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The Effect of Chelating Agents on the Foliar Sorption of Zinc Fertilizers

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ABSTRACT

This study investigated the sorption of chelated zinc (Zn) fertilizers applied to plant foliage. *n*-Octanol water partition coefficients showed that rhamnolipid formed lipophilic complexes with Zn. It was hypothesized that the lipophilic complex would be better absorbed by leaves than $ZnSO_4$ because leaf cuticles are primarily hydrophobic. Cuticle/water partition coefficients, which were measured using enzymatically isolated Valencia orange (*Citrus sinensis*) cuticles, showed that a chelating polymer called polyethylenimine and rhamnolipid increased Zn sorption to adaxial cuticle surfaces by 2-fold and 5-fold, respectively. The chelate EDTA reduced cuticle sorption of Zn to 17% of the $ZnSO_4$ control. The rate of Zn diffusion across isolated adaxial cuticles was measured by dialysis. In addition, Zn sorption by cotton (*Gossypium hirsutum* L.) foliage was measured by spraying chelated Zn solutions on the foliage before washing the leaves with a rainfall simulator at set intervals. The results showed that the rate of Zn diffusion across *C. sinensis* cuticles was not related to the cuticle/water partition coefficients of the chelate solution. Nor did the formation of lipophilic Zn-rhamnolipid complexes increase Zn diffusion across the primarily hydrophobic cuticle. Six hours after application to *G. hirsutum* leaves, rhamnolipid, EDTA and polyethylenimine had reduced the rate of Zn sorption by 71, 74 and 81% of the $ZnSO_4$ control, respectively, which suggested that these chelates may increase the risk of Zn-fertilizer runoff during rainfall events. The reduction in Zn sorption with chelate application was probably due to the size selectivity of aqueous pores that are present within leaf cuticles and the efficiency with which aqueous pores transport ionic Zn^{2+}.

Keywords: cuticle, dialysis, EDTA, lipophilic, polyethylenimine, rhamnolipid

INTRODUCTION

Millions of hectares of the world's arable landmass are deficient in plant-available Zn (Graham 2008). 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 fertilizer Zn can significantly increase crop yields and the concentration of Zn in agricultural produce (Cakmak *et al*. 1999; Cakmak 2002). However, on many soils, rapid adsorption and fixation reactions can substantially reduce the efficacy of trace element fertilizers (Higgs and Burton 1955; Moraghan and Mascagni 1991; Brennan and Bolland 2003). 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 primarily a hydrophobic layer, comprised of high molecular weight biopolymers such as cutins and suberins, and hydrophobic C_{14}-C_{72} epicuticular waxes (Holloway 1993). However, physiological studies have identified polar aqueous pores, which appear to facilitate the absorption of charged ions into leaf epidermal cells (Schonherr 2000). Aqueous pores are thought to be mainly derived from hydroxyl, amino, and carboxyl groups (Schonherr 2006).

Lipophilic compounds may be readily sorbed via hydrophobic pathways in plant cuticles (Schonherr and Riederer 1989; Baur *et al*. 1997; Liu 2004). However, trace element fertilizers 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 trace element ions by plants.

Trace elements such as Zn, Cu, Fe and Mn, mixed with ethylenediamine tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminedi(o-hydroxyphenylacetic) acid (EDDHA), among others, are commonly sold for foliar application. Recent research has shown that chelating agents may reduce the rate of Fe diffusion across cuticles (Schonherr *et al*. 2005), probably due to the size selectivity of aqueous pores (Schonherr and Schreiber 2004; Popp *et al*. 2005). 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.

