The uptake of different formulations of foliar applied micronutrients by *Citrus sinensis* (L.) Osbeck cv. Valencia

by

Surita Marizaan van der Merwe

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Supervisor: Mr J.T. Vahrmeijer
Co-supervisor: Prof. P.J. Robbertse

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Department of Plant Production and Soil Science

Abstract

Perennial fruit crops like citrus can benefit from foliar nutrition since deep roots can deplete the soil and soil amendments other than fertigation are not easily applied without damaging roots. Increases in production costs are urging farmers to be more cost effective in supplying their crops with the necessary micronutrients. Different formulations (amino acid, chelated or inorganic complexes) for micronutrients exist and, when used as foliar sprays, more information is needed on: i) the most effective and cost effective formulation for uptake in citrus, ii) the highest concentrations for each formulation for the most efficient application and iii) the influence of contact time between micronutrient and citrus leaves on nutrient uptake. Plant species, organ types and developmental stages of the plant organ may also result in differences in cuticle ultrastructure that influences the uptake of foliar applied chemicals.

Potted 'Midknight' trees on Carizzo Citrange rootstock were used to evaluate the uptake of foliar applied micronutrients. All experiments were done in a greenhouse at the University of Pretoria. For the main experiment different formulations, concentrations and times of sampling for foliar applied manganese (Mn), zinc (Zn), copper (Cu), iron (Fe), boron (B) and molybdenum (Mo) were evaluated. The

treated leaves, as well as the leaf directly above and below the treated leaves were sampled. The most effective and cost effective formulation and concentration were determined by applying B: H_3BO_3 (2X) and Mo: $Na_2MoO_4.2H_2O$ (4X) as a multiple of the recommended concentration of the FSSA (Fertiliser Society of South Africa) (2003) and Mn: $MnSO_4$ (4X), Zn: $ZnSO_4$ (4X), Cu: $CuSO_4$ (4X) and Fe: $FeSO_4$ (0.5X) as a multiple of the recommended concentration of the manufacturer of the amino acids used. The multiple concentrations are given in parenthesis. The optimal times of sampling after application to determine the time at which maximum uptake occurred for the different elements were: Cu – 24 h, Mo & B – 48 h, Mn & Zn – 96 h, Fe – 192 h. The translocation of Mn, B and Mo occurred to the leaf directly above and below the treated leaf, while Cu, Zn and Fe did not translocate readily, indicating that multiple seasonal applications may be necessary. A scanning electron microscopic study (SEM) was done on leaf surfaces of lemon, grapefruit, mandarin, navel and orange trees. The physical appearance of the wax on the leaf surfaces was visually inspected and no considerable differences between the different citrus species, for the same age leaves, were found that may affect penetration of foliar applied micronutrients. Mature leaves had larger amounts of surface wax than young leaves. Cuticle thicknesses for the different citrus species differed among the leaf age and the abaxial and adaxial leaf surfaces. 'Bahianinha' navel, 'Satsuma' mandarin and 'Star Ruby' grapefruit leaves had thicker cuticles than Valencia leaves in most cases. 'Washington' navel and 'Genoa' lemon leaves had thinner cuticles than that of Valencia. Cuticle thickness may therefore have an influence on differences in the uptake of foliar applied products between citrus species.

Keywords: Foliar application, micronutrients, citrus, formulation, leaf cuticles, SEM.

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Chapter 1   General background

'Valencia' is regarded as a cultivar of *Citrus sinensis* in the title of this dissertation. However, in literature, Valencia is also subdivided into cultivars like 'Midknight', 'Delta' and others. The experiments on nutrient uptake in this dissertation were performed on 'Midknight' and in the text this name was used. Before continuing with the introduction, it is therefore necessary to explain this controversy by supplying some information about the history and nomenclature of *Citrus sinensis.*

The taxonomy of citrus is a complicated and controversial matter, due to the long period of cultivation, wide dispersion, large amount of bud mutations and sexual compatibility with related genera (Nicolosi *et al*., 2000). Various classification systems for citrus have been suggested. Scora (1975) and Barrett & Rhodes (1976) suggested that only three 'basic' true species of citrus exist within the subgenus Citrus: citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), and pummelo (*C. maxima* L. Osbeck). Scora (1988) later included a fourth true species, *C. halimi*. Hybridysation of the four basic true species or closely related genera and mainly natural mutations resulted in the other cultivated species (such as orange, grapefruit, lemon and lime) originating within Citrus. As part of cultivated citrus, sweet orange (*C. sinensis* (L.) Osbeck) resulted as a natural hybrid between pummelo and mandarin (Barrett & Rhodes, 1976) and does not show high genetic diversity (Luro *et al*., 1995; Novelli *et al.*, 2006). A study by Uzun *et al*., (2009) found the genetic similarity of oranges, including 'Washington Navel', 'Valencia', 'Moro', 'Shamouti' and 'Pineapple', among many others, to be more than 98%, while some were genetically identical.   From seven Valencia cultivars examined, only 'Midknight' differed

genetically from the other Valencia cultivars. However, it is not disputed that 'Midknight' is closely related and probably derived from 'Valencia' (Fang & Roose, 1997). The cultivar epithet 'Midknight' itself was validly published with a description and may be considered fully established (Hodgson, 1967). It therefore seems to meet every requirement to be considered a distinct, valid cultivar, but at this time there is no published, formal Group name to use when referring to various "Valencia oranges" as a cohesive assemblage of cultivars (Hodgson, 1967).

Because the original 'Valencia' orange remains a distinct variety and is reportedly still cultivated, the name 'Valencia' should not be used to denote a horticultural Group, although using a Group epithet may be the most suitable way to refer to the assemblage of cultivars commonly known as 'Valencia oranges'. The 'Valencia' cultivar was introduced from the Azores by the English nurseryman Thomas Rivers, who catalogued and sold it as 'Excelsior' in the United States of America in the 1800's. Clones purchased from Rivers were renamed 'River's Late' or Hart's Tardiff', but were later renamed as 'Valencia Late' (Hodgson, 1967). It finally became widely known only as 'Valencia', representing this cultivar worldwide. Clones are still found with the name 'Valencia Late' but, if correctly identified, may be indistinguishable from 'Valencia'. Various bud sports, nucellar seedlings and other derivatives that are different to the parent clone have received cultivar names as well and is commonly known as "Valencia oranges" (Hodgson, 1967). Reference in the title made to *Citrus sinensis* (L.) Osbeck cv. Valencia should therefore be seen as a group name like navel, lemon and grapefruit, each with their separate cultivars.

The South African citrus industry produced nearly 1.5 million tons oranges valued at about R 4 037 million in the 2010/2011 season. Of these, 135 131 tons were sold on the 19 major fresh markets across South Africa, 279 449 tons were destined for

processing and 879 950 tons exported of which 771 000 tons were Valencia and mid-season oranges (88%) from the Limpopo province (Department of Agriculture, Forestry and Fisheries, 2011).

The production area for export citrus in South Africa amounts to approximately 73 000 ha. From these orchards, a total of 51 407 000 cartons (15 kg each) of Valencia and mid-season oranges were passed for export by the Perishable Products Export Control Board (PPECB). These cartons were exported mainly to the Middle East (24%) and Northern Europe (24%) (Citrus Growers Association of South Africa, 2014).

Valencia oranges (*Citrus* 'Valencia') trees have large leaf areas. A three-year-old tree ("Campbell" strain of Valencia orange) grown under normal cultivation practices was calculated to have a total leaf area of approximately 34.44 m^2 from 16 419 leaves, and a 12-year-old tree has a leaf area of 146.09 m^2 from 92 708 leaves and a crown volume of 32.78 m^3. A 29-year-old tree has approximately 290 000 leaves (Turrell, 1961). The number of leaves on a citrus tree can be reduced by pruning and natural defoliating forces such as desiccating winds and extreme hot or cold weather conditions. For a 12-year-old tree to bear one kg of fruit, it would require 2.6 m^2 of leaf area (Turrell, 1961). It was not mentioned whether these trees were pruned as part of the normal cultivation practice. Leaves are alternately arranged, unifoliolate with pinnate-reticulate venation and can be retained on the citrus tree for more than two years (Scott *et al*., 1948).

The phenomenon of 'wet deposition', where plants acquired sulphur (S), nitrogen (N), magnesium (Mg) and copper (Cu) through rains that were enriched with these elements by industrial gases has been recorded in England as long ago as 1852.

That aboveground plant parts have the ability to absorb nutrients has been known for more than a century and foliar fertilisation has been practised for just as long (Franke, 1961; Fernandez & Eichert, 2009).

Perennial fruit crops such as citrus can benefit greatly from foliar nutrition, since deep roots can deplete the soil nutrients over time (Abd-Allah, 2006). Soil amendments (excluding fertigation) are not easily applied without damaging the roots and crop growth responses to soil applied nutrients may be too slow when correcting a deficiency. Foliar applied nutrients can be utilised much faster and can correct some deficiencies more rapidly than soil applications (Neumann, 1988). Crops respond to soil applications in five to six days under favourable climatic conditions and to foliar applications in three to four days (Fageria *et al*., 2009).

Foliar nutrition can be advantageous during phenological stages such as flowering and fruit development where the percentage fruit set can increase with foliar applied boron (B) (Abd-Allah, 2006). Foliar fertilisation can also be an economical method of nutrient supply, especially when micronutrients are mixed and sprayed with compatible fungicides and insecticides (Kannan, 2010; Fageria *et al*., 2009; Rashid, 2006). Foliar fertilisation may be especially beneficial if the nutrients are phloem-mobile, because smaller amounts of the nutrient can be applied to leaves instead of large amounts to the soil. The nutritional status of the fruit crop and the quality are also enhanced by foliar fertilisation. However, foliar feeding should not be seen as a practice that replaces the soil application of fertilisers, but rather as a supplementary measure (Kannan, 2010).

