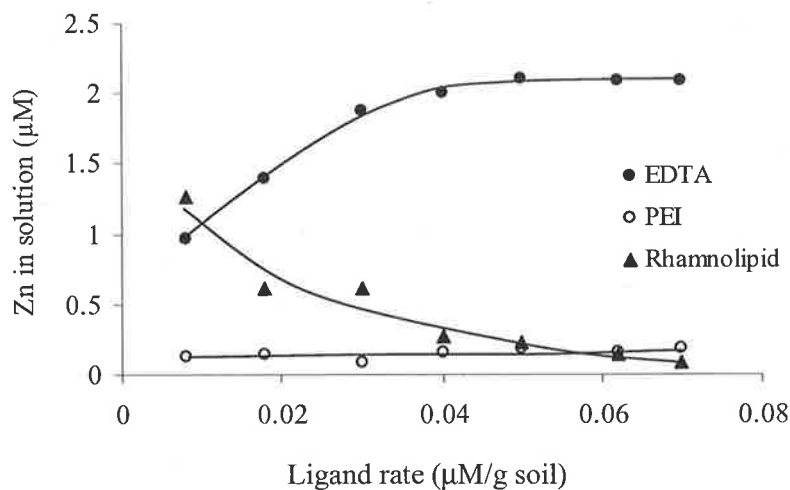


Figure 3.3. Effect of EDTA, PEI and rhamnolipid on solution concentrations of Zn in Streaky Bay Soil.

The labile Zn pool consists of both solution and surface exchangeable Zn pools (Hamon et al. 2002). My hypothesis was that PEI and rhamnolipid may have increased the size of the exchangeable Zn pool in soil, thereby accounting for their availability to canola.

Zinc E_c and E_a values were measured to differentiate between the exchangeable and fixed pools of Zn in Birchip and Streaky Bay soils. In addition, the measurements provided important information about the partitioning of native soil Zn in each of these Zn pools.

On Birchip soil, PEI significantly ($P \leq 0.05$) increased the concentration of exchangeable Zn (Figure 3.4), which resulted in an increase in the size of the labile Zn pool (E_a) with PEI addition (Figure 3.5). Only $1.2 \mu\text{g Zn/g soil}$ was applied as

fertiliser. Therefore over 90% of the exchangeable Zn was derived from the soils native Zn pool. PEI did not significantly increase the size of the exchangeable or labile Zn pools on the highly calcareous Streaky Bay soil (Figure 3.6, Figure 3.7), possibly due to the high concentration of competing Ca^{2+} ions on this soil. These results help to explain why PEI increased Zn absorption by canola on the Birchip soil, but not the Streaky Bay soil (Figure 3.1).

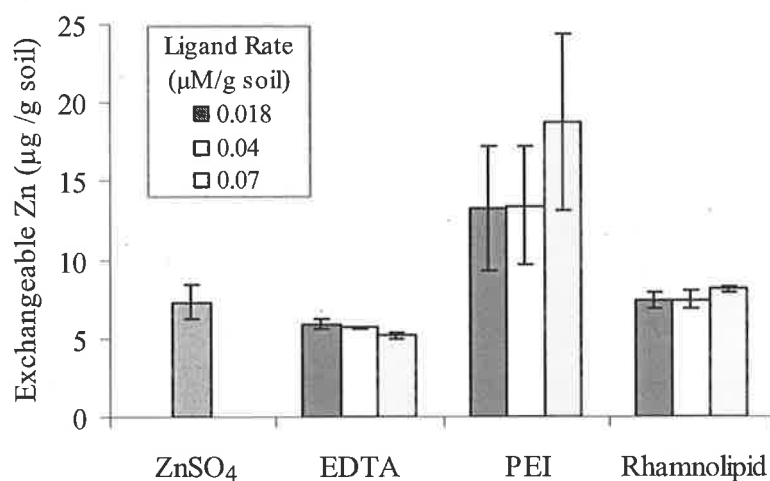


Figure 3.4. Zinc Ee values on Birchip soil as affected by ligand type and concentration (± 1 S.E.).

Rhamnolipid did not significantly increase the size of the exchangeable Zn pool (Ee) or total labile Zn pool (Ea) on either soil (Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7). These results were surprising because the rhamnolipid fertiliser was highly effective, significantly increasing Zn absorption by canola on both soils (Figure 3.1).

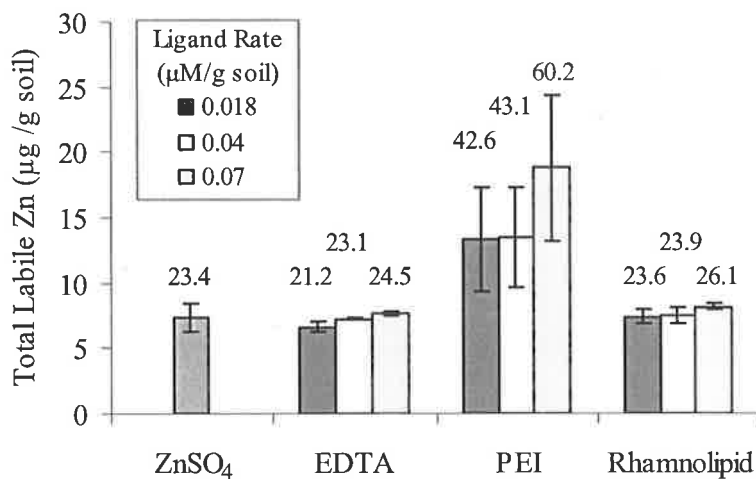


Figure 3.5. Zinc Ea values on Birchip soil as affected by ligand type and concentration (± 1 S.E.). Numbers above bars are Ea % of total soil Zn.

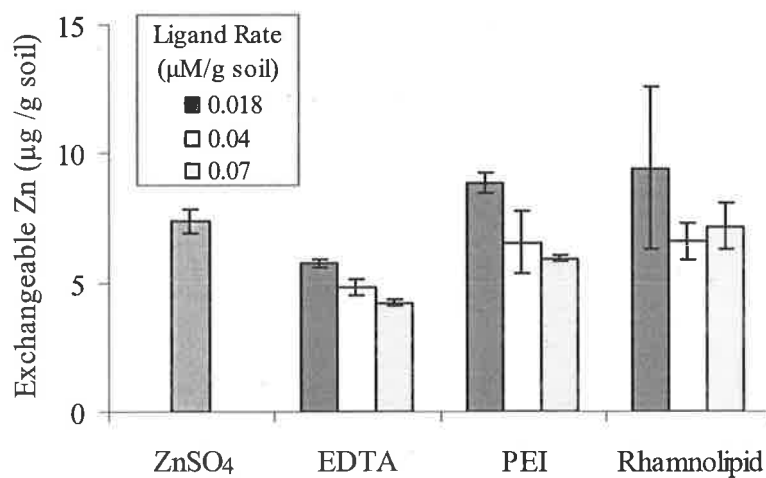


Figure 3.6. Zinc Ee values on Streaky Bay soil as affected by ligand type and concentration (± 1 S.E.).

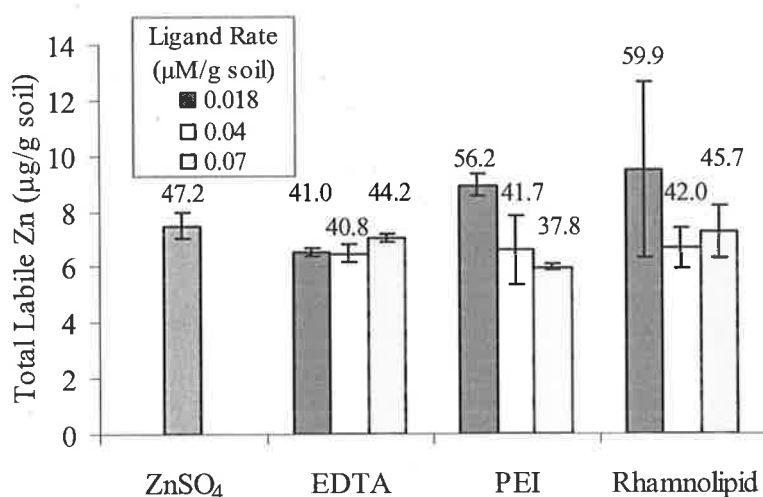


Figure 3.7. Zinc Ea values on Streaky Bay soil as affected by ligand type and concentration (± 1 S.E.). Numbers above bars are Ea values expressed as a percent of total soil Zn.

EDTA did not significantly ($P > 0.05$) increase the size of the exchangeable or total labile Zn pools on either soil (Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7). However, EDTA did increase Zn partitioning to the solution phase (Table 3.2, Figure 3.2, Figure 3.3).

At the highest application rate, PEI mobilised $11.8 \mu\text{g Zn/g soil}$ from the native Zn pool in Birchip soil (Figure 3.8). This native Zn was derived from the 'fixed', i.e. non-readily exchangeable, pool of Zn in the Birchip soil. Neither EDTA nor rhamnolipid mobilised appreciable Zn from the native Zn pool.

Table 3.2. Soluble Zn (Cs) and adsorption coefficients (Kd) of Zn in Birchip and Streaky Bay soils.