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 sorption by live cotton (*Gossypium hirsutum* L.) leaves. *Citrus sinensis* was chosen to provide astomatous adaxial leaf cuticles. Three chelating agents were chosen for this study; EDTA, polyethylenimine (PEI) and rhamnolipid. EDTA has been used in fertilizer products for many decades. PEI is based on a reoccurring $[-CH_2-CH_2-NH-]^n$ monomer and has a charge density of 16-20 meq/g (BASF 1996). PEI is of interest in fertilizers because of the polymer's high complexing capacity for metal ions (von Harpe *et al*. 2000) and its unique positive charge (Remy *et al*. 1998). Rhamnolipid is a biosurfactant produced by *Pseudomonas aeruginosa*. Rhamnolipids have been used to complex metal ions in soils and are of interest in fertilizers

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because they are non-toxic and readily biodegradable (Herman *et al*. 1995; Frazer 2000; Ochoa-Loza *et al*. 2001).

MATERIALS AND METHODS

Chelants

The Jeneil Biosurfactant Company (Saukville, WI, USA) supplied a 25% rhamnolipid solution that contained equal proportions of R1 (504 amu) and R2 (650 amu) rhamnolipids. BASF (Germany) supplied a highly branched 50% PEI solution with an average molecular weight of 800 amu. Sub-samples of both products were digested in concentrated HNO_3 and analyzed 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. Tetrasodium EDTA was purchased from Sigma Aldrich (St. Louis, Mo, USA).

Enzymatic isolation of citrus leaf cuticles

Valencia orange (*Citrus sinensis*) leaf cuticles were collected from the adaxial surface by enzymatic isolation and used in fertilizer sorption and diffusion experiments. Leaves were collected from the same branch and on the same day and 14 mm diameter 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 pectintranseliminase, polygalacturonase and pectinesterase from *Aspergillus niger*. The solution contained 1 mM sodium azide to reduce microbial activity and 20 mM 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; cuticle separation was observed after 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 light microscope at 400X magnification. Isolated cuticles were also resin embedded 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 0.05 mM cacodylate buffer for 15 min, before being placed in 1% osmium tetroxide for 2 h. The cuticles were then rinsed for 1 min in deionised water, and kept overnight in 0.5% uranyl acetate. Dehydration was done in 30, 50, 70, 80, 95 and 100% alcohols for 10 min each. The cuticles were placed in propylene oxide, twice, for 15 min each before being transferred to a 50% solution of propylene oxide and 50% Spurr embedding medium for 2 h. 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.

Partition coefficients

n-Octanol/water partition coefficients ($K_{o/w}$) and cuticle/water partition coefficients ($K_{c/w}$) were measured to establish the affinity of each chelated Zn solution for hydrophobic phases and the excised cuticles. *n*-Octanol/water partition coefficients were determined using the shake-flask method. Twenty mL solutions were prepared containing of 1.5 mM $ZnSO_4.7H_2O$ and 20 mM rhamnolipid, PEI or EDTA. High chelate rates were used to ensure that the majority of the metal was in the complexed state. In addition, a series of 1 mM $ZnSO_4.7H_2O$ solutions was prepared with (mmol L^{-1}) 0, 0.1, 0.24, 0.5, 1, 1.5, 2, and 2.5 of rhamnolipid. Each solution was buffered at pH 6.0 with 2 mM KMES (2-morpholinoethanesulphonic acid, 50% as potassium salt). Two mL of *n*-octanol was added to each vial before they were shaken end-over-end for 24 h. Following shaking, 3 mL of solution was removed from the aqueous phase and digested in concentrated HNO_3 before analysis for total Zn by ICP-AES. The concentration of each metal partitioned in the octanol phase was determined by mass balance. Each treatment was replicated in triplicate. *n*-Octanol/water partition coefficients were calculated according to equation [1].

$$K_{o/w} = \frac{C_O}{C_W} \qquad [1]$$

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

To measure cuticle/water partition coefficients, pre-weighed isolated cuticles were immersed in 1 mM $ZnSO_4.7H_2O$ solutions, either as the sulfate salt or chelated by EDTA (1 mmol L^{-1}), PEI (0.5 mmol L^{-1}) and rhamnolipid (1.5 mmol L^{-1}). Complex formation was calculated 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 h the cuticles were removed, rinsed in double deionised water, digested in concentrated HNO_3 and analyzed for total metal concentration by ICP-AES. All treatments were replicated four times. The cuticle/water partition coefficient ($K_{c/w}$) was calculated from:

$$K_{c/w} = \frac{\text{cuticle Zn (mg kg}^{-1})}{\text{solution Zn (mg kg}^{-1})} \qquad [2]$$