1.1   Problem statement

Differences in formulations of foliar applied solutions affect penetration of foliar applied nutrients (Fageria *et al*., 2009). The response from foliar sprays is very inconsistent due to a lack of knowledge of the many factors related to foliar applied solutions (Fernandez & Eichert, 2009). Because plants only need small amounts of micronutrients, foliar application of micronutrients is not only used when plants are deficient, but it is also applied preventatively as part of planned production practices, especially for elements that are less mobile in the phloem and cannot be transported as readily in the plant tissue like calcium (Ca), boron, iron (Fe), manganese (Mn) or zinc (Zn). Applying nutrients foliarly can also be very efficient as an alternative method of nutrient application method to plants in peak nutrient demand and plants growing in soils with low nutrient availability (Fernandez & Eichert, 2009). Additionally, foliar applications allow farmers to apply other agrochemicals in the same spray operation, saving time, energy, labour and costs.

Different formulations for micronutrients exist. These formulations include amino acid chelate (AA) and chelate or inorganic (SO_4^{2-}, NO_3^-, Cl^-, etc.) complexes and, when these complexes are used as foliar sprays, more information regarding the following is needed to determine maximum uptake of foliar applications:

i.   Differences in leaf surface, leaf structure and leaf age between different citrus cultivars.

ii.   The highest element uptake for the concentration range applied to the leaves.

iii.   The influence of contact time between the micronutrient solution and leaf surface for highest element uptake of each micronutrient formulation applied to the leaves.

iv.   The most effective and most cost effective formulation for micronutrient uptake in citrus.

1.2   Hypothesis

Leaf surface and cuticle thickness varies for different citrus groups (lemons, grapefruit, Valencia, navels and mandarins) and leaf age (Tukey, 1970, Bondada *et al*., 2001). The uptake of foliar applied micronutrients on non-bearing potted 'Midknight' trees is influenced by:

i.   Formulation. The AA formulated micronutrients are the most efficient but least cost-effective formulation.

ii.   Application concentration. Micronutrient uptake is concentration dependent with the highest concentrations of each micronutrient for each formulation resulting in the highest uptake.

iii.   Contact time between the micronutrient and leaf surface. A longer contact time will result in higher quantities of the respective micronutrients being absorbed by the plant.

Data obtained from these experiments will indicate which micronutrient formulation, application concentration and contact time will result in the highest uptake in the leaves of *Citrus sinensis* 'Midknight'.

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1.3   Aim and objectives

Scanning electron microscope (SEM) images of the cross section and surfaces of different age leaves from lemons, grapefruit, Valencias, navels and mandarins will be sampled and the cuticle thickness evaluated. Potted, non-bearing *Citrus sinensis* 'Midknight' trees on Carizzo Citrange rootstock will be treated with different formulations (AA, chelate, sulphate) of Cu, Fe, Zn, Mn, B (only boric acid) and molybdenum (Mo) (only sodium molybdate) at five different concentrations and the nutrient concentration of the treated leaves as well as the leaves above and below the treated leaves will be measured after different contact times to determine the:

i.   Highest element uptake for the concentration range applied to the leaves.

ii.   Contact time needed between the micronutrient solution and leaf surface for highest element uptake of each micronutrient formulation applied to the leaves.

iii.   Most efficient and cost effectiveness of different formulated micronutrients applied foliarly.

Chapter 2　　Literature study

2.1　　Introduction

Mengel & Kirkby (1987) stated that living plant material consists of 70% water, 27% organic matter and 3% minerals.　Although mineral elements only occur in such relative small quantities, they are essential for the production of organic matter. Essential elements have been defined as those elements which fit the following criteria: (1) the element is required for the plant to complete its life cycle, (2) the function of the element cannot be replaced by any other mineral element and (3) the element must be directly involved in plant metabolism (Arnon & Stout, 1939).　The 17 essential plant nutrients have been divided into macronutrients (carbon (C), hydrogen (H), oxygen (O), N, phosphorus (P), potassium (K), Ca, Mg and S) and micronutrients (Zn, Cu, Fe, Mn, B, Mo, chlorine (Cl) and nickel (Ni)) (Brady & Weil, 2002).　Essential element concentrations in plants are expressed as percentages or gram per kilogram ($g.kg^{-1}$) for macronutrients and parts per million (ppm) or milligrams per kilogram ($mg.kg^{-1}$) for micronutrients.　These concentrations will vary with growth conditions, plant species, time of sampling and nutrient accessibility (Fageria *et al*., 2009).　Only minute quantities of micronutrients are needed, maximum 0.1 $kg.ton^{-1}$ of fresh product, but these are just as important for plant growth as macronutrients (Fageria *et al*., 2009).

Plant nutrition can be defined as the totality of the relationships which the plant has with chemical elements and compounds and which occur both within the plant and at its interface with the external environment (Martin Prevel *et al*., 1984).

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Nutrients can be supplied to plants via soil and foliar applications. Soil application is most commonly used to supply macronutrients, which are absorbed by the roots and mainly translocated via the xylem to the above ground plant parts (Fageria *et al.,* 2009). Micronutrients are commonly supplied via foliar application, since higher plants are also able to absorb nutrients when applied to their leaf surfaces in appropriate concentrations (Fageria *et al.,* 2009).

When a solution is deposited on the leaf surface via foliar application, droplets rest on the waxy cuticle and need to penetrate the cuticle proper (a semi-hydrophilic cutin layer) (Figure 2.1). The solution then penetrates the pectin layer, which is composed of polysaccharides, after which it can move through the cell wall (Mengel, 2002). The solution then moves through the plasma membrane into the free space of the cell and can then be translocated to other cells via the plasmodesmata (channels connecting adjacent cells) and into the xylem and phloem. The penetration into the free space of the cell and translocation between cells are largely driven by diffusion and to a lesser extent by mass flow (Mengel, 2002).

Once these elements have been taken up by the plant, they may be chelated inside the plant. How efficiently the plant uses the applied nutrient once it is inside the plant, depends on solute translocation in the plant which occurs through either 'apoplastic movement' or 'symplastic movement' (Mengel, 2002).

Apoplastic movement is the passive movement of solutes exclusively through the cell wall without crossing over any plasma membranes, and usually involves the xylem (Flowers & Yeo, 2007) (Figure 2.2). The apoplast is the continuous system of cell walls and intercellular air spaces in plant tissues. In the apoplast, solute transport is driven by diffusion and mass flow. When transported via mass flow,
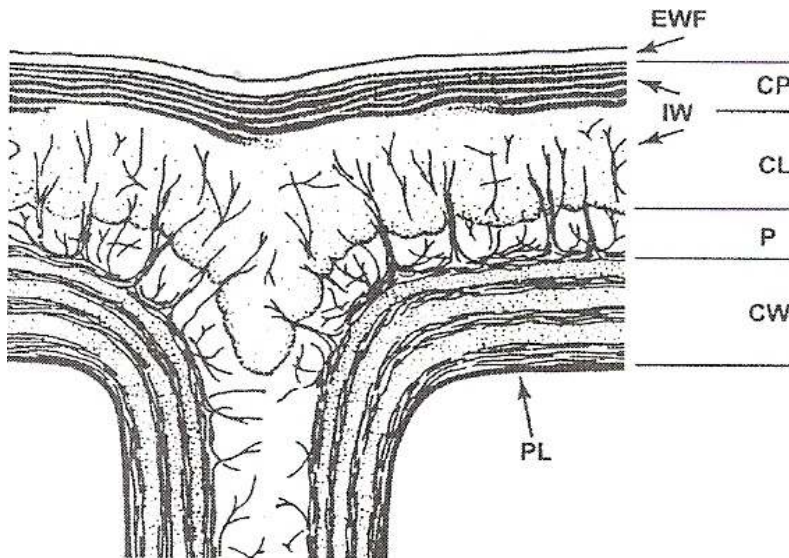
Figure 2.1: Schematic cross-section of the outer parts of a generalized plant epidermis devoid of wax crystals (CP, cuticle proper; CL, cuticular layer; CW, cell wall; EWF, epicuticular wax film; IW, intracuticular waxes; P, pectinaceous layer and middle lamella; PL, plasma membrane) (Jetter *et al.*, 2000).

solutes move in conjunction with transpiration water, which may aid the distribution of solutes in the leaf tissue. However, this may also cause a significant loss of water from the apoplast, and as a consequence limit diffusion (Mengel, 2002). The nutrient concentration gradient between the apoplast and the solutes sprayed on the leaf surface greatly determines the diffusion rate of the solutes into the symplast. The cell membrane has many transporters and channels (uptake systems) for certain nutrients and a constant gradient is created to propel nutrients from the apoplast to the symplast. This happens especially when the nutrient is in demand and the uptake of that nutrient is not limited by low temperatures (Mengel, 2002).