Soil	Fertilizer	Chelate Rate ($\mu\text{M/g soil}$)	C_s ($\mu\text{g Zn/g soil}$)	Kd
Birchip	ZnSO ₄	-	.030 \pm .005	242.6 \pm 5.1
	Zn EDTA	0.018	.687 \pm .018	8.6 \pm 0.3
		0.04	1.463 \pm .012	3.9 \pm 0.0
		0.07	2.420 \pm .026	2.1 \pm 0.1
	Zn PEI	0.018	.051 \pm .012	253.8 \pm 17.0
		0.04	.053 \pm .016	263.5 \pm 13.5
		0.07	.068 \pm .018	268.8 \pm 13.5
	Zn rhamnolipid	0.018	.033 \pm .005	226.4 \pm 16.1
		0.04	.034 \pm .002	217.0 \pm 1.7
		0.07	.037 \pm .002	222.1 \pm 9.1
Streaky Bay	ZnSO ₄	-	.089 \pm .008	82.9 \pm 2.3
	Zn EDTA	0.018	.767 \pm .009	7.5 \pm 0.2
		0.04	1.667 \pm .009	2.9 \pm 0.2
		0.07	2.797 \pm .024	1.5 \pm 0.0
	Zn PEI	0.018	.075 \pm .006	118.9 \pm 8.7
		0.04	.049 \pm .009	131.9 \pm 2.6
		0.07	.051 \pm .002	118.1 \pm 6.1
	Zn rhamnolipid	0.018	.066 \pm .021	141.8 \pm 6.3
		0.04	.054 \pm .005	122.2 \pm 4.2
		0.07	.066 \pm .010	110.3 \pm 4.4

The measured E_a of unfertilised soil was very small on the Streaky Bay soil (2.1 $\mu\text{g Zn/g soil}$). This low value produced inaccurate E_n values on Streaky Bay soil (Figure 3.9); it was very unlikely that the addition of ZnSO₄ caused the net mobilisation of native soil Zn.

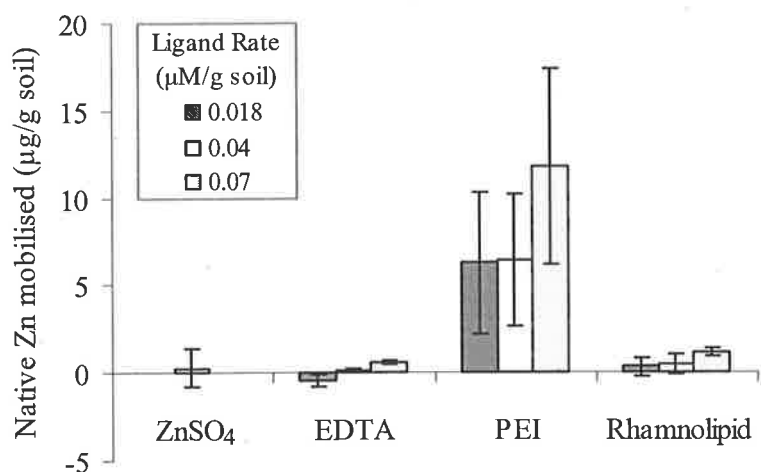


Figure 3.8. Native soil Zn mobilised (positive En) into the labile pool on Birchip soil (± 1 S.E.). Negative En values indicate no net mobilisation and some fixation of fertiliser Zn.

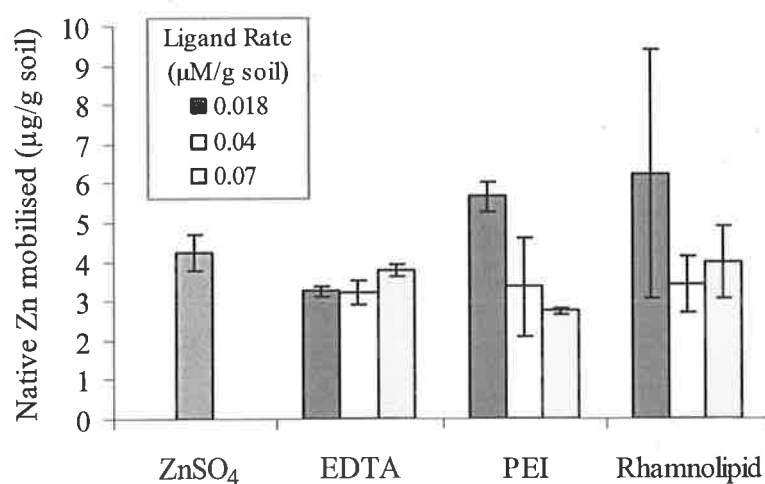


Figure 3.9. Native soil Zn mobilised into the labile pool on Streaky Bay soil (± 1 S.E.).

Zinc adsorption isotherms (K_d) were significantly ($P \leq 0.05$) affected by chelate application (Table 3.2). PEI and rhamnolipid increased Zn K_d compared with the chelate-free control, which confirmed that they increased Zn adsorption to the soil

solid phase (Table 3.2). The exception was Zn-rhamnolipid on Birchip soil, which had a slightly lower K_d than $ZnSO_4$. PEI did slightly increase the concentration of solution Zn on Birchip soil, despite its higher K_d (Table 3.2). This can be explained by the dramatic increase in exchangeable soil Zn when PEI was applied to the soil (Figure 3.3). Soil adsorption of rhamnolipid was reported previously (Noordman et al. 2000).

EDTA dramatically reduced Zn K_d on both soils. These results were in agreement with our earlier data, which showed that EDTA significantly increased the concentration of Zn in the solution phase (Figure 3.2, Figure 3.3). These results suggest that EDTA could increase Zn leaching from the soil, possibly leading to a decline in soil fertility and environmental degradation in high rainfall environments or under irrigation.

DISCUSSION

According to the literature, chelating agents increase plant absorption of trace element fertilisers by preventing their sorption and precipitation and facilitating their transport to plant roots (Lindsay 1974; Norvell 1972; Wallace 1983). It is well known that chelates forming anionic metal complexes, such as EDTA, reduce trace element absorption by plants in solution culture (Marschner 1995). However, some authors have presumed that root exclusion is unimportant to the overall effectiveness of chelates in soil, relative to the importance of trace element solubilisation and transport (Lindsay 1974; Wallace 1983).

These presumptions were challenged by this experimental work. EDTA significantly increased the soil solution concentrations of Zn through the formation of anionic ZnEDTA²⁻ complexes. Therefore, EDTA would have increased Zn diffusion to the rhizosphere. However, EDTA did not increase Zn uptake by canola on either soil. The ineffectiveness of EDTA on alkaline soils has previously been blamed on metal-chelate dissociation (Wallace and Wallace 1983) and competition with Ca²⁺ ions for metal binding sites on the chelate (Lindsay 1974). However, in this study, EDTA substantially increased solution Zn concentrations on both alkaline soils (Figure 3.2, Figure 3.3), which showed that ZnEDTA²⁻ remained relatively stable. This study suggested that the absorption of ZnEDTA²⁻ complexes was limited by exclusion at the root surface. A number of previous studies also found that EDTA complexes were not readily absorbed by roots (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995; McLaughlin et al. 1997).

Chelates are usually applied to improve trace element transport to the rhizosphere (Lindsay 1974; Wallace 1983). On Birchip soil, PEI produced a small but significant increase in solution Zn compared with ZnSO₄ alone (Table 3.1). The small increase in solution Zn may be responsible for the higher Zn uptake by canola following PEI application (Figure 3.1).

Rhamnolipid significantly increased Zn availability to canola on both soils. However, rhamnolipid did not increase the size of the labile Zn pool or appear to enhance Zn diffusion on either soil (low concentrations of solution Zn) (Figure 3.5, Figure 3.7, Table 3.1). Moreover, rhamnolipid increased Zn sorption to the soil solid phase (Figure 3.2, Figure 3.3). According to conventional wisdom, soil sorption should

reduce Zn uptake by canola as solution concentrations of Zn should decrease (assuming no change in the size of the labile pool). These results support my earlier hypothesis that the lipophilic properties of rhamnolipid may have facilitated Zn absorption into the root symplast by providing an alternative pathway across the hydrophobic root membrane (Chapter 2). This hypothesis was tested further in Chapter 4.

In Chapter 2, I hypothesised that Ca^{2+} ions might displace Zn^{2+} ions complexed by rhamnolipid, due to the low stability of Zn-rhamnolipid complexes and the abundance of Ca^{2+} in calcareous soils. In this study, rhamnolipid significantly increased Zn absorption by canola (Figure 3.1). Therefore, cation exchange, if problematic, may have proceeded slowly enough not to render the rhamnolipid fertiliser ineffective.

The very low K_d of metal-EDTA complexes (Table 3.2) suggested that EDTA might exacerbate trace element leaching from the soil, possibly leading to a decline in soil fertility and environmental degradation in high rainfall environments or under irrigation (Thayalakumaran et al. 2003; Wu et al. 2004). Rhamnolipid and PEI would be less likely to generate these environmental losses due to their adsorption to soil particles and/or soil organic matter (Table 3.2).