Diffusion of fertilizers 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 100 mL of 1 mM of $ZnSO_4$ either chelate buffered or as the sulfate salt and adjusted to pH 6.0 with NaOH or HCl. The chelates tested were EDTA (1 mmol L^{-1}), rhamnolipid (1.5 mmol L^{-1}) and PEI (0.5 mmol L^{-1}). All treatments were replicated four times. Acceptor solutions contained 0.6 mL of double deionised water only.

Isolated adaxial cuticles were super-glued to 1 cm 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 diffusion 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 µL was removed after 0.5, 2, 4, 7, 12 and 24 h. 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 using a graphite furnace atomic absorption spectrometer (PerkinElmer, AAnalyst 600) after acidifying the solutions with HNO_3. Cumulative Zn diffusion across the isolated cuticles was calculated and transformed to a logarithmic linear model. The data was analyzed by analysis of variance (ANOVA) using Genstat 8 and differences between the treatment means were determined by least significant difference ($P \leq 0.05$). 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.

Sorption of foliar-applied Zn by cotton plants

This experiment measured the rate of Zn sorption by cotton (cv. 'DPL444BR') plants. One plant per 1.5 L pot was grown in Sun-

gro™ sunshine mix #1 (Sungro Horticultural Distribution Inc., Bellevue, WA.) in the glasshouse under a mixture of natural and artificial light (12 h day^{-1}). Plants were watered every second day with Zn-free half-strength Hoagland's solution.

Five weeks after emergence, 1 mM Zn fertilizer treatments were sprayed on the foliage using a CO_2 pressurized backpack sprayer, calibrated to deliver 95 L ha^{-1}. Zinc fertilizer solutions were applied as either the sulphate salt ($ZnSO_4.7H_2O$) or were chelate buffered using EDTA (1 mmol L^{-1}), rhamnolipid (1.5 mmol L^{-1}) or PEI (0.5 mmol L^{-1}). A rainfall simulator (Humphry *et al*. 2002) applied 12.5 mm of water to the plants over 30 minutes to simulate field conditions where rainfall can wash foliar-applied fertilizer off of the leaves. Zinc not removed by rainfall was considered to be sorbed by the leaf. Rainfall was applied at 0 (no rainfall control), 1, 3, 6 and 12 h after Zn solutions were spayed onto the foliage. Each fertilizer by rainfall treatment was replicated four times.

Following each rainfall period, plant leaves were harvested, dried and ground before 1 g of the leaf material was digested in concentrated HNO_3 and analyzed by ICP-AES for Zn. The ratio of Zn sorbed ([Zn sorbed]/[Zn applied]) was calculated and analyzed by ANOVA as a completely randomized design, using the type of fertilizer, time after application and a fertilizer by time interaction as the treatment factors tested. Differences between the treatment means were determined by Least Significant Difference ($P \leq 0.05$). 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 ANOVA.

RESULTS

Cuticle ultrastructure and surface morphology

The light micrographs of the isolated cuticles clearly showed the presence of epidermal cell walls embedded within the cuticular matrix of the adaxial surface of *C. sinensis* leaves (**Fig. 1**). The adaxial cuticles, those used during the dialysis and $K_{c/w}$ experiments, did not contain stomatal openings through which the micronutrient fertilizers could have readily diffused (**Fig. 1**). Therefore, Zn present in the acceptor cell must have diffused through the cuticular matrix or via aqueous pores. In *C. sinensis* leaves, stomata are confined to the abaxial leaf surface. TEM cross-sections showed that there were no intact epidermal cells following enzymatic isolation, nor were there cracks or tears in the cuticular tissue through which Zn could have readily diffused (**Fig. 2**).