The symplast consists of the entire network of cell cytoplasm interconnected via plasmodesmata (Mengel, 2002). Plasmodesmata connect adjacent protoplasts through cylindrical pores (microscopic channels) in the cell wall with diameters of 20-60 nm and allow small molecules, water and nutrients, to freely diffuse from one cell

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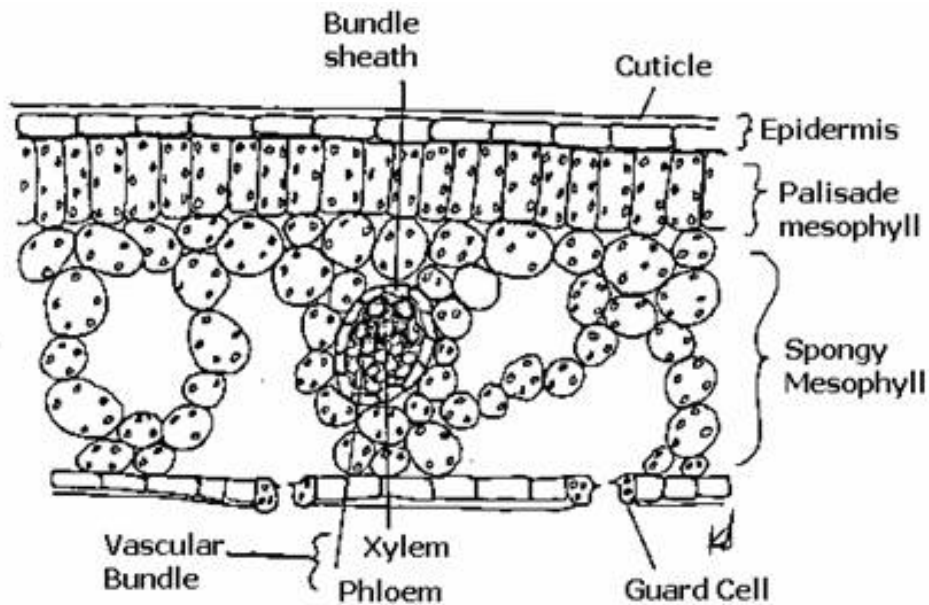
Figure 2.2: Diagram of cross section of a leaf (Wizznotes, 2011).

to another. Nutrients are also transported over long distances in the phloem tissue by first entering the sieve tube-cells or the companion cells through the plasmodesmata. This is mainly relevant for the nutrients which are phloem-mobile (Mengel, 2002). Larger molecules, such as proteins, will not be able to pass through the plasmodesmata. These plasmodesmata aid in transport and cell-to-cell communication (Mengel, 2002).

The symplastic pathway is of greater importance where foliar nutrition is concerned. When some nutrients in the plant become deficient, it is transported from older to younger leaves e.g. N, P, K, Mg, Mo, Zn and occasionally B (Brown & Shelp, 1997; Mengel, 2002). Nutrients which are not translocated from older foliage include Ca, S, Mn and Cu and are not as mobile in the phloem (Mengel, 2002).

Phloem-mobility of nutrients is an important consideration when treating nutrient deficient plants. Phloem-mobile elements are said to move independently down

concentration gradients from sources to sinks (from mature leaves to immature or non-photosynthesising plant parts) (Weinbaum, 1988).

The foliar spray application process consists of interconnected factors that influence its uptake, such as active ingredient formulation, spray solution atomization, spray transportation to the leaf surface, droplet size, the retention and spreading on the surface of the leaf, formation of residues on the leaf surface and leaf penetration/ uptake (Brazee *et al.,* 2004; Fernandez & Eichert, 2009). Demand and potential for adequate nutrient supply through foliar applications varies among essential nutrients, and may be influenced by cropping intensity, tree vigour and the nutrient status of the plant (Weinbaum, 1988).

Leaf immersion is another foliar application method which can be used with success in foliar uptake studies (Jyung & Wittwer, 1964). When immersing leaves, the external environment is consistent for the entire leaf and the solution concentration is also maintained. Humidity, transpiration and translocation complications are minimised and temperature is better controlled, so that less variables remain which increases the reproducibility of the experiment (Jyung & Wittwer, 1964). Although Kaindl (1961) stated that immersing or dipping leaves into solutions can change the epidermal structure, or interfere with normal gas exchange, Sacher (1959), showed for leaf slices of the terrestrial species *Mesembryanthemum* spp. and *Rhoeo discolor* that their permeability integrity are retained, even after five days of immersion, since the air spaces were not waterlogged and cells were still able to undergo plasmolysis and deplasmolysis. Jyung & Wittwer (1964) could reproduce foliar uptake results for the first time using the leaf immersion technique.

2.2 Fick's law and factors influencing foliar uptake

Cuticular penetration, following a foliar application, is the diffusion from an aqueous donor (applied solution) to an aqueous receiver (the apoplast) across the cuticle (membrane) (Bukovac & Petracek, 1993) and therefore Fick's first law, that states that diffusion across a membrane is proportional to the concentration gradient that exists over that membrane, is applicable (Flowers & Yeo, 2007). The concentration of a leaf applied compound in the epidermal apoplast will depend on physiological factors such as how mobile that compound is in the plant phloem and rate of uptake through diffusion by the epidermal and mesophyll cells, according to Fick's law (Fernandez & Eichert, 2009). Factors that influence the elements in solution will also play a role, since nutrient uptake from gaseous states or solid states are restricted to a few elements (three) or not possible (Mengel, 2002). Since very small amounts (15-20%) of total foliar applied nutrients penetrate the leaf surface (FSSA, 2003), the concentration range of leaf applied solutes needs to be much higher than those of soil applied solutes to be effective (Fernandez & Eichert, 2009). This is because a small amount of the applied product is absorbed by the leaf and increased foliar uptake needs to be facilitated with high application concentrations. However, too high concentrations may damage leaves and nutrient uptake can actually decrease. This risk may always be present when working with foliar applications.

2.2.1 Environmental factors

The behaviour of the spray solution on the leaf surface, foliar uptake and distribution processes in the plant are affected by environmental factors such as temperature and relative humidity (Ramsey *et al.,* 2005). A continuous interaction between these

factors is present under field conditions, so that predictions on uptake and effectiveness of foliar sprays are difficult (Fernandez & Eichert, 2009). According to Bukovac & Petracek (1993), diffusion of applied solutes through the cuticle will only be possible for 30-45 minutes under field conditions, since uptake depends on factors that affect drying time, such as type of additives, applied solution volume and environmental conditions.

Temperature can be a determining factor when considering water or solute penetration through the leaf cuticle. In Figure 2.3 it is shown that water movement through the leaf cuticle increases with temperature for the species indicated. However, temperature should be considered together with relative humidity, since high temperatures with low relative humidity may cause leaf-applied solutes to dry on the leaf surface, resulting in less solute uptake (Fernandez & Eichert, 2009). Increased temperatures will also not induce increased solute penetration indefinitely, since too high temperatures may damage leaves. Under field conditions citrus leaves may reach temperatures of between 35 - 37.5 °C (Jifon & Syvertsen, 2001), although optimal leaf temperature for citrus is reported as 25 - 30 °C (Kriedemann, 1968), so that reduced solute penetration may be expected at temperatures above optimal for specific species. Organic solutes may penetrate the cuticle faster when temperatures increase (Riederer & Schreiber, 2001).

According to Midwest Laboratories (1994), the best time for applying foliar fertilisation is in the early morning (before 9 AM) or the late afternoon (after 6 PM) when meteorological conditions favour tissue permeability.

Relative humidity influences foliar uptake by influencing the rate of cuticular hydration and the evaporation rate of foliar applied compounds, which impacts

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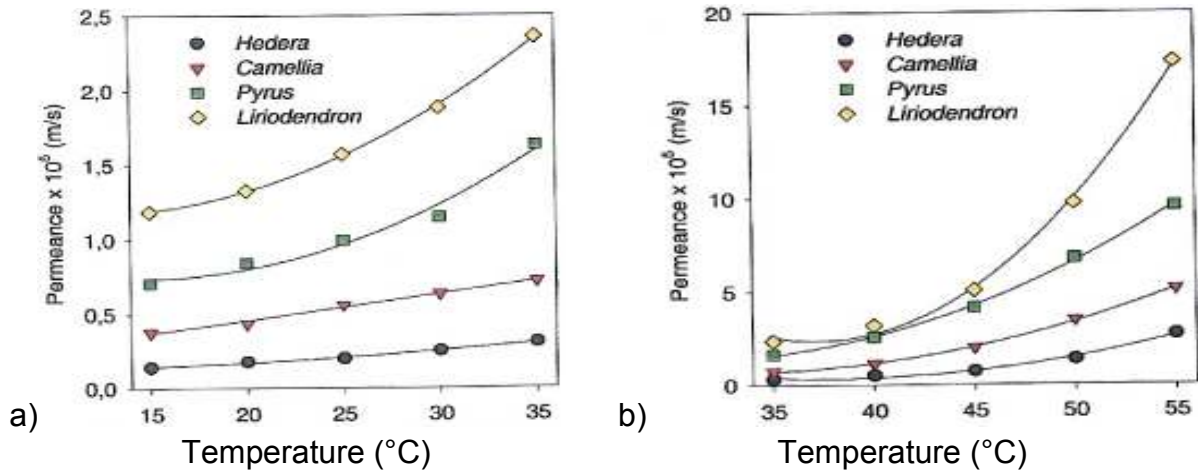
Figure 2.3: Influence of temperature on the permeance of water through the leaf cuticles for selected plant species. Low (a) and high (b) temperature ranges are shown for leaf cuticles from *Hedera helix, Camellia sinensis, Pyrus communis,* and *Liriodendron tulpifera* (Riederer & Schreiber, 2001).

diffusion into the leaf that can only occur when nutrients are in liquid state. Relative humidity should not be too high or low when foliar application is made so that the solution does not dry out too quickly and is then not taken up by the plant. The hydration of cuticles decreases when air humidity decreases which reduces the permeability of the hydrophilic solutes. The water content of the cuticle also increases sharply when relative air humidity approaches 100% (Fernandez & Eichert, 2009).

Wind not only plays a role in reducing the time for an element to stay in solution but may also cause spray drift which can result in uneven foliar coverage of foliar applied micronutrients. It is also important that when foliar applications of micronutrients are made a sufficient period of no rain is needed to be effectively absorbed by the leaves (Fageria *et al*., 2009).