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Chapter 4. Absorption kinetics of Zn from chelate-buffered solutions

INTRODUCTION

Chelate fertiliser research has traditionally focussed on products that solubilise trace element ions and increase their concentrations in the soil solution (Lindsay 1974; Norvell 1972). The plants ability to absorb the chelated trace elements from the soil solution was thought to be of minor importance compared with the chelate's effect on trace element transport to roots (Wallace 1983). These presumptions have lead to products such as EDTA receiving widespread use in soil-applied fertilisers, despite the inability of plants to absorb EDTA-chelated ions in solution culture experiments (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995). More recently, the Free Ion Activity Model (FIAM) has been applied to explain the dynamics of metal-chelate absorption by plants (Parker and Pedler 1997). The model states that only the free metal ion will be absorbed across root membranes into the symplast. Thus, the absorption of chelated Zn would first required complex dissociation. The importance of metal-chelate dissociation for micronutrient absorption by plants was recently demonstrated by Degryse et al. (2006a; 2006b). Degryse et al. (2006a) correlated Zn uptake from chelate buffered solutions with the desorption constant of the metal-chelate complex.

In the literature review (Chapter 1), I hypothesised that the value of chelated fertilisers was limited by the plants inability to absorb chelated trace elements from the soil solution. Experiments undertaken in Chapter 3 provided supporting evidence for this hypothesis, as EDTA strongly increased soil solution concentrations of Zn but did not increase plant absorption of the trace element ion.

Two novel chelates, PEI and rhamnolipid, significantly increased Zn absorption by canola, even though they reduced soil solution concentrations of Zn. PEI, but not rhamnolipid, increased the total labile pool of Zn in the soil, which probably accounted for the effectiveness of the PEI-containing fertiliser. These results led me to hypothesise that the lipophilic properties of rhamnolipid facilitated Zn absorption into the root symplast, thereby accounting for efficacy of the rhamnolipid-containing fertiliser despite the reduction in soil solution concentrations. The presence of a lipophilic absorption pathway would counter the FIAM.

There exists a positive relationship between cation loading of the root apoplast and plant nutrient absorption (Asher and Ozanne 1961). PEI, having a positive charge and a high complexing capacity for metal ions, may have increased trace element absorption by 'loading' the root apoplast with Zn. Alternatively, PEI may have been absorbed into the cytoplasm, as PEI has previously been used as a non-viral vector for gene transfer into biological cells (Grosse et al. 2006; von Harpe et al. 2000). The published literature suggests that PEI may be absorbed into the cell cytoplasm by endocytosis (Bieber et al. 2002).

The aim of this study was to investigate these hypotheses by comparing the rate of Zn absorption from solutions buffered with rhamnolipid, PEI and EDTA. Zinc absorption into the root symplast was measured, as was Zn translocation to canola shoots.

MATERIALS AND METHODS

Pre-treatment of canola seedlings

Canola seedlings (*Brassica napus* var. Pinnacle) were pre-germinated on filter paper moistened with deionised water. On day 6, the seedlings were transferred to complete nutrient solution and moved to a controlled environment growth chamber (10 h dark at 15°C, 14 h light at 20°C, 41% humidity). The nutrient solution contained Ca (3.55 mM), Mg (1.45mM), NO₃⁻ (8.1mM), H₂PO₄⁻ (0.2mM), Cl (10µM), Na (1.1mM), K (1.2mM), SO₄ (1.45mM), H₃BO₃ (30µM), MoO₄²⁻ (0.2µM), FeEDDHA (25µM), Mn (10µM), Zn (1µM), Cu (1µM), buffered at pH 6.0 with 2mM MES (2-morpholinoethanesulphonic acid, 50% as potassium salt) (Kupper et al. 2000). After 14 days, the canola plants, three per pot, were transferred to pre-treatment solution for 24 hours. Pre-treatment solution contained 2mM NaMES (pH 6.0) and 0.5mM CaCl₂. Following pre-treatment, the plants were used in the ⁶⁵Zn uptake experiments.

Apoplastic and symplastic uptake of ⁶⁵Zn from ice-cold and 20°C solutions:

Canola seedlings were transferred to 4°C or 20°C uptake solutions containing 2mM NaMES (pH 6.0), 0.5mM CaCl₂ and 10 µM ZnCl₂ as either the metal salt or complexed with 10µM EDTA, 20µM rhamnolipid or 5µM PEI. Ligand concentrations differed in accordance with their metal complexing capacities and stability constants. A relatively high Zn concentration was chosen to ensure that uptake was measurable over the relatively short absorption period (30 minutes). Uptake solutions were spiked with ⁶⁵Zn to give 0.037 MBq/L. Vials containing 4°C solutions were packed within ice throughout the duration of the experiment. Each treatment was replicated 3 times.

After 30 minutes the canola roots were removed from the uptake solutions and rinsed with MilliQ water. Roots used to measure symplastic absorption of Zn, at both 20°C and 4°C, were transferred to ice-cold desorption solutions for 30 minutes in order to desorb apoplastically bound Zn. Desorption solutions contained 2mM NaMES (pH 6.0), 5mM CaCl₂ and 60µM ZnCl₂. The concentration of K⁺ remaining in the uptake solutions was measured using ICP-OES. Potassium efflux was used to test for potential loss of membrane integrity due to the presence of chelating agents in the uptake solutions.

Canola plants were separated into roots and shoots, blotted dry and weighed. Roots were transferred into radioactivity counting vials, to which 4ml of 5M HNO₃ was added. Samples were left overnight to solubilise the cell contents before the ⁶⁵Zn contents of roots were determined by gamma spectroscopy.

The amount of treatment Zn retained within the root apoplast was calculated from the difference in ⁶⁵Zn contents of desorbed and non-desorbed roots and using the specific activity of treatment Zn.

Absorption and translocation of chelate buffered Zn

Pre-treated canola seedlings were transferred to uptake solutions containing 2mM KMES (pH 6.0), 0.5mM CaCl₂ and 1µM ZnCl₂ as either the metal salt or complexed with EDTA, rhamnolipid or PEI. Ligand rates were (µM): EDTA 1, 0.8, 0.6, 0.4, 0.2, 0; rhamnolipid 10, 7.5, 5.5, 3.5, 1.5, 0; PEI 0.4, 0.3, 0.2, 0.1, 0.05, 0. Uptake solutions were spiked with ⁶⁵Zn to give 0.037 MBq/L.

After a 24 hour uptake period in a controlled environment growth chamber (10 h dark at 15°C, 14 h light at 20°C, 41% humidity), canola shoots were harvested and transferred to radioactivity counting vials, to which 10ml of 5M HNO₃ was added. Samples were left overnight to solubilise the cell contents before the ⁶⁵Zn contents of shoots were determined by gamma spectroscopy.

The degree of complex formation in each of the uptake solutions was measured using mercury drop anodic stripping voltammetry (ASV). A standard curve was prepared by diluting 10µg/ml ± 0.05µg/ml Zn High-Purity Standard (CAT# ICP-MS-KIT-A 2% HNO₃) with MilliQ water plus 22.4mg KCl to achieve final Zn concentrations of (µg/L) 20, 10, 5, 2.5, 0.5 and 0. Each solution was prepared in acid washed Teflon™ electrochemical cells with a final solution volume of 10.2ml. Purified nitrogen was passed through the solution for 15 seconds. Deposition of the electrolyte was performed in stirred solution at -1.2V for 3 minutes. Following deposition, the stirrer was switched off for 20 seconds, before the voltamperogram was recorded between -1.2V and +0.4V. ASV analysis was performed twice on each solution using a fresh mercury drop each time. The standard curve was replicated 4 times (Figure 4.1)

Three millilitres of each Zn uptake solution was diluted to 10.2ml with MilliQ water plus 22.4mg of KCl to yield total Zn concentrations of 19.2µg/L in each solution, within the range of the standard curve for Zn. Voltammetric analysis of the uptake solutions was performed using the method employed for standard curve determination.

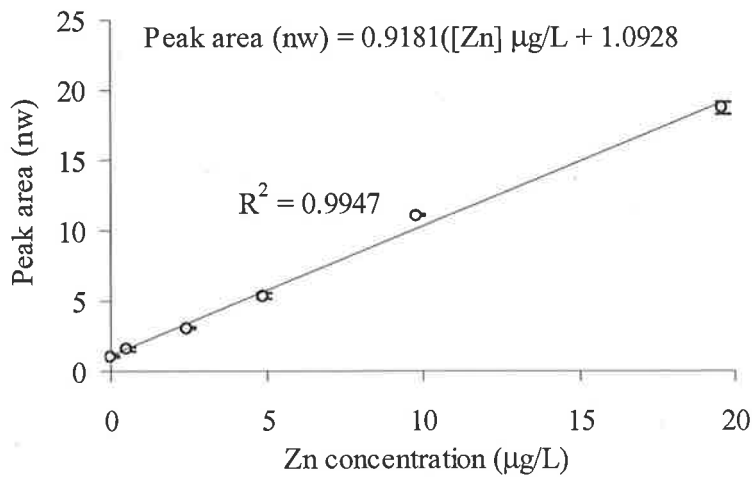


Figure 4.1. Standard curve for ASV-labile Zn.