Partition coefficients

Rhamnolipid formed lipophilic (positive $K_{o/w}$) complexes with Zn (**Fig. 3**). The amount of Zn able to dissolve into the hydrophobic *n*-octanol layer was proportional to the rhamnolipid concentration (**Fig. 4**). These results indicate that rhamnolipids form uncharged complexes with Zn. In the absence of rhamnolipid, or when complexed by EDTA or PEI, Zn remained in the water phase, probably due to the polarity of Zn^{2+}, $ZnEDTA^{2-}$ and Zn-PEI^{n+}.

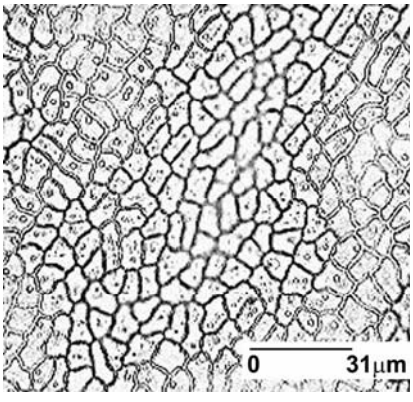

Fig. 1 Light micrograph of isolated adaxial cuticle showing epidermal cells in a surface view (X400).

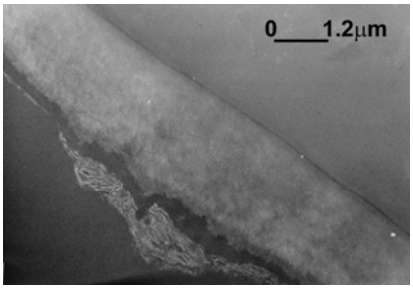

Fig. 2 Transmission electron micrograph of isolated adaxial cuticle showing epicuticular wax (EW) (outer morphological surface) and inner morphological surface (IMS) (X13000).

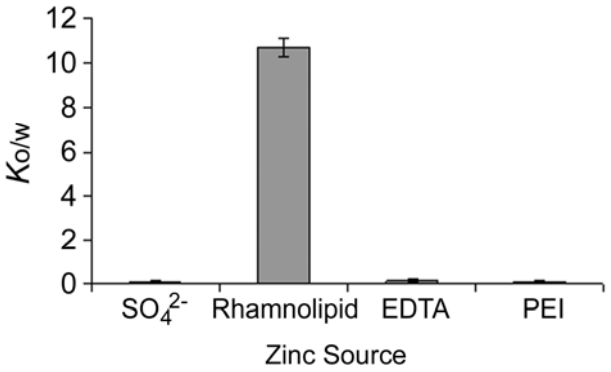

Fig. 3 The effect of EDTA, rhamnolipid and PEI on the *n*-octanol/water partition coefficients of Zn. Error bars represent one standard error of the mean.

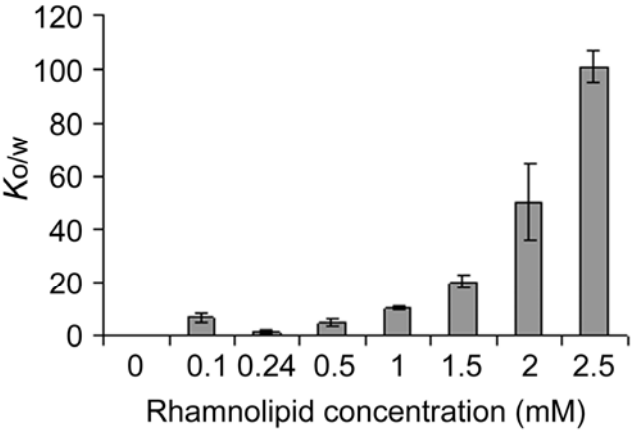

Fig. 4 Zinc *n*-octanol/water partition coefficients with varying rhamnolipid concentrations. Error bars represent one standard error of the mean.