2.2.2 The use of adjuvants

Adjuvants are substances added to the spray solution that improves the performance of the primary sprayed product and which may affect the driving forces of foliar uptake (concentration difference between the donor and receiver solutions), cuticle permeability as well as the mobility of solutes in cuticles. Solute adhesion and leaf coverage can be increased by adding adjuvants that enhance the physical-chemical characteristics of the applied solution, including pH, surface tension (increased wettability) or reducing evaporation from leaves. Adjuvants used in foliar applied solutions include wetter-spreaders (improves wettability of the leaf surface), stickers (builders and extenders), stabilisation agents (UV filters) and pH buffers (Midwest Laboratories, 1994).

The isoelectric points (pH at which a certain peptide has no net charge) of plant cuticles is approximately three, so that pH fluctuations will change the ion exchange capacity of cuticles. A cuticle will have a negative charge if it has a pH above three and will therefore be selectively permeable to cations when a solution is applied to the leaf. On the other hand, a cuticle will be positively charged if it has a pH lower than three and will then be selectively permeable to anions (Schönherr & Huber, 1977). Buffering agents are added to the foliar applied solutions to minimize the effect of pH on the uptake of the applied elements (Midwest Laboratories, 1994).

Water adhesion to the leaf is reduced by surface roughness and the extent of the leaf's hydrophobicity. This may also increase the angle between leaf surface and water droplets. Tukey (1970) found that wetting of citrus leaves were problematical due to the smooth waxy cuticle but were less susceptible to nutrient leaching by rain as a result. The leaf wettability challenge is addressed by adding surfactants and

stickers to the sprayed solution. Surfactants decrease the angle between the spray droplets and the leaf surface, so that the leaf area in contact with the applied solution increases and therefore also possible uptake (Schönherr & Bukovac, 1972). Stickers increase the adhesion of spray droplets to the leaf surface and reduce runoff of the spray solution (Midwest Laboratories, 1994), while humectants increase the spray drying time, allowing longer time for uptake (Fernandez & Eichert, 2009).

2.2.3 Molecule size of the solute

Molecular sizes may influence the uptake of foliar applied products due to molecule mobility, pore polarity and size and restrictions by cuticular spaces (Fernandez & Eichert, 2009).

Uptake of foliar applied solutes is affected by the molecule size of the solute since cuticles contain pores of certain maximum sizes and molecules therefore penetrate selectively (Figure 2.4) (Fernandez & Eichert, 2009). Larger molecules have a much smaller relative mobility than molecules with a smaller molecular mass. Mobility is also influenced by the hydrophobicity of the molecule (Figure 2.4).

Schönherr (1976) observed the exclusion of large hydrophilic solutes from cuticular membranes due to size and took it as evidence that pores within the cuticles are polar. These polar pores are generated through water molecules that adsorb to polar entities in the cuticular membrane (Schönherr, 2000). However, polar pores are not visible due to unachievable microscopic resolution as yet and Schreiber (2005) has stated five points in argument favouring polar pore existence: penetration of ions is reported independent of (i) temperature and (ii) plasticisers (iii) and weakly

affected by wax extraction, (iv) is affected by humidity and (v) is reported as less size selective than the lipophilic path. This assumes that the size of permeating molecules will be limited to the cross sectional area of the water clusters moving from the leaf surface through the cuticle.

The hydrocarbon network of the cutin matrix, in which the lipophilic molecules diffuse, functions as a molecular filter so that molecules too large for the cuticular spaces will be prevented from this pathway (Luque *et al*., 1995). A rather broad range of polar pore sizes, 0.3 to 2.4 nm, have been published, although this may be due to the methods used or differences in plant species. Pores with a radius of 1 nm would be able to let molecules such as sucrose and chelated micronutrients through, but pores with a radius of 0.3 nm would not let these molecules through (Fernandez & Eichert, 2009).
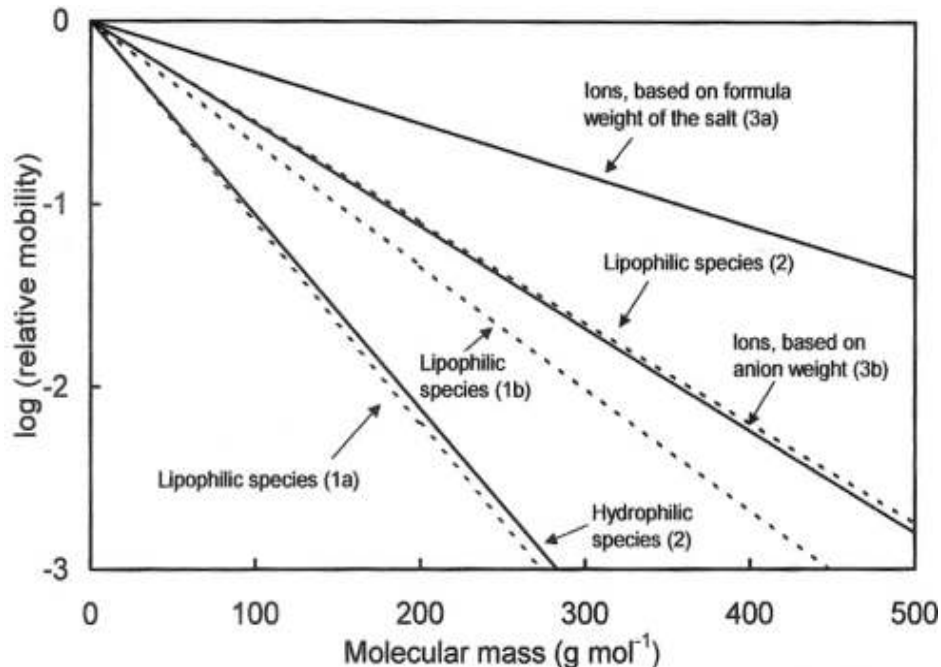
Figure 2.4: The correlation between the molecular mass of hydrophilic (solid lines) and lipophilic (broken lines) solutes and their relative mobility over astomatous isolated cuticles of different plant species *(Populus alba, Hedera helix, Populus canescens)* from different studies (1, 2 and 3). Mobility of molecule with zero molecular weight was set to zero for comparability (Fernandez & Eichert, 2009).

2.2.4 Leaf structure and leaf surfaces

Over the years leaves have evolved to create stomata for gas exchange between the atmosphere and the plant and a barrier in the form of a cuticle to prevent water loss. Air is easily diffused into the spongy mesophyll via the open stomata. It can then move as CO_2 and O_2 across the plasma membrane into the cytosol. For water soluble nutrients sprayed on the leaf surface, the barriers to overcome before entering the cytosol are more complex (Mengel, 2002) (Figure 2.1).

In Figure 2.1 a schematic illustration of the outer parts of the leaf epidermis is given. The outer surface of the leaf, including stomatal pores and epidermal trichomes (epidermal hairs) is covered by a cuticle (Kannan, 2010), under which is situated the primary cell wall. Underneath this lies the plasma membrane and cytosol (Franke, 1967). Solutes need to cross the cuticle, which poses a significant barrier for hydrophilic solutes, where after it can move into the apoplast (free space of the cell wall). This space is largely filled with atmospheric gases and not, as in the case of roots, with water (Lohaus *et al*., 2001). Diffusion of solutes can only be facilitated by the water-filled part of the apoplast and this diffusion is restricted further due to a large portion of this water being bound strongly to other cell wall structures (Mengel, 2002). Once in the symplast, solutes move through the palisade and spongy mesophyll cells via plasmodesmata to enter the vascular bundle, containing the xylem and phloem (Mengel, 2002).

The controversy regarding the uptake mechanisms of solutes through stomatal pores has been a long standing debate (Fernandez & Eichert, 2009). Although it was earlier believed that solutes cannot enter the stomata (Adam, 1948; Schönherr & Bukovac 1972), more recent results provide evidence for penetration of solutes by

open stomata (Eichert & Burkhardt, 2001; Eichert *et al*., 2008; Burkhardt *et al*., 2012; Singh & Khan, 2012). However, in practice, foliar applications are frequently made under conditions of high humidity and when evaporation of applied droplets is low. Under such conditions most stomata are closed, so that applied solutes have to penetrate the cuticle (Mengel, 2002). Penetration of foliar applied solutes can be influenced by guard and accessory cells of stomata as preferential entry sites for especially organic molecules (Franke, 1967).

Epicuticular waxes

According to Buschhaus & Jetter (2011), plant cuticles consist of two components, cuticular wax and cutin, and are differentiated according to their solubility in organic solvents. Cuticular wax can be dissolved with a lipophilic solvent, such as chloroform or hexane, while cutin is not extractable by lipophilic solvent, as it has a polymer structure of hydroxylated fatty acids and glycerol. Cuticular wax can further be divided into epicuticular waxes and intracuticular waxes. Epicuticular waxes, in the form of rodlets or other wax crystalline structures (Nawrath, 2006), are defined as "hydrophobic compounds" on the surface of the leaf which can give certain leaves a grey colour (Post-Beittenmiller, 1996). Intracuticular waxes are embedded into the cutin polymer matrix (Post-Beittenmiller, 1996) and are accountable for the non-electrolyte diffusion and the barrier properties of the layer. These waxes form a tough barrier for hydrophilic molecules such as plant nutrients (Figure 2.1). Both epicuticular and intracuticular waxes can vary in thickness and composition, with different results regarding water loss (Boyer *et al*., 1997; Riederer & Schreiber, 2001).

Tukey (1970) found that the leaching of nutrients from citrus leaves due to rain is restricted, because the leaves have a smooth waxy cuticle that is difficult to wet. Young leaves have an almost unbroken cover of epicuticular waxes. When leaves become older, the wax layer may crack and develop deletions, become physically damaged by wind abrasion and microbial action. These areas on leaves can become ideal sites for foliar nutrients to penetrate leaf surfaces (Fernandez & Eichert, 2009) and Mengel, (2002) found that nutrients, which are applied foliarly, will enter the cuticle easier as the leaves gets older.