RESULTS

Cold temperatures suppress active absorption pathways in plant roots (Lasat et al. 1996; Lombi et al. 2001). Therefore, data from the root absorption experiments, undertaken in 4°C and 20°C solutions, provided a comparison between metabolically mediated and passive Zn absorption pathways. At both temperatures, Zn absorption was strongly affected by chelation. EDTA significantly reduced both the concentration of Zn in the root apoplast as well as Zn absorbed into the symplast at 4°C and 20°C (Table 4.1, Figure 4.2). There was a slight discrepancy in the EDTA data, as Zn uptake was higher in desorbed roots (symplastic Zn) than those rinsed in deionised water (total root Zn). However, in both cases, EDTA substantially reduced Zn absorption.

Table 4.1. Zinc accumulation in canola roots from 10 μ M Zn solutions at 4°C (\pm 1 S.E.).

Fertiliser	Total root Zn (μ g Zn/g root)	Symplastic Zn (μ g Zn/g root)	Apoplastic Zn (μ g Zn/g root)
ZnCl ₂	16.98 \pm 1.07	1.45 \pm 0.06	15.53
Zn EDTA	0.14 \pm 0.01	0.63 \pm 0.02	-
Zn PEI	17.34 \pm 1.53	2.06 \pm 0.18	15.28
Zn rhamnolipid	15.79 \pm 1.78	2.00 \pm 0.09	13.79

At 4°C, PEI and rhamnolipid significantly ($P \leq 0.05$) increased the absorption of Zn into the root symplast above that of ZnCl₂ alone (Table 4.1). Therefore, PEI and rhamnolipid may have facilitated Zn absorption via non-metabolically mediated absorption pathways. There was negligible K⁺ efflux into uptake solution during the absorption period, which suggested that neither PEI nor rhamnolipid caused any significant damage to the root membrane structure (data not shown).

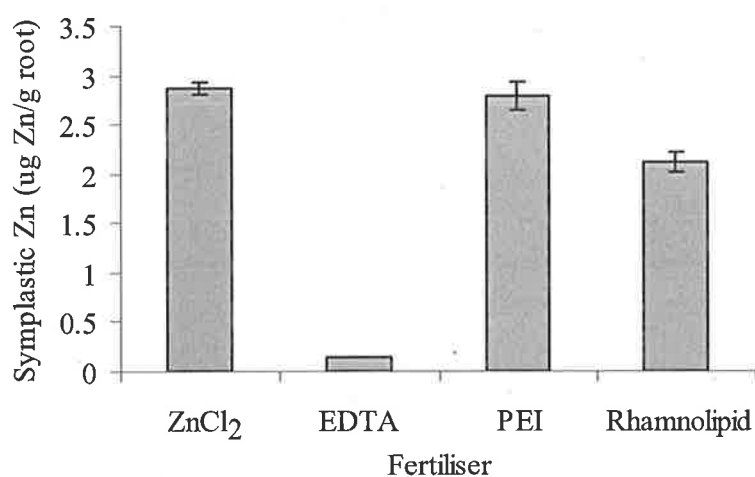


Figure 4.2. Absorption of Zn into the symplast of canola roots at 20°C (\pm 1 S.E.).

At 20°C, the symplastic absorption of ZnCl₂ and ZnPEI increased substantially over those at 4°C (Figure 4.2), thus indicating that the absorption of ZnPEI was at least partially governed by metabolically mediated uptake pathways. There was no significant difference ($P>0.05$) in the absorption of Zn-rhamnolipid in 4°C and 20°C solutions (Table 4.1, Figure 4.2). Either the absorption of Zn-rhamnolipid did not follow active uptake pathways or the surfactant properties of rhamnolipid may have induced a toxic effect (not manifest by K⁺ efflux) on the roots at the rates applied.

There was no significant difference ($P>0.05$) in total root Zn (apoplast plus symplast) when roots were supplied with ZnCl₂, Zn-PEI or Zn-rhamnolipid (Table 4.1). PEI did not increase the concentration of Zn in the apoplast compared with ZnCl₂ alone. Thus, there was no evidence to support the hypothesis that PEI increased apoplastic loading of Zn.

Anodic stripping voltammetry differentiated between chelated and ASV-labile Zn for EDTA- and PEI-buffered solutions (Figure 4.3). The amount of Zn associated with EDTA varied between 0.77 and 1.39 moles Zn/mole EDTA, decreasing with increasing concentrations of EDTA. EDTA is known to complex Zn with a 1:1 molar ratio (Norvell 1991). Therefore, it is highly likely that the electrical current dissociated some of the ZnEDTA²⁻. Our results indicate that dissociation occurred when the molar fraction was ≥ 0.4 moles EDTA/mole Zn.

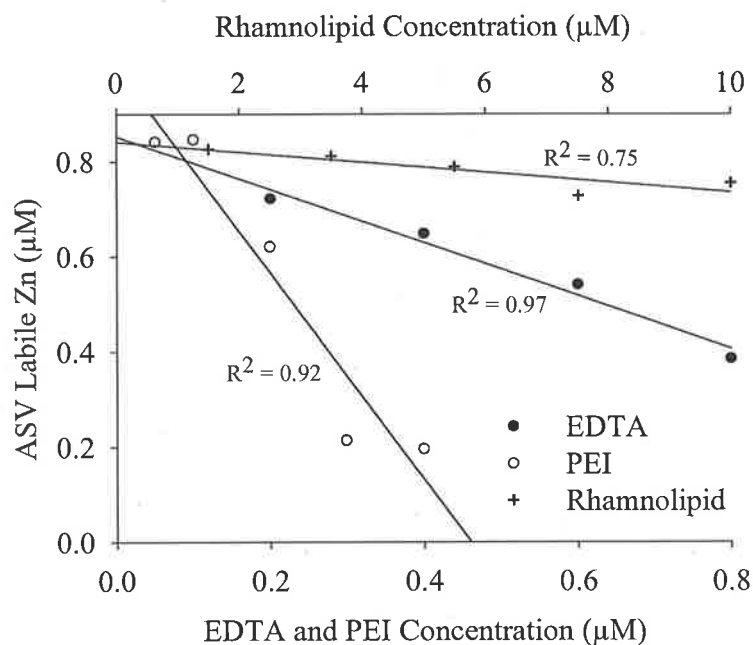


Figure 4.3. ASV-labile Zn in chelate-buffered uptake solutions.

There was a moderately strong relationship ($R^2 = 0.75$) between rhamnolipid concentration and ASV-labile Zn (Figure 4.3). However, the analysis was not considered reliable due to the low Zn-rhamnolipid stability constant and the probability of Zn-rhamnolipid dissociation during ASV analysis. ASV-labile Zn was unusually high for the rates of rhamnolipid used; up to 10:1 molar ratio with Zn (Figure 4.3). Metal-chelate dissociation during ASV analysis has been reported for complexes with stability constants of $\text{Log } K > 18$ (Tao et al. 1999), even with very short deposition times. Zinc-rhamnolipid complexes were reported to have stability constants of only $\text{Log } K = 5.62$ (Ochoa-Loza et al. 2001). Therefore, ASV-labile Zn could not be used to predict Zn accumulation by shoots (Figure 4.4).

Absorption of ZnEDTA^{2-} followed the general trend predicted by the FIAM, in that Zn accumulation in shoots decreased with EDTA addition to the uptake solution

(Figure 4.4). Clearly, this was in response to ZnEDTA^{2-} exclusion at the root surface (Figure 4.2). In contrast to the FIAM, Zn complexed by PEI was readily accumulated in canola shoots (Figure 4.4). The root absorption study showed that PEI facilitated Zn absorption into the root symplast. The shoot accumulation data confirmed these results and showed that the absorbed Zn could be readily translocated to the shoot biomass.

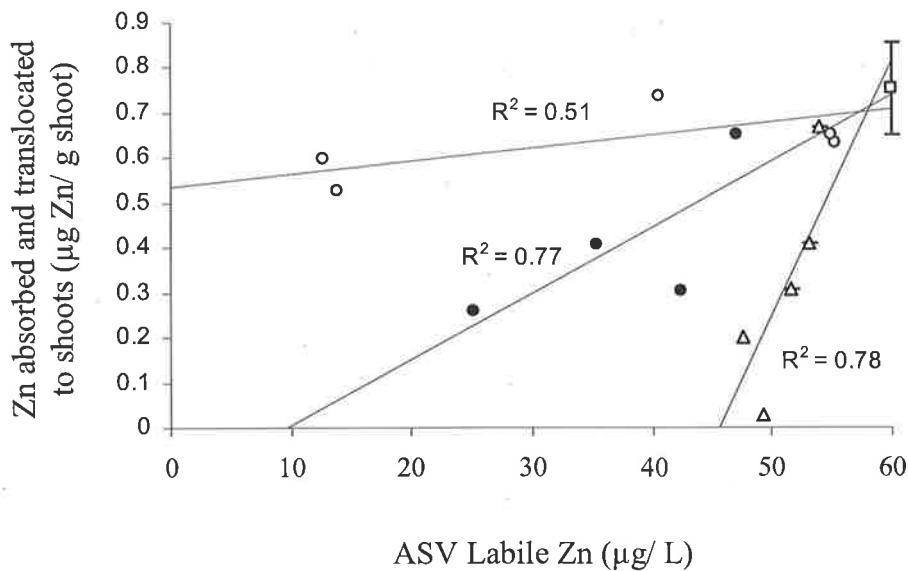


Figure 4.4. Zn accumulation in shoots versus ASV-labile Zn in uptake solutions buffered with PEI (o), EDTA (●), rhamnolipid (△) or chelate free (□) (± 1 S.E.).