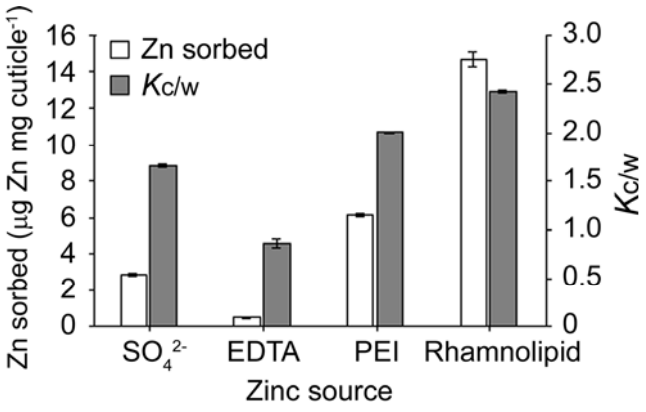

Fig. 5 Zn sorption and partition coefficients ($K_{c/w}$) in isolated *Citrus sinensis* leaf cuticles. Error bars represent one standard error of the mean.

Cuticle/water partition coefficients showed that polyethylenimine and rhamnolipid increased Zn sorption to adaxial cuticle surfaces by 2- and 5-fold, respectively (**Fig. 5**). The chelate EDTA reduced cuticle sorption of Zn to 17% of the $ZnSO_4$ control (**Fig. 5**).

Fertilizer diffusion 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 across isolated citrus leaf cuticles (**Fig. 6**). There was no significant difference ($P > 0.05$) in the rate of Zn diffusion from $ZnSO_4$, Zn-PEI and Zn-rhamnolipid, despite large differences in the $K_{c/w}$ of Zn from each of these sources. The lipophilic properties of Zn-rhamnolipid did not enhance Zn diffusion across isolated cuticles.

All three chelating agents significantly ($P \leq 0.05$) reduced Zn sorption by *G. hirsutum* leaves (**Fig. 7**). Zinc-sulfate was rapidly absorbed by cotton leaves; 80% of the applied Zn was absorbed within the first hour of application. Zinc complexed by PEI was absorbed more rapidly ($P \leq 0.05$) than Zn complexed by EDTA or rhamnolipid (**Fig. 7**). Sorption of Zn-rhamnolipid, which had a high $K_{c/w}$, was not significantly different to Zn-EDTA, which had the lowest $K_{c/w}$. Moreover, the lipophilic properties of Zn-rhamnolipid did not enhance Zn sorption by cotton cuticles. According to these results, all of the chelates reduced Zn sorption by leaves and the rate of Zn sorption was not directly influenced by the $K_{c/w}$ or the $K_{o/w}$ of the fertilizer material.

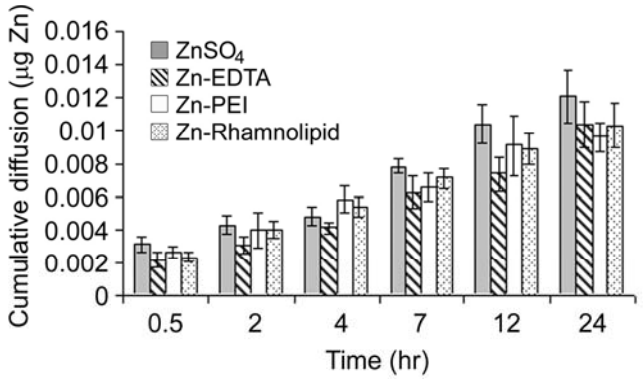

Fig. 6 Time-dependent Zn diffusion across isolated *Citrus sinensis* adaxial leaf cuticles. Error bars represent one standard error of the mean.

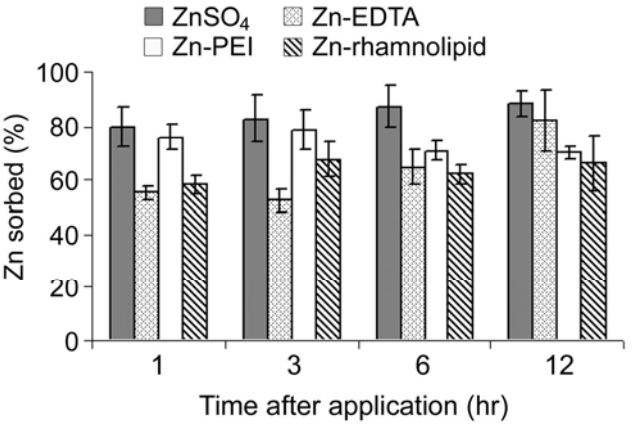

Fig. 7 Time-dependent absorption of foliar applied Zn fertilizers by *Gossypium hirsutum* L. foliage. Error bars represent one standard error of the mean.