Cuticle structure

The cuticle is mainly composed of cutin and wax plates fixed in the frame work of the cutin. According to Mengel (2002) the cutin is "a polymeric network of C_{16} and C_{18} hydroxy fatty acids in which the carboxylic group is esterified with the hydroxyl group". The sites of esterification are hydrophilic (-CO-O-) and allow hydrophilic solutes to diffuse across the cutin layer to enable contact with the primary cell wall. Therefore cutin is not considered an important barrier for hydrophilic solute penetration (DiTomaso, 1999). However, lipophilic compounds can also, after moving through the epicuticular waxes, penetrate the cutin readily, due to the presence of embedded waxes in the cutin.

Two features can be distinguished in cuticles, the first being wax crystals that extend beyond the epicuticular layer into the atmosphere and can vary extensively as shown with SEM images (Buschhaus & Jetter, 2011). The second feature is the nano-scale lamellae that are situated in the intracuticular layer, as documented by TEM images (transmission electron microscopy) (Buschhaus & Jetter, 2011).

Cell wall structure

The primary cell wall (Figure 2.5) consists of a matrix of peptic polysaccharides (Thimm *et al*., 2009) with pectins (hetropolysaccharides) interacting with the surface of cellulose microfibrils, and with xyloglucans (XG). Only a limited number of XG segments are incorporated into the microfibrils, giving XG partial rigidity and heterogeneous dynamics (Dick-Perez *et al*., 2011).
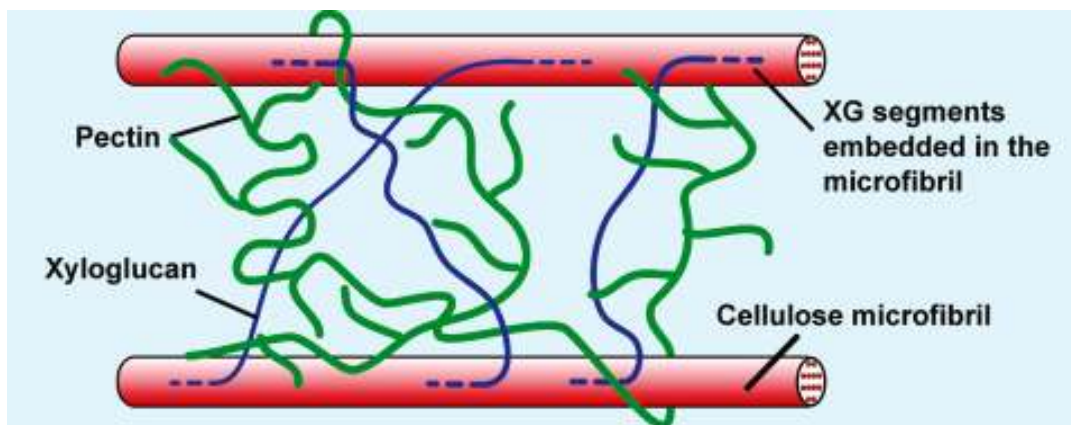
Figure 2.5: A new structural model of plant cell walls. Green lines indicate pectin chains, solid blue lines indicate xyloglucans and broken blue lines indicate embedded xyloglucans. Red tubes represent cellulose microfibrils (Dick-Perez *et al*., 2011).

Three major classes of polysaccharides are present in the primary cell wall: (1) cellulose, whose microfibrils make up the rigid framework of the cell wall, (2) hemicellulose, consisting of mainly XG, (3) pectins which include homogalacturonan (HG) and rhamnogalacturonan. The three types of polysaccharides interact in one network (Dick-Perez *et al*., 2011).

Cell walls also include structural and glycoproteins; the primary cell wall components are therefore predominantly hydrophilic (Dick-Perez *et al*., 2011). Polar and non-polar molecules are able to penetrate the cellulose. However, compounds with sizes

of 700 mw (molecular weight) and larger will not be able to diffuse through the cell wall (DiTomaso, 1999).

The primary cell wall's porosity comes from the pectic matrix that contains pores with a diameter of ±5 nm. If one considers that K^+, Ca^{2+} and sucrose have diameters of 0.66 nm, 0.82 nm and 1.0 nm respectively, the pectic matrix may represent a barrier for larger molecules such as chelated compounds. The pectic matrix also contains indiffusible anions forming Donnan sites that can trap cations, particularly Ca^{2+}. Two rhamnogalacturonan strands bridged by borate esters may also form Donnan sites (Carpita *et al*., 1996).

The secondary cell wall, if present, lies between the primary cell wall and the plasmalemma; it is mainly cellulose, impregnated with other compounds such as lignin embedded therein. This cell wall is not considered an obstacle for hydrophilic solutes (Mengel, 2002).

According to Schreiber *et al*., (1996) the foliar uptake mechanism of organics from diluted aqueous solutions may be differentiated into four consecutive steps: (1) surface desorption, (2) dissolution in cutin and/or wax, creating the transport limiting barrier, (3) movement over the transport limiting barrier and flanking polymer matrix through diffusion and (4) solute desorption or transfer from the cuticular membrane into the apoplast of the cell wall. This foliar uptake mechanism supports the biphasic uptake kinetics, which consists of surface adsorption (of the organic chemicals to the interface between the waxy leaf surface and the aqueous solution) and cuticular penetration (through diffusion over the cuticle's waxy transport barrier) (Schreiber & Schönherr, 1992). Schreiber *et al*., (1996) then concluded that the total applied amount of lipophilic solute, it being a xenobiotic compound or agrochemical, does

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not necessarily add up to the same amount entering the mesophyll. After uptake has commenced for a short period of time, the leaf surface will still contain the majority of the substance. However, after a longer period the larger part of the substance will have diffused into the leaf interior.

Leaf cuticle thickness, weight and wax content of *Citrus sinensis* 'Valencia' leaves has been measured by Leece (1976), where cuticle thickness was found to be 4.2 ± 0.1 μm for the adaxial cuticle, and 3.9 ± 0.2 μm for the abaxial cuticle (Table 2.1). The cuticular membrane is less of a barrier to foliar uptake than the waxes, and the surface waxes, which form a continuous layer over the leaf surface, constitutes a more formidable barrier than the embedded waxes. Foliar applied products tend to penetrate better through the abaxial than adaxial surfaces of leaves since the abaxial surface contains more preferred sites of entry, such as guard and accessory cells, as can be seen in Figure 2.6 (Leece, 1976).

Epidermal cells are multilateral in outline and rather uniform in size. The external walls are cutinized and covered with wax. These waxes are secreted by minuscule canals not unlike those found in the rind of citrus fruit. Once treated with IKI-H_2SO_4, the canals are shown to have a lining of protoplasmic threads resembling plasmodesmata. Maintenance of the waxy layer is presumably due to protoplasmic activity within these channels (Scott *et al*., 1948). Bally (1999) removed the epicuticular wax from mango fruit surface by agitating for three minutes in chloroform and then drying the chloroform solution to be able to study the cutin layer of the fruit. It was found that stomata occur on the abaxial side of the citrus leaf with no stomata seen on the adaxial epidermis (Scott *et al*., 1948; Saeed *et al*., 2010).

Table 2.1: Thickness, weight and wax content of cuticles isolated from orange leaves. Leaf area was 29 ± 2 cm^2 for orange. Values given are means ± standard error (Adapted from Leece, 1976).

Cuticle details	Orange
Cuticle thickness	**(µm)**
Adaxial cuticle	4.2 ± 0.1
Abaxial cuticle	3.9 ± 0.2
Cuticle weight	**(µg cm^{-2})**
Adaxial cuticle	448 ± 2
Abaxial cuticle	430 ± 4
Wax-free cuticular membrane	**(µg cm^{-2})**
Adaxial cuticle	387 ± 3
Abaxial cuticle	370 ± 3
Surface wax	**(µg cm^{-2})**
Adaxial cuticle	18 ± 1
Abaxial cuticle	12 ± 1
Embedded wax	**(µg cm^{-2})**
Adaxial cuticle	43 ± 3
Abaxial cuticle	48 ± 5
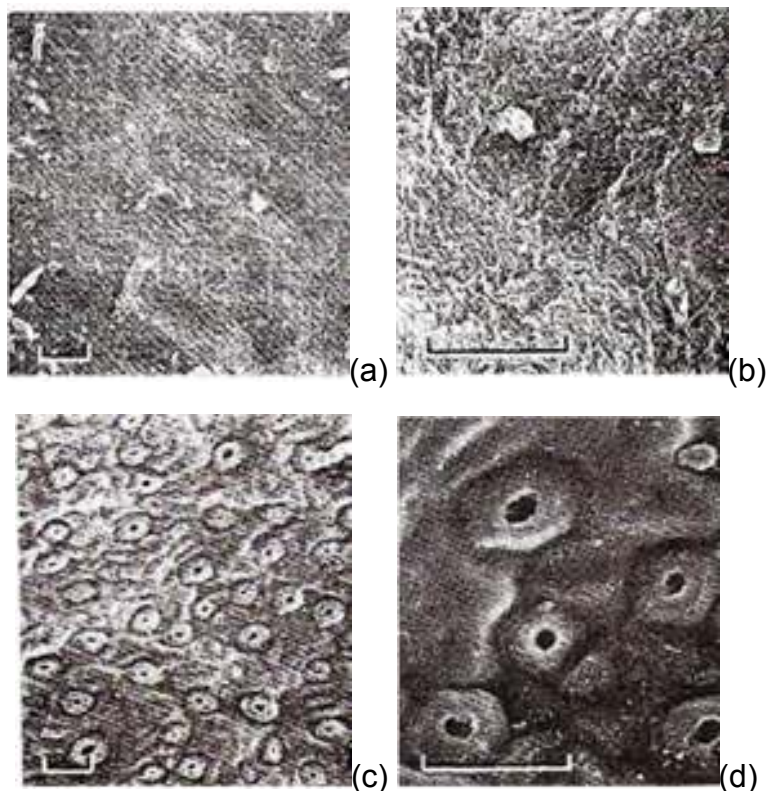
Figure 2.6: Scanning electron micrographs of adaxial (a and b) and abaxial (c and d) leaf surfaces of 'Valencia'. Scale bars 20 µm (Adapted from Leece, 1976).