In the root absorption experiments, the symplast Zn content was measured by desorbing apoplastic Zn with 5mM CaCl_2 and 60 μM ZnCl_2 . An important assumption was that negligible ^{65}Zn remained in the root apoplast following the desorption period. The desorption efficiency may have been different for each compound, depending on the affinity of the complexes for the cell wall, membrane surface and

the general root free space. Shoot accumulation of ^{65}Zn provided a more reliable measure of fertiliser absorption. Hence, the fact that Zn from PEI-buffered solutions readily accumulated in plant shoots confirmed that the trace element ion had indeed been readily absorbed into the root symplast during the earlier experiments.

DISCUSSION

The FIAM states that only the free metal ion will be absorbed across root membranes into the symplast. According to the model, chelated ions must dissociate from the ligand prior to absorption (Parker and Pedler 1997). In this study, Zn complexed by PEI or rhamnolipid was absorbed more readily than the free metal ion in 4°C solutions. Chelate dissociation would have yielded Zn^{2+} and the rate of absorption would not have been greater than the ZnCl_2 control. Therefore, chelate dissociation at the root surface, explained by the reciprocal of the metal-chelate stability constants, did not explain differences in Zn availability from each of these sources.

Anionic ligands may enhance cationic metal absorption by overcoming diffusion limitations to specific uptake sites in the root apoplast (Smolders and McLaughlin 1996; Degryse et al. 2006a, b). However, this effect was probably negligible because the absorption experiments were undertaken at 4°C, which would have suppressed absorption through specific uptake sites in the plasma membrane. Thus, the data suggests that PEI and rhamnolipid facilitated the passive absorption of Zn into the root symplast. For rhamnolipid, these results were consistent with our understanding of the lipophilic absorption and may help to explain why the absorption of Zn-rhamnolipid from alkaline and calcareous soils was not proportional to the

concentrations of Zn in the soil solutions (Chapter 3). The rapid absorption of ZnPEI was unexpected, due to the polarity of these complexes and the large molecular weight of PEI. One possible explanation was that the root desorption solution did not efficiently remove apoplastically bound Zn-PEI. If true, this could explain the higher apparent uptake of Zn from solutions containing Zn-PEI complexes.

Zinc from PEI-buffered solutions was readily accumulated in canola shoots, which showed that the fertiliser was readily absorbed and translocated to plant shoots. This result corroborates the previous experiment and suggests that the apparent increased absorption observed in the short-term uptake experiment was not due to an inefficient desorption procedure during root washing. The experimental data did not show whether ZnPEI was absorbed intact, or whether PEI simply facilitated absorption at the root surface. Therefore, one cannot conclude whether the PEI was also translocated into the canola shoots.

EDTA significantly reduced Zn absorption by canola roots (Figure 4.2). Consequently, EDTA also suppressed the accumulation of Zn in plant shoots (Figure 4.4). Previous authors have also shown that trace element-EDTA complexes are not readily absorbed by plants (Marschner 1995; McLaughlin et al. 1997; Smolders and McLaughlin 1996). The fact that very little Zn was associated with the root apoplast showed that EDTA had a higher association constant for Zn than the root cation exchange sites and thus the ZnEDTA^{2-} complex did not dissociate (Table 4.1). Moreover, the complex may have been electrostatically repelled from the predominantly anionic apoplast. These results explain why EDTA did not increase Zn

absorption by canola on alkaline and calcareous soils despite strongly increasing the concentration of Zn in the soil solution phases (Chapter 3).

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Chapter 5. The effect of chelating agents on the foliar sorption of trace element trace element fertilisers

INTRODUCTION

Millions of hectares of the world's arable landmass are deficient in plant available Zn. The World Health Organization ranked Zn deficiency as the 5th most important cause of illness and diseases in developing countries, caused primarily by inadequate dietary Zn intake (Edejer et al. 2002). Adequate use of fertiliser Zn can significantly increase crop yields and the concentration of Zn in agricultural produce (Cakmak 2002; Cakmak et al. 1999). However, on many soils, rapid adsorption and fixation reactions can substantially reduce the efficacy of trace element fertilisers (Brennan and Bolland 2003; Higgs and Burton 1955; Moraghan and Mascagni 1991). Therefore, farmers regularly apply trace elements to crop foliage (Reuter et al. 1988). Foliar applications have also been successfully used to increase the nutritional value of food and fodder crops (Belak et al. 1970).

The leaf cuticle is the primary barrier for foliar nutrient absorption. The cuticle is a hydrophobic layer, comprised of high molecular weight biopolymers such as cutins and suberins, and hydrophobic C₁₄-C₇₂ epicuticular waxes (Holloway 1993). Physiological studies have identified polar aqueous pores, which may facilitate the absorption of charged ions into leaf epidermal cells (Schonherr 2000). Nutrient sorption via aqueous pores is a relatively slow process, however, as the cuticle still represents the primary barrier for foliar nutrient absorption (Schonherr et al. 2005).

Lipophilic compounds may be readily sorbed via hydrophobic pathways in plant cuticles (Baur et al. 1997; Liu 2004; Schonherr and Riederer 1989). However, trace element fertilisers are generally polar and absorbed via aqueous pores in the cuticle. Therefore, the hydrophobic pathway has received very little attention with regard to the absorption of fertiliser ions by plants.

Chelated trace elements such as Cu, Fe, Mn, and Zn mixed with EDTA, DTPA and EDDHA, among others, are commonly sold for foliar application. Recent research has shown that chelating agents can reduce the rate of Fe diffusion across cuticles (Schonherr et al. 2005), probably due to the size selectivity of aqueous pores (Popp et al. 2005; Schonherr and Schreiber 2004). However, Basiouny and Biggs (1976) reported that EDTA increased the rate of Fe diffusion across isolated citrus leaf cuticles. The effect of chelates on the foliar sorption of other trace elements has not been adequately studied.

In Chapter 2, rhamnolipid formed lipophilic complexes with trace element ions. Because the cuticle is hydrophobic, lipophilic chelates could potentially increase trace element penetration of cuticles via the hydrophobic pathway. However, the effect of lipophilic chelates on trace element sorption by cuticles has not been tested.

The aims of this study were to investigate whether chelating agents would affect Zn sorption and diffusion across isolated Valencia orange (*Citrus sinensis*) cuticles and Zn uptake by live cotton leaves. EDTA, PEI and rhamnolipid were chosen for this study because they form anionic, cationic and lipophilic complexes with Zn, respectively.

MATERIALS AND METHODS

Enzymatic isolation of citrus leaf cuticles

Citrus leaf cuticles were collected by enzymatic isolation and used in fertiliser sorption and diffusion experiments. Leaves from the same branch and on the same day were collected from Valencia orange (*Citrus sinensis*) and 14mm leaf disks were removed using a cork borer, avoiding major veins. Cuticles were excised from the leaf disks by immersing them in a 6% pectinase solution (Sigma-Aldrich P2736, 3405 units/ml), which contained mainly pectintranseliminase, polygalacturonase and pectinesterase from *Aspergillus niger*. The solution contained 1mM sodium azide to reduce microbial activity and 20mM citric acid, adjusted to pH 3.8 with NaOH (Schonherr and Riederer 1986). Leaf disks remained in the enzymatic solution under dark conditions until the cuticles completely separated from the leaf tissue (approximately 21 days). Isolation was undertaken without agitation. The isolated cuticles were carefully removed, rinsed thoroughly in double deionised water until they were free of cellular debris. Cuticles were not air dried, nor stored for long periods of time as this caused the cuticle to become extremely fragile. Cuticles were used directly following isolation so that they structurally reflected living tissue. Previous studies have shown that cuticular waxes undergo structural changes when they are dehydrated and stored for extended periods (Geyer and Schonherr 1990; Kirsch et al. 1997).

Cuticle Ultrastructure

Isolated cuticles were mounted on glass slides and viewed under a compound light microscope at 400 × magnification. Isolated cuticles were also embedded in resin for

transmission electron microscopy (TEM). The cuticles were fixed, under vacuum, in Karnovsky's fixative solution for two days. The cuticles were rinsed twice in 0.05M cacodylate buffer for 15 minutes, before being placed in 1% osmium tetroxide for 2 hours. The cuticles were then rinsed for 1 minute in deionised water, and kept overnight in 0.5% uranyl acetate. Dehydration was done in 30, 50, 70, 80, 95 and 100% alcohol solutions for 10 minutes each. The cuticles were placed in propylene oxide, twice, for 15 minutes each before being transferred to a 50% solution of propylene oxide and 50% Spurr embedding medium for 2 hours. The cuticles were then placed in 100% Spurr embedding medium overnight. The next day, cuticles were poured into blocks and placed in a 70°C oven overnight before they were cross-sectioned and analysed by TEM at 3300 × magnification and 13000 × magnification.