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 *et al*. (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 this study, rhamnolipid and PEI significantly increased the $K_{c/w}$ of Zn compared with $ZnSO_4$ alone. The high sorption rate of Zn-rhamnolipid was probably due to the lipophilic properties of these complexes (**Fig. 3**), which may have allowed Zn to penetrate the hydrophobic matrix. PEI contains cationic amine groups (von Harpe *et al*. 2000) 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.

The chelate EDTA significantly reduced Zn sorption by adaxial *Citrus sinensis* cuticles (**Fig. 5**). The Zn-EDTA complex is a very stable anionic species (Martell and Smith 1976). Therefore, EDTA probably reduced Zn associations with the cuticles anionic aqueous pores by electrostatic repulsion, as well as the outer surfaces of the cuticles and/or residual leaf tissue.

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

Although rhmnolipid and PEI significantly increased the $K_{c/w}$ of Zn, they did not increase Zn diffusion across isolated cuticles and decreased Zn sorption by intact *G. hirsutum* leaves. Therefore, there was no discernable relationship between the $K_{c/w}$ of fertilizer solutions and Zn permeability through the leaf cuticles. For living *G. hirsutum* leaves, this could be explained by the rapid absorption of Zn^{2+} ions via the aqueous pathway (Popp *et al*. 2005).

The high molecular weights of EDTA and PEI may have reduced Zn sorption by *G. hirsutum* leaves due to the size selectivity of aqueous pores in the cuticular matrix. For example, Schonherr and Schreiber (2004) measured a 13-fold decrease in the permeability of hydrophilic compounds through *Populus canescens* cuticles with increasing molecular weight from 100 g mol^{-1} to 500 g mol^{-1}. In a similar study with *Hedera helix* L. cuticles, Popp *et al*. (2005) measured a reduction in the mobility of hydrophilic compounds with molar volumes above 110 cm^3 mol^{-1}. The molar volume of $Na_2ZnEDTA$ is 170 cm^3 mol^{-1} (Hovey and Tremaine 1985) and the average molecular weight of PEI was 800 g mol^{-1}, which may explain why both of these chelants hindered Zn sorption.

Previous studies have shown that lipophilic compounds are readily sorbed by cuticles (Schonherr and Riederer 1989; Baur *et al*. 1997; Liu 2004), probably by association with high molecular weight biopolymers and epicuticular waxes (Holloway 1993). However, for low molecular weight compounds, mobility may be higher in the hydrophilic pathways of cuticles (Popp *et al*. 2005). In this study, rhamnolipid formed lipophilic complexes with Zn that were sorbed less readily by *G. hirsutum* leaves than $ZnSO_4$ alone. These results are in agreement with those published by Popp *et al*. (2005) and they reiterate the efficiency with which aqueous pores transport ionic Zn^{2+}.

CONCLUSIONS

In this study, EDTA, PEI and rhamnolipid did not improve Zn diffusion across *Citrus sinensis* cuticles and reduced Zn sorption by *Gossypium hirsutum* leaves. Reduced diffusion and sorption may have been due to size exclusion in aqueous pores, which are considered responsible for transporting ionic Zn^{2+}. Rhamnolipid formed lipophilic complexes with Zn but did not improve Zn uptake across the waxy leaf cuticles. This study showed that EDTA, PEI and rhamnolipid hindered Zn transport across leaf cuticles compared with the application of $ZnSO_4$ alone. Future research should test

the efficacy of foliar applied chelated trace elements under field conditions.

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