2.3   Nutrient formulations and foliar uptake

Organic and inorganic ions as well as un-dissociated molecules can penetrate cuticular membranes (Franke, 1967) from where it can enter the mesophyll cells. Ion penetration through the cuticle is determined by the type of charge, ion radius and the ion's ability to adhere to the cuticle.   According to Schönherr (2001), the movement of ions into plant leaves can only be favoured by the choice of electrolyte carrier that ideally should have a low molecular weight and high solubility.

Cu, Fe, Zn and Mn sulphates are the most common used inorganic micronutrients due to favourable physical properties such as solubility (Mortvedt, 1991). Although these formulations contain plant available S, the amount of S applied to the leaves in recommended doses are so low that additional S application may be necessary (Mortvedt, 1991).

The term 'chelate' was derived from the Greek word "chela" meaning "claw" (Buckman & Brady, 1964) and a chelate is used to form a complex, usually a ring structure, with a metal cation through coordinate or ionic bonds. Chelates can be synthetically manufactured, but are also produced naturally where metal ions complexes with sugars or citric acid. According to Mengel & Kirkby (1987), Fe, Cu, Zn and Mn can often be found in the plant in a chelated form.

The most common synthetically manufactured chelate is the EDTA (ethylene-diamine-tetraacetic acid) molecule which is a polyprotic acid. There are two amine groups present with lone pair electrons as well as four carboxylic acid groups (Figure 2.7). This molecule is synthesized industrially from ethylediamine, formaldehyde and a cyanide source such as HCN or NaCN and by 2007 more than 100 000 tons were produced worldwide per year (Sinex, 2007).
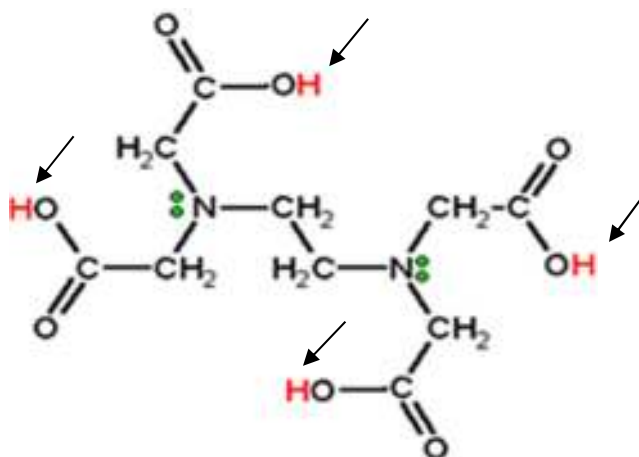
Figure 2.7: The classic structural formula of EDTA (dots indicate lone electrons, while arrows indicate acidic hydrogen ions) (Adapted from Sinex, 2007).

According to Sinex (2007) EDTA is extensively used in the food industry as a sequestering agent to remove a metallic taste, for blocking further reactions or even to stabilise foaming in commercial beer. It is also used in the medical world to treat lead poisoning and added to blood stocked in blood banks as an anticoagulant. Cu-EDTA with its blue colour is used in many shampoos and cosmetics (as disodium EDTA-copper). Since chelates are used so extensively, it has also found application in agriculture as part of fertiliser sources commonly used for foliar applications. It has been reported that non-chelated sources of micronutrients may not be as efficient as chelated sources, although chelated sources can be much more expensive and less accessible to crop producers (Fageria *et al*., 2009).

Farmers have been using AA compounds for more than 30 years, since beneficial responses have been seen on several crops. This may be due to the biostimulant action of the AA's under various stress conditions (Heuer, 2003). Sanchez-Sanchez *et al.,* (2002) hypothesised that AA's may contribute to improve Fe uptake in plants by acting as chelators that function as efficient carriers of Fe into the plants. AA's may also improve the metabolism of plants and also increase cell membrane

permeability, stimulation of H^+-ATPase and Fe^{3+}-chelate reductase activity (Cerdan et al., 2009).

According to Mortvedt (1991), few reports have been published on direct comparisons between inorganic, chelated and organically complexed micronutrients on various crop responses. Hergert et al., (1984) conducted an experiment where five different Zn compounds (ZnO, Zn-EDTA, $ZnSO_4$, $ZnSO_4$-NH_3 complex and $Zn(NO_3)_2$-UAN) were soil-applied in banded applications together with a 10-15-0 NPK fertiliser. This was done for maize on a Zn deficient calcareous soil (pH 7.8). Results show that at the lowest level (0.11 kg.ha^{-1}) Zn-EDTA was more effective than $ZnSO_4$ and complexes of $ZnSO_4$, but at higher rates (1.12 kg.ha^{-1} and 3.36 kg.ha^{-1}) of Zn, the different Zn compounds performed equally effective. Mortvedt (1991) commented on above trial, stating that Zn-EDTA may not be the most economical to apply, even if lowest rates of Zn-EDTA were the most effective since Zn-EDTA is more costly per unit of Zn.

2.4 Micronutrients

Fruit-bearing plants respond well to optimal mineral nutrient applications. This includes a positive influence on fruit quality, as well as good root, fruit and vegetative growth (Mengel & Kirkby, 1987).

2.4.1 Manganese

Even though Chesworth (1991) reported that Mn is present in the soil at approximately 600 mg.kg^{-1}. A wide concentration range of Mn exists between

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different soils and the availability is influenced mainly by pH, organic matter, soil moisture content and type of soil and primary minerals (Mengel & Kirkby, 1987).

According to Draycott & Farley (1973), when comparing soil applied Mn silicates and Mn oxides with Mn foliar sprays in Mn deficient soils, the soil treatment did not prevent Mn deficiency in sugar beet, while the foliar application succeeded in curing the deficiency and increasing the sugar yields. When Mn is applied to soils, it can be adsorbed rapidly (in minutes to seconds) to negatively charged surfaces in the soil and become unavailable to plants (Silber et al., 2009).

Ozaki (1955) stated that a sprayed solution of $MnSO_4$, rather than Mn chelates, will cure a Mn deficiency and because Mn is less mobile in the plant, more than one application might be necessary in one season to obtain desired Mn concentrations in the plant (Fageria et al., 2009).

Citrus is sensitive to Mn deficiency (Mengel & Kirkby, 1987) and in Table 2.2 the optimum as well as high and low nutrient concentrations for leaf analysis are given.

Mn resembles Mg in its biochemical functions, where it mostly has functions in activating or deactivating enzymes, but is also important in the Hill reaction of photosynthesis (Mengel & Kirkby, 1987; Taiz & Zeiger, 1991).

Mn mobility resembles also that of Ca^{2+}, in that it is poorly mobilised and has low phloem-mobility, although foliar applied Mn^{2+} can alleviate Mn deficiency symptoms (Fageria et al., 2009). In the case of Ca^{2+}, deficiency symptoms are present in the fruit and for Mn^{2+}, the symptoms appear on the leaves as interveinal chlorosis, so foliar applications will alleviate this deficiency more effectively. Deficiency symptoms may often develop in crops growing under soil conditions of high organic matter

Table 2.2: Guidelines for interpretation of orange tree leaf analysis based on four- to six-month old spring flush leaves from fruiting terminals (adapted from Quaggio *et al.,* 2010 as cited by Mattos *et al.*, 2012).

Nutrient in mg.kg^{-1}	Low	Optimum	High
B	<80	80-160	>160
Cu	<10	10 to 20	>20
Fe	<49	50-120	>200
Mn	<34	35-50	>100
Zn	<34	35-50	>100
Mo	<2	2 to10	>10

levels and high pH soils since Mn compounds are then less soluble (Mengel & Kirkby, 1987).

Fageria *et al.*, (2009) stated that at least two foliar sprays of $MnSO_4$ are necessary in the growth season on soybeans because it shows low mobility in the phloem and is not remobilised well. In a study conducted by Tariq *et al.*, (2007), leaves of sweet orange, 'Blood Red' variety, were sprayed (three times in 15 days) with combinations of 0.4 kg.ha^{-1} Zn as $ZnSO_4.7H_2O$, 0.2 kg.ha^{-1} of $MnSO_4.3H_2O$, and 0.04 kg.ha^{-1} B as H_3BO_3 in 400 L water with urea and a surfactant. The Zn concentrations in the leaves (sampled before and after spraying) increased significantly from 14.80 µg.g^{-1} to 87.33 µg.g^{-1} when Zn was sprayed without Mn and B, while concentrations of Mn increased from 49.07 µg.g^{-1} to 85.87 µg.g^{-1} when sprayed only with urea and the surfactant. When Zn was applied together with Mn, B, urea and the surfactant, leaf Zn concentrations increased from 19.70 mg.kg^{-1} to 79.30 mg.kg^{-1}, and was not significantly different from the treatment applied without Mn and B. Leaf Mn concentrations increased from 35.44 mg.kg^{-1} to 48.57 mg.kg^{-1} while B concentrations did not increase (Tariq *et al.*, 2007). A study by Embleton *et al.*, (1988) showed that Mn sprayed on 'Washington' navel leaves had an economic impact with fruit showing

less green colour on the fruit peel and higher juice percentage. Fruit from Mn treated trees also had a higher TSS (Total Soluble Solids):acid ratio and thinner peels than untreated trees.