Cuticle Water Partition Coefficients

Pre-weighed isolated cuticles were immersed in 1mM ZnSO₄.7H₂O solutions, either as the sulphate salt or chelated by EDTA (1 mM), PEI (0.5 mM) and rhamnolipid (1.5 mM). Ligand concentrations were determined by GEOCHEM-PC for EDTA and rhamnolipid and varied depending on the stability constant and stoichiometry of complex formation. For GEOCHEM-PC modeling, Zn-rhamnolipid stability constants were derived from the literature (Ochoa-Loza et al. 2001). At least 90% of the Zn was complexed at the rates applied. After 48 hours the cuticles were removed, rinsed in double deionised water, digested in concentrated HNO₃ and analyzed for total metal concentration by ICP-OES. All treatments were replicated four times. The cuticle/water partition coefficient ($K_{c/w}$) was calculated from:

$$K_{c/w} = \frac{\text{cuticle Zn (mg/kg)}}{\text{solution Zn (mg/kg)}} \quad [1]$$

Dialysis of fertilisers across isolated cuticles

The rate of Zn diffusion across isolated cuticles was measured by dialysis (Yamada et al. 1964). All solutions were prepared in acid-washed glassware rinsed three times with double deionised water. Donor solutions contained 100ml of 1mM of ZnSO₄ either chelate buffered or as the sulphate salt and adjusted to pH 6.0 with NaOH or HCl. The chelates tested were EDTA (1mM), rhamnolipid (1.5mM) and a highly branched 800MW polyethyleneimine (PEI) obtained from BASF (0.5mM). All treatments were replicated four times. Acceptor solutions contained 0.6ml of double deionised water only.

Isolated cuticles were glued to 1cm diameter rigid polypropylene tubing so that the outer cuticle surface faced the donor solution and the inner cuticle surface faced the acceptor solution (Yamada et al. 1964). Glue was only applied to the outer perimeter of the cuticle so that the entire cuticle surface facing the acceptor cell was free of glue or glue residue. Nitrogen gas was gently bubbled through the donor cell to stir the solution and prevent the formation of a metal depletion zone next to the surface of the leaf cuticle.

The average flow rate (F) of Zn through the cuticle was measured by periodic sampling of the acceptor solution during the absorption period (Schonherr and Riederer 1989). From the acceptor solution, 400 μ l was removed after 0.5, 2, 4, 7, 12 and 24 hours. The volume of solution removed from the acceptor cell was replaced with double deionised water to maintain a constant acceptor solution volume. Total Zn in the acceptor solutions was determined by GFAAS after acidifying the solutions

with HNO₃. Cumulative Zn diffusion across the isolated cuticles was calculated and transformed to a logarithmic linear model. The data was analysed by analysis of variance (ANOVA) and differences between the treatment means were determined by least significant difference (LSD) at $P \leq 0.05$. Residual distribution and residual versus fitted plots were checked for normality and even scatter, respectively, to ensure that the data met the main assumptions of the analysis.

The permeance (P) was a measure of the conductance of the cuticle (m/sec) for a given fertiliser, independent of the thickness of the cuticle (Schonherr and Riederer 1989). P was calculated from the flow rate of the fertiliser (F), the exposed area of the cuticle (A) and the Zn concentration gradient (ΔC) between the donor and acceptor solutions (Schonherr and Riederer 1989):

$$P (\text{m s}^{-1}) = \frac{F (\text{mol s}^{-1})}{A (\text{m}^2) \Delta C (\text{mol m}^{-3})} \quad [2]$$

Sorption of foliar-applied Zn by cotton plants

This experiment measured the rate of Zn sorption by live leaf tissue. Cotton plants (DPL444BR), one plant per pot, were grown in Sungro™ sunshine mix #1 in the glasshouse under a mixture of natural and artificial light (12 hours per day). Plants were watered every second day with Zn-free half-strength Hoaglands solution.

Five weeks after emergence, 1mM Zn fertiliser treatments were sprayed on the foliage using a CO₂ pressurized backpack sprayer, calibrated to deliver 95L H₂O per ha. Zinc fertiliser solutions were applied as either the sulphate salt (ZnSO₄.7H₂O) or were

chelate buffered using EDTA (1mM), rhamnolipid (1.5mM) or PEI (0.5mM). A rainfall simulator (Humphry et al. 2002) applied 12.5ml of water to the plants over 30 minutes to simulate field conditions where rainfall can wash foliar applied fertiliser off of the leaves. Rainfall was applied at 0 (no rainfall control), 1, 3, 6 and 12 hours after fertiliser application. Each fertiliser by rainfall treatment was replicated four times.

Following each rainfall period, plant leaves were harvested, dried and ground before 1g of the leaf material was digested in concentrated HNO₃ and analysed by ICP-OES for Zn. The ratio of Zn sorbed ([Zn sorbed]/[Zn applied]) was calculated and analysed by ANOVA. Differences between the treatment means were determined by LSD. Residual distribution and residual versus fitted plots were checked for normality and even scatter, respectively, to ensure that the data met the main assumptions of the ANOVA.

RESULTS

Cuticle Ultrastructure

The compound micrographs of the isolated cuticles clearly showed the presence of epidermal cell walls and nuclei embedded within the cuticular matrix (Figure 5.1). The adaxial cuticles, those used during the dialysis and $K_{c/w}$ experiments, did not contain stomatal openings through which the micronutrient fertilisers could have readily diffused (Figure 5.1a). Therefore, Zn present in the acceptor cell must have diffused through the cuticular matrix or via aqueous pores. Stomata were confined to the abaxial leaf surface (Figure 5.1b).

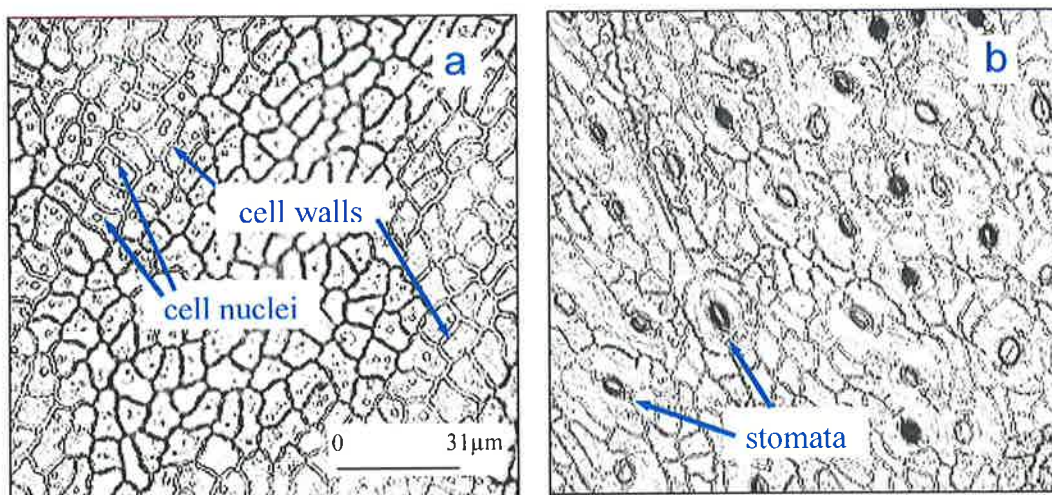


Figure 5.1. Light microscopy of isolated Valencia (*Citrus sinensis*) leaf cuticles at 400 \times magnification showing embedded cellular structure for (a) astomatous adaxial cuticle and (b) abaxial cuticle with stomates.

TEM cross-sections showed that the enzymatic isolation, followed by cuticle washing, did not remove all of the residual cell tissue (Figure 5.2). There were no intact epidermal cells (possibly due to the TEM fixation process), though some cell wall tissue, invisible to the naked eye, remained (Figure 5.2). There was no evidence of cracking or tears in any of the cuticular tissue through which Zn could have readily diffused.

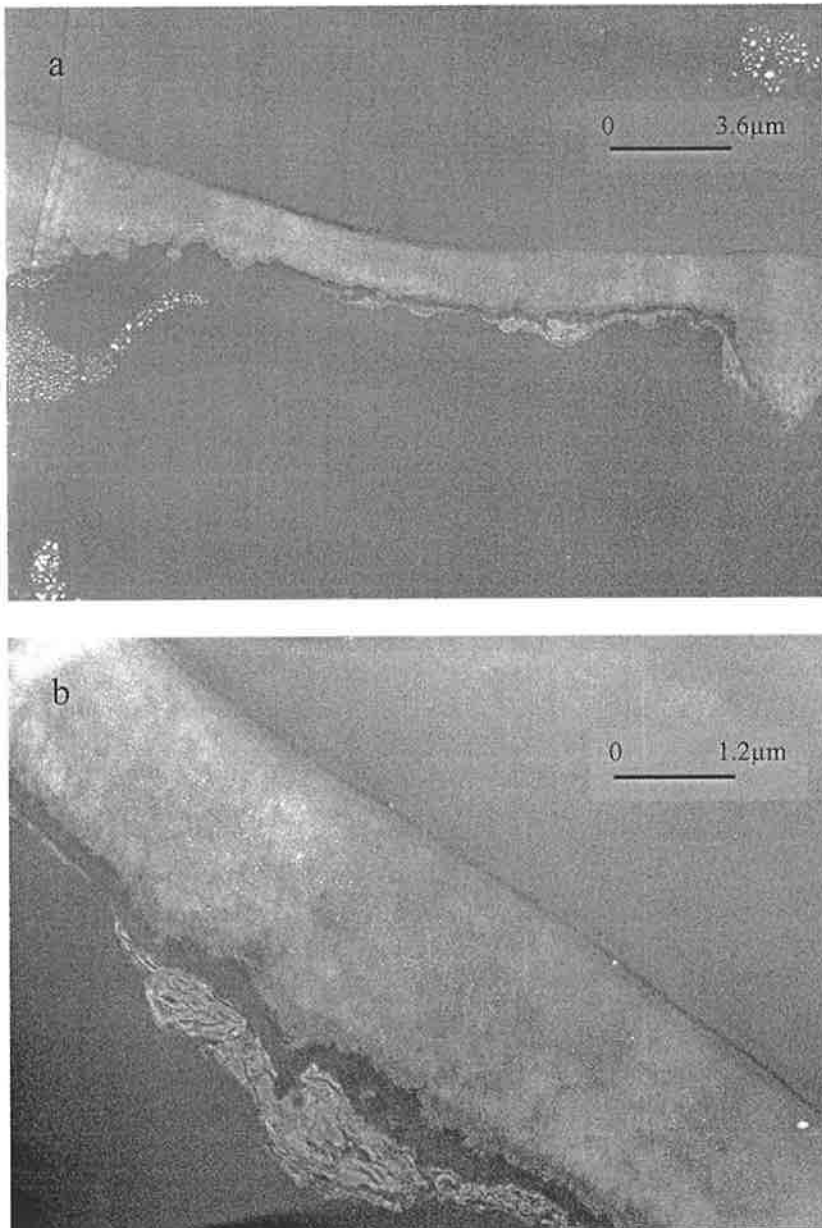


Figure 5.2. TEM cross sections of isolated Valencia (*Citrus sinensis*) leaf cuticles at (a) 3300 × magnification and (b) 13000 × magnification.

Cuticle Water Partition Coefficients

PEI and rhamnolipid significantly ($P \leq 0.05$) increased Zn sorption by isolated cuticles (Figure 5.3). The high sorption rate of Zn-rhamnolipid was probably due to the lipophilic properties of these complexes (Chapter 2), which may have allowed Zn to penetrate the hydrophobic matrix. PEI is a polar cationic polymer and may have

increased the $K_{c/w}$ of Zn by associating with anionic aqueous pores, by sorption with residual epidermal cell tissue and/or adsorption to the outer cuticle surface (von Harpe et al. 2000).

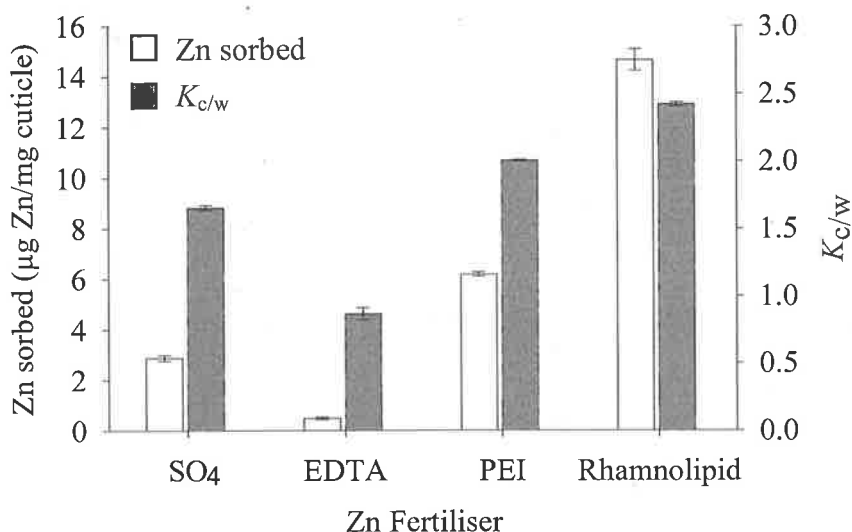


Figure 5.3. Zn sorption and partition coefficients ($K_{c/w}$) in isolated Valencia (*Citrus sinensis*) leaf cuticles (± 1 S.E.).

EDTA substantially reduced Zn sorption by cuticles, compared with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ alone. The ZnEDTA complex is a very stable anionic species (Martell and Smith 1976). Therefore, EDTA probably reduced Zn associations with the cuticles aqueous pores, which are anionic, the outer surfaces of the cuticles and/or residual leaf tissue.

Dialysis of fertilisers across isolated cuticles and sorption by cotton leaves

In the dialysis experiment, chelation by EDTA significantly ($P \leq 0.05$) reduced the rate of Zn diffusion into the acceptor cells (Figure 5.4). These data support the hypothesis that low $K_{c/w}$ fertilisers diffuse across cuticles more slowly than high $K_{c/w}$ fertilisers. However, there was no significant difference ($P > 0.05$) in the rate of Zn diffusion from

ZnSO₄, PEI and rhamnolipid, despite large differences in the $K_{c/w}$ of Zn from each of these sources. There was no significant difference ($P>0.05$) in the flow rate (F) or permeance (P) of the fertilisers through the isolated cuticles (Table 1).

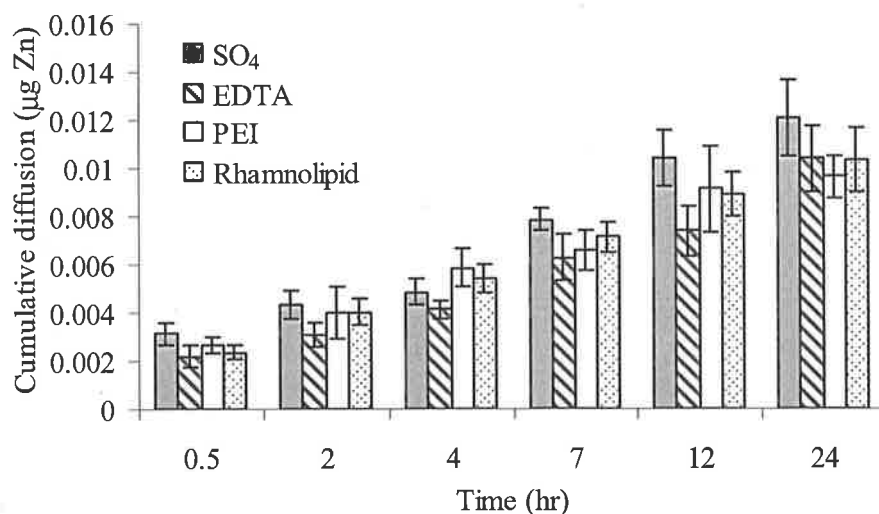


Figure 5.4. Zn concentration in acceptor solutions following diffusion across isolated Valencia (*Citrus sinensis*) adaxial leaf cuticles (± 1 S.E.).

All three chelating agents significantly ($P\leq 0.05$) reduced Zn sorption by cotton leaves (Figure 5.5). Significantly ($P\leq 0.05$) more Zn was sorbed using PEI than when EDTA or rhamnolipid was applied to the leaves (Figure 5.5). Sorption of Zn-rhamnolipid, which had a high $K_{c/w}$, was not significantly different to Zn-EDTA, which had the lowest $K_{c/w}$. According to these results, all of the chelates reduced Zn sorption by leaves and the rate of sorption was not directly influenced by the $K_{c/w}$ of the fertiliser material.

Table 1. Flow rate (F) and Permeance (P) of Zn fertilisers through isolated Valencia (*Citrus sinensis*) leaf cuticles.

Fertiliser	F (mol/ s)	$\text{Log}_{10}P$ (m/ s)
ZnSO ₄	1.59×10^{-13}	2.02×10^{-6}
Zn-EDTA	1.19×10^{-13}	1.51×10^{-6}
Zn-PEI	1.44×10^{-13}	1.84×10^{-6}
Zn-rhamnolipid	1.39×10^{-13}	1.76×10^{-6}

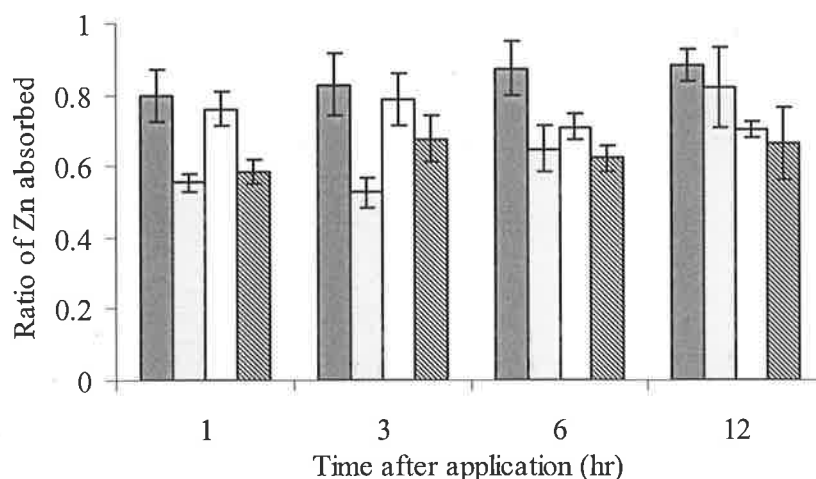


Figure 5.5. Time-dependent sorption of foliar applied Zn fertilisers by cotton plants. Fertilisers applied were ■ ZnSO₄, ▨ Zn-EDTA, □ Zn-PEI and ▩ Zn-rhamnolipid (± 1 S.E.).