2.4.2 Zinc

Although the Zn content of the lithosphere generally ranges between 10-250 mg.kg^{-1} (Buckman & Brady, 1964), it is widely reported from soil and leaf analysis that Zn is deficient for citrus throughout the world and in South Africa, making it the single most deficient nutrient for citrus worldwide (Srivastava & Singh, 2005b). In most soils, citrus does not absorb adequate amounts of Zn, since uptake is negatively affected by any unfavourable condition that affects the root system negatively. Zn deficiency in most soils still cannot be explained fully other than by pH dependence (Srivastava & Singh, 2005b). For Zn to be readily available to plants, a pH less than seven is preferred (Mengel, 2002).

It is widely reported that foliar application of Zn increases the Zn content of crops. In a citrus and coffee trial, $ZnSO_4$ was more efficient than chelated Zn in increasing the Zn concentration in the leaves in a quarter of the field trials (Rosolem & Sacramento, 2001). According to Modaihsh (1997) the foliar uptake and translocation of commercial chelated Zn products applied to wheat grain was not better than those of inorganic Zn salts. Fox & Guerinot (1998) stated that once Zn is inside the plant it can be stored in the vacuoles of leaf cells, thereby preventing the build-up of toxic levels of Zn in the cytoplasm. Once in the plant the final distribution of Zn is not dependent on whether Zn was supplied through the leaves or roots (Srivastava & Singh, 2005b).

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Zn exists in the plant only as Zn^{2+} and function as a divalent cation by forming tetrahedral chelates with different organic compounds and couples enzymes with related substrates. This element is necessary in the plant since it plays an important role in many enzymatic reactions and has a structural role in numerous proteins and is also significant in reducing fruit drop and granulation in citrus (Srivastava & Singh, 2005b). Srivastava & Singh, (2005b) found that citrus production decreases markedly as a result of Zn deficiency long (two to three years) before symptoms appear. Zn deficient plants show metabolic changes which may affect carbohydrates, proteins and auxins and also impair membrane integrity. Visual symptoms of Zn deficiency include rosetting, or shortened internodes, and decreased leaf expansion or 'little leaf'. Symptoms may appear in conjunction with chlorosis of young leaves (Romheld & Marschner, 1991; Taiz & Zeiger, 1991).

Boaretto *et al*., (2002) conducted a study to determine the effect of Zn foliar fertilisation on foliar Zn concentration. The conventional treatment consists of a micronutrient formulation, recommended for citrus, containing 1.32 kg.ha^{-1} Zn, 1.0 kg.ha^{-1} Mo, 0.272 kg.ha^{-1} B and 4.5 kg.ha^{-1} N and was applied biannually, at flowering and vegetative flushing. He concluded that the sprays increased foliar Zn content from deficiency to 85 mg.kg^{-1}, which is within the adequacy range of 25-200 mg.kg^{-1} for old leaves (Jones *et al.,* 1991).

Mann & Takker (1983) reported that Zn foliar sprays had no beneficial influence on yield when sprayed on leaves with a Zn content of 16 mg.kg^{-1}, although it elevated the leaf Zn content to 25 mg.kg^{-1}. However, Haribabu & Rajput (1982) showed that Kagzi lime increased flowering as a result of 2,4-D treatment alone, and in combination, when followed with Zn treatment (0.3% and 0.6% $ZnSO_4$). This also considerably reduced the period of flowering and fruit drop. Foliar application of

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0.5% ZnSO$_4$ to 18-year-old 'Darjeeling' mandarin (*Citrus reticulata* Blanco) trees resulted in higher yields than untreated trees. Sharma *et al*., (1999) reported that foliar applications of 0.4% Zn-EDTA to 5-year old seedless lemon (*Citrus limon* Burm.) increased leaf Zn concentrations from 14.3 mg.kg^{-1} to 30.3 mg.kg^{-1}, and increased fruit yield from 9.3 kg.tree^{-1} to 12.3 kg.tree^{-1}. Devi *et al*., (1997) also reported that leaf Zn concentration in *Citrus sinensis* 'Sathgudi' (sweet orange) trees increased from 11.9 mg.kg^{-1} to 45.3 mg.kg^{-1} when treated with 0.5% ZnSO$_4$. A corresponding increase in fruit yield was also observed from 12.54 kg.tree^{-1} to 30.4 kg.tree^{-1}.

Extensive research has been conducted on foliar applications of ZnSO$_4$ and Zn-EDTA. In most cases, Zn concentrations in the leaf increased with Zn application of either ZnSO$_4$ or Zn-EDTA and variation in results from these trials may be due to the different varieties of citrus used and the various conditions under which the trials were conducted.

2.4.3 Copper

Cu is present in the soil at approximately 25 mg.kg^{-1} (Buckman & Brady, 1964). More Cu is complexed by organic matter than any other micronutrient cation and Cu is also strongly adsorbed on inorganic exchange sites in the soil that limits availability for plant uptake (Mengel & Kirkby, 1987). In soils with low pH the plant availability of Cu may increase due to competition for the exchange sites by H$^+$ and the increase in solubility of Cu (Mengel & Kirkby, 1987).

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Guidelines for Cu concentrations in citrus leaves are given as 10 - 20 mg.kg^{-1} for four - six month-old spring leaves (Table 2.2) (Quaggio *et al*., (2010) as cited by Mattos *et al*., 2012). Foliar application of $CuSO_4$ has led to leaf scorching on many occasions, and this may be because Cu^{2+} ions are strongly bound to the apoplast (Mengel, 2002).

Cu is essential for many processes in plants including the electron transfer reactions in respiration and photosynthesis, cell wall lignification and superoxide radical detoxification. The reaction, $R\text{-}COO^- + Cu^{2+} \rightarrow R\text{-}COOCu^+$, where carboxylic groups of cell walls bind with Cu^{2+} is commonly the cause of restricted Cu^{2+} diffusion to uptake sites in the plasma membrane (Mengel, 2002).

Cu deficiency can be detected as vigorous growth of dark green leaves with occasionally necrotic spots, as well as unusually vigorous twig growth. Necrotic spots may first appear on tips of younger leaves and later develop to include the leaf margins. Malformed and twisted leaves may also occur (Zekri & Obreza, 2009).

2.4.4 Iron

Fe constitutes about 5% of the earth's crust in weight, but soluble Fe is very low if compared to the total Fe content (Mengel & Kirkby, 1987). Fe can be present in the soil as a chelate or in ionic form. The different inorganic soluble forms of Fe are Fe^{3+}, $Fe(OH)_2^+$, $FeOH^{2+}$ and Fe^{2+}. In soils, the solubility of Fe is lower at a higher pH (pH 7.4-8.5, KCl), so that in acid soils inorganic Fe has a higher solubility than in calcareous soils (Mengel & Kirkby, 1987).

Fe is readily absorbed when applied as FeSO$_4$, since Fe^{2+} can pass through the plasmalemma and be used in other physiological processes (Fox & Guerinot, 1998). However, Fe is trivalent when applied foliarly as a chelate, and must be reduced to Fe^{2+} before it can enter the cytosol. The reduction reaction will be inhibited when the pH of the apoplast is high. This occurs if the plant has been supplied with nitrate as the only nitrogen source (Kosegarten *et al*., 1998). Since Fe is not very mobile in the phloem, it is not taken up well by the older leaves or transported to developing leaves and should ideally not be sprayed on older leaves (Kosegarten *et al*., 1999). In herbaceous plants and citrus shoots, foliar applied Fe is translocated towards newly developing leaves by the phloem and xylem (Brown *et al*., 1965; Rediske & Biddulph, 1953). In some cases, Fe-chelates were translocated better by citrus plants than Fe-salts (Fernandez *et al*., 2005). Chelated iron (Fe-EDTA, Fe-EDDHA and Fe-EDDS) applied foliarly may be more available to the plant since chelation keeps it in a soluble form, and chelated Fe may translocate to other plant parts more readily (Ylivainio *et al*., 2004; Cerdan *et al*., 2009). Abadia *et al*., (2002), however, concluded that Fe^{2+} salts and Fe^{3+} chelates are equally effective in many cases.

Fe forms part of some metalloenzymes in the plant, such as catalase and peroxidase (Bollivar & Beale, 1996) and is important in the formation of chlorophyll and ferredoxin, which is involved in the oxidation-reduction reactions in the plant (Brady & Weil, 2002).

When Fe is applied at too high concentrations, it may damage the leaf and penetration into the leaf will also decrease (Fernandez & Eichert, 2009; Cerdan *et al*., 2009). Deficiency symptoms of Fe include interveinal chlorosis on the younger leaves due to low mobility of Fe and because Fe is required for the synthesis of chlorophyll (Taiz & Zeiger, 1991). Often leaves may show chlorosis but not be Fe

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deficient; Fe concentrations in the plant may therefore not be a good indication of its physiological Fe status (Mengel, 2002). Fe chlorosis in leaves can be linked to nitrate uptake (Kosegarten et al., 1999): when foliar applied NO_3^- is absorbed, the leaf apoplast pH increases because of NO_3^-/H^+ co-transport. When the pH rises, Fe^{3+} reductase activity is constrained, and uptake of Fe^{2+} into the cytosol is then restricted (Table 2.3). The process of Fe uptake was demonstrated by Kosegarten et al., (1999). The Fe-chelates bind to the plasmalemma, then a reduction of Fe^{3+} to Fe^{2+} follows, where the chelate complex is broken up, and Fe^{2+} is then taken up. Re-greening occurred when leaves, with sufficient Fe concentrations, were sprayed with a substance that lowered the pH of the leaf apoplast, because Fe^{3+} reductase is activated in the plasmalemma that results in Fe^{2+} to be taken up in the cytosol via the apoplast (Mengel, 2002).

Literature is not conclusive regarding an optimal Fe concentration threshold for the supply of sufficient concentrations of Fe; ranges varied between 1 - 29 mM Fe that were applied to plants (Rombola et al., 2000). Foliar Fe applications are widely applied as chelates (Ylivainio et al., 2004; Cerdan et al., 2009).

Table 2.3: Fe^{3+} reduction % in intact leaves of sunflower in relation to apoplast pH of xylem vessels (Kosegarten et al., 1999).

Xylem Apoplast pH	Fe^{3+} Reduction %
≤ 5.0 (Light)	100
5.44 ± 0.07	98 ± 5
5.92 ± 0.43	78 ± 15
6.86 ± 0.62	54 ± 20
7.71 ± 0.41	22 ± 11

2.4.5 Boron

B is an essential element which abundance is reported as 15 mg.kg^{-1} in the earth crust (Chesworth, 1991). It is reported that B is mainly present in the soil solution at pH 7 as undissociated boric acid [$B(OH)_3$] and at higher pH values as dissociated $B(OH)_4^-$ (Romheld & Marschner, 1991). B is, according to Mengel & Kirkby (1987), the micronutrient of the most importance with regards to high quality fruit yields, including citrus. In the soil B availability is limited to plants because it is not readily soluble in soil water and percolation to deeper levels in the soil is common (Brady & Weil, 2002). In addition, soil applications of $B(OH)_4^-$ can be strongly adsorbed by clay minerals at a high soil pH (Brady & Weil, 2002).

B forms strong complexes in cell walls and plays a role in the formation, lignification and stabilisation of cell walls as well as xylem differentiation. B is essential for formation of the pectic framework at the primary cell walls of meristematic tissue. It is frequently applied at flowering to enhance pollen grain germination and pollen tube elongation, especially in plants where B is transported via the phloem. Since pollination is affected positively, the percentage fruit set can increase with foliar B application (Abd-Allah, 2006). Deficiency symptoms for B are retarded growth at the terminal buds and youngest leaves or even necrosis, deformed leaf blades and shorter internodes. Citrus fruit may have a decreased pulp/peel ratio (Romheld & Marschner, 1991), with gum-soaked spots in the fruit albedo, lumpiness and hard, dry fruit (Smith & Reuther, 1949).

According to Brown & Shelp (1997), B is distributed passively to other plant parts via the transpiration stream after being absorbed as a molecule. B is relatively mobile in the apoplast and may therefore be effective as foliar application (Mengel, 2002).

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According to Neumann (1988), B is translocated readily in the xylem but not necessarily in the phloem and this means that developing leaves may be exclusively dependent on xylem for incoming supplies of B, together with Ca and nitrate (NO_3^-) (also classified as relatively phloem immobile). B mobility differs widely among plant species and some plants may distribute B much better than others (Brown & Shelp, 1997). This difference in mobility is species dependent, where species that use sorbitol as a primary translocated photosynthate will render the foliar applied B more mobile in the plant. In a study on the phloem sap composition of *Citrus sinensis* 'Pineapple' (sweet orange) sorbitol was not found to be present in the phloem sap (Hijaz & Killiny, 2014) so that it can be assumed that B will not be relatively phloem mobile in citrus plants, but is still mobile in the xylem (transpiration stream) (Neumann, 1988).

The application of B as a foliar spray yields better results in perennial crops such as vines, nuts and fruit orchards (Martens & Westermann, 1991) and boric acid is thus commonly used in foliar applications. However, the range between B deficiency and toxicity is very narrow and toxicity symptoms such as physical injury on the fruit may appear that will decrease its marketability (Chutichudet & Chutichudet, 2009). Abd-Allah (2006) sprayed boric acid (300 mg.kg^{-1}) in different mixtures with K_2HPO_4 (1%) and calcium chelate (0.5%) on navel oranges at full bloom. Although Ca and B concentrations in leaves were not reported, he presence of boric acid, regardless of concentration, with K_2HPO_4 increased the N, P and K content of the leaves when compared to the control treatment. Two treatments containing boric acid (boric acid + K_2HPO_4 and boric acid + calcium chelate) also increased fruit set per branch in the first two seasons and fruit weight in the first season, while the boric acid + calcium chelate treatment yielded the highest number of fruits per tree.

In some citrus, foliar applied B may result in a thinner peel but a larger fruit diameter (Foroughi et al., 1973). In a study by Dong et al., (2009) on $Citrus$ $sinensis$ 'Cara Cara' navel trees, it was found that B, when sprayed on the foliage, increased the fruit quality by increasing total dietary fibre and restrained gene expression and activities of polygalacturonase, pectinesterase and β–galactosidase which regulates dietary fibre content and enhances the tissue structure of the segment membrane. The most efficient applications were made with two different treatments, the first containing 1 g.kg^{-1} B + 2 g.kg^{-1} Ca and the second treatment containing 2 g.kg^{-1} B + 1 g.kg^{-1} Ca. The quality of citrus fruit may be enhanced with foliar applied B (Mengel & Kirkby, 1987), as well as pollination and fruit set of the crop (Abd-Allah, 2006).

Chutichudet & Chutichudet (2009) conducted an experiment comparing foliar sprays of borax ($B_4O.2Na.10H_2O$) and boric acid ($B(OH)_3$) at four concentrations (0, 0.0625, 0.125 and 0.1875%) on lettuce. The plant height and bush size – the most important parameters – increased at concentrations of 0.0625% boric acid and B application at higher rates had detrimental effects on lettuce growth. However, crop responses to B fertilisation were inconsistent due to different soils and other environmental factors during foliar application (Martens & Westermann, 1991).

2.4.6 Molybdenum

According to Swaine (1955) most agricultural soils contain between 0.6-3.5 mg.kg^{-1} Mo, of which approximately 0.2 mg.kg^{-1} is available, but values may vary according to primary minerals and soil forming processes (Srivastava & Singh, 2007). The adsorption of Mo to soil particles will decrease as the pH increases, since it exists as an anion. This results in increased Mo availability with higher pH (Stout et $al.,$ 1951).

Mo is absorbed by plants in the form of molybdate (MoO_4^{2-}), and may be taken up by the plants in excess without showing toxicity symptoms. Mo is needed in the plant as components of two major enzymes, nitrate reductase and nitrogenase. Where other nutrient elements are accumulated in the veins and midribs of leaves, Mo is collected in interveinal areas of the leaf (Stout & Meagher, 1948).

Mo deficiency symptoms include older leaves showing interveinal chlorosis and necrosis (Taiz & Zeiger, 1991) and is commonly called yellow spot. Symptoms may develop as water soaked spots on citrus leaves early in summer, and the characteristic bright yellow spots only on older leaves after the summer growth flush. Citrus fruits may show large spots resembling sunburn when Mo deficiency is very severe (Srivastava & Singh, 2007). Deficiencies are usually seen on acidic soils and are easily cured by spraying sodium molybdate or ammonium molybdate on the plant's foliage (Zekri & Obreza, 2009).

2.5   Economic aspects of foliar sprays

The ultimate objective of citrus nutrient management is to optimise financial returns for the producer, while minimising the environmental impact due unnecessary and excessive applications. Usually the cost of actually applying nutrient sprays in the orchard is not high, since applications are combined with other regular foliar treatments (Abadia *et al.*, 2002; Weinbaum, 1988). Since micronutrients requirements of plants are lower than those of macronutrients, the micronutrient fertiliser applications are approached differently to macronutrients. Two main approaches are followed: i) maintenance or 'insurance' application and ii) 'prescription' application. Maintenance applications are made whether or not the

crop requires the micronutrients and are usually not based on soil or leaf analysis results. These applications may result in over-application (wastage) of certain nutrients, but is easier to manage, since leaf and soil analyses are not required. 'Prescription' applications are only performed when soil and leaf analyses indicate that nutrient concentrations are too low for optimal production. This programme is more intense than maintenance applications since leaf and soil analyses are required, together with formulations of different fertiliser grades, for each orchard. However, the prescription approach may prove to be more economical to the producers (Mortvedt, 1991).

Chelates have been used for more than 50 years to correct micronutrient deficiencies by applying them to the soil. However, these products have become too expensive for general use on economic crops, and therefore have been mostly applied to crops that provide a good cash return to producers (Mortvedt, 1991). Other fertiliser sources have been used in countries where crop producers do not have access to these chelates or cannot justify them economically. Companies with interest in chelated nutrient sources have been known to emphasise the constraints regarding foliar applications of inorganic nutrient sources (Abadia *et al*., 2002; Fageria *et al*., 2009). Abadia *et al.,* (2002) conducted a study on the Spanish fertiliser market, finding that Fe salts were as effective as Fe chelated compounds to increase leaf chlorophyll concentrations, but were much cheaper than the chelated products. In 2000, the Spanish market consisted mostly of synthetic chelates in liquid formulation, especially EDTA. Of the organic complexes, the AA's, lignosulphates, humic and fulvic acids were present in most products. Although a range of products were available on the Spanish market, claims on their efficiency

were not always based on solid scientific evidence and that market may well be driven by commercial interest (Abadia *et al*., 2002).

Chapter 3   Materials and methods

Three year old *Citrus sinensis* 'Midknight' trees grafted on Carrizo Citrange rootstock were used in this study. Sixty trees were transplanted into 10 L plastic containers filled with a sand:coir (1:1) growing medium mix and kept under greenhouse conditions on the Experimental farm of the University of Pretoria (25° 45' 02'' S 28° 15' 22'' E). Each tree received 200 ml of Hoagland nr.2 solution each, prepared without micronutrients, shortly after transplant. The growing medium was analysed for physical and chemical properties and results are given in Table 3.1. Trees were irrigated every other day and received 7 g limestone ammonium