DISCUSSION

Citrus sinensis cuticles were enzymatically isolated and used to measure cuticle water partition coefficients ($K_{c/w}$) and the rate of Zn diffusion through cuticular membranes. Kirsch (1997) showed that the permeance of cuticles from *Primus laurocerasus*, *Ginkgo biloba* and *Juglans regia* were not significantly altered by enzymatic isolation. Cold storage reduced the water permeability of *Citrus aurantium* cuticles

(Geyer and Schonherr 1990). Therefore, in this study, cuticles were used immediately following isolation so that their permeances better reflected those of intact leaf cuticles.

In structurally homogenous cuticles, the permeability constant (p) is the product of the $K_{c/w}$ and the diffusion coefficient (D) (Crank 1957; Schonherr and Riederer 1989).

$$p \text{ (m}^2/\text{s)} = K_{c/w} \times D \text{ (m}^2/\text{s)} \quad [3]$$

Thus compounds with high $K_{c/w}$'s should readily permeate homogenous cuticles. Plant leaf cuticles are not structurally homogenous and contain aqueous pores through which polar solutes may be absorbed. In this study, rhamnolipid and PEI significantly increased the $K_{c/w}$ of Zn compared with ZnSO_4 alone, but did not increase Zn diffusion across isolated cuticles and decreased Zn sorption by cotton leaves. Therefore, there was no discernable relationship between the $K_{c/w}$ of fertiliser solutions and Zn permeability. This could be explained by the rapid sorption of Zn^{2+} ions via the aqueous pathway (Popp et al. 2005) or by Zn adsorption to the inner cuticle surfaces and residual cell tissue during $K_{c/w}$ measurement. This effect may have caused an overestimation of the $K_{c/w}$ of the fertiliser solutions. Yamada (1964) also found that ion sorption to inner cuticular surfaces was considerable.

The chelating agents did not increase the rate of Zn diffusion across isolated cuticles and inhibited Zn sorption by live cotton plants. The high molecular weights of these chelating agents probably reduced Zn sorption due to the size selectivity of the cuticular matrix (Popp et al. 2005; Schonherr and Schreiber 2004). These results suggest that chelating agents should not be incorporated into Zn foliar sprays, particularly given the high cost of these chelates to farmers.

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Chapter 6. Conclusions and Future Directions

Fate in soil and absorption by plants

This study showed that Zn-PEI and Zn-rhamnolipid were more effective fertilisers than ZnEDTA²⁻ or ZnSO₄ on alkaline soils. The following discussion explains why, with respect to the data collected.

PEI substantially increased the size of the labile Zn pool in the non-calcareous soil, which helped to explain why PEI increased Zn absorption by canola, even though much of the labile Zn was partitioned in the exchangeable pool, rather than the soil solution. The mobilisation of native soil Zn was probably enhanced by the large metal complexing-capacity of the polymer. PEI did not increase the size of the Zn labile pool in the highly calcareous soil, probably due to competition with Ca²⁺. Nor did PEI significantly ($P > 0.05$) increase Zn uptake by canola on the calcareous soil. It should be noted that Zn-PEI increased Zn uptake by canola, compared with ZnSO₄, at the 90% confidence level and was more effective than Zn-EDTA at the 95% confidence level on the calcareous Streaky Bay soil.

In solution culture, PEI facilitated Zn absorption by canola roots, an effect that probably also contributed to the efficacy of Zn-PEI applied to soil. The exact way in which PEI facilitated Zn absorption was not elucidated, though it is possible that PEI adhered to the plasma membrane by electrostatic interaction (Futami et al. 2005) and was absorbed via polyamine carriers in the plasma membrane (Soulet et al. 2004) or by endocytosis (Bieber et al. 2002; Soulet et al. 2004). Numerous polyamine absorption sites have been located in biological membranes and PEI may have been

absorbed via one of these (Igarashi et al. 2001; Sharpe and Seidel 2005). The exact way in which PEI facilitated trace element absorption should be the subject of future investigations.

In summary, the efficacy of PEI was attributed to the polymer's high complexing capacity for metal ions, its effect on the size of the labile Zn pool in soil and its ability to facilitate Zn absorption by canola roots.

The rhamnolipid-based product was the most effective Zn fertiliser on both soils even though rhamnolipid increased Zn adsorption to the soil solid phase and did not significantly increase the size of the labile Zn pool in either soil. In solution culture, rhamnolipid facilitated trace element absorption by canola, probably via a novel lipophilic absorption pathway, which this study suggested was a passive process. It seems probable that the high bioavailability of Zn-rhamnolipid accounted for the efficacy of this fertiliser on the alkaline and calcareous soils used in this study.

This study did not determine whether the canola plants absorbed intact Zn-PEI and Zn-rhamnolipid complexes, or whether the complexes dissociated prior to Zn absorption; this could be the subject of a future investigation.

At the highest application rate, EDTA significantly ($P \leq 0.05$) increased the size of the labile Zn pool on both soils. At lower application rates, such as those used in the pot experiment, EDTA significantly ($P \leq 0.05$) increased the concentration of Zn partitioned in the soil solution phase. Chelating agents usually increase trace element absorption by increasing ion solubilisation and transport to the rhizosphere (Treeby et

al. 1989; Wallace 1983). However, in this study, ZnEDTA^{2-} was ineffective on both soils, which suggested that the plant roots could not readily absorb the ZnEDTA^{2-} complex from soil solutions. This limitation was confirmed in solution culture experiments when EDTA strongly reduced the rate of Zn absorption by canola. Other authors have also found that EDTA, and other chelates, reduce trace element absorption by plants grown in solution culture experiments (Halvorson and Lindsay 1977; Malzer and Barber 1976; Marschner 1995). However, when applied to soil, the importance of root exclusion has rarely been critically evaluated. In fact, Wallace (1983) implied that the exclusion of EDTA by roots in solution culture experiments did not translate to the soil environment. This study showed that, on alkaline soils, chelates that increased the rate of trace element absorption into the root symplast were significantly more effective than EDTA, which was not readily absorbed by canola roots.

Product cost is the primary reason that chelating agents have not received widespread use in agriculture (Wallace 1983). Relatively low metal complexing-capacities of conventional chelates such as EDTA and DTPA have necessitated high chelate application rates. This study has shown that there is a genuine opportunity to reduce chelate application rates by using polymers with extremely high complexing-capacities for trace element ions, which could ultimately improve the affordability of chelate use. Moreover, there are opportunities to improve the performance of chelated fertilisers by overcoming a key limitation of the conventional products, i.e. root exclusion of the chelated nutrient. By reducing application rates and increasing product performance, chelated fertilisers may become more affordable in the future.

Foliar absorption

In this study, chelating agents reduced the absorption of foliar-applied Zn. Rhamnolipid, the lipophilic sequestering agent, generated a fourfold increase in Zn sorption by enzymatically excised *Citrus sinensis* cuticles but did not significantly increase Zn absorption by live leaf tissue. Thus, there was no clear relationship between cuticle water partition coefficients ($K_{c/w}$) and the rate of Zn diffusion across isolated cuticles.

This study generated further evidence that chelating agents should not be incorporated in foliar-applied trace element fertilisers. There was no evidence to suggest that lipophilic trace element fertilisers would be absorbed more readily than the metal SO_4^{2-} salt alone. However, this subject warrants further investigation in order to differentiate between Zn trapped within the cuticle versus the intracellular absorption of various Zn chelates. One potential method would be to study Zn concentrations and transport into non-treated parts of the plant following the foliar application of chelated Zn fertilisers.

Environmental considerations

EDTA is highly mobile in soils and may increase trace element leaching, which could potentially contaminate aquifers and nearby water-bodies, and/or cause a decline in soil fertility (Thayalakumaran et al. 2003; Wu et al. 2004). The risk of environmental degradation is probably mainly limited to high rainfall environments or under intensively irrigated agriculture. As discussed in the Literature Review (Chapter 1), EDTA has been detected in several European waterbodies, though much of the EDTA load probably originated from industrial effluents and wastewater treatment plants

(Eklund et al. 2002; Geschke and Zehring 1997; Nakashima and Yagi 1991; Pietsch et al. 1995). In this study, PEI and rhamnolipid increased Zn adsorption to the soil solid phase. Therefore, they would be far less likely to leach from soils than EDTA-based fertilisers.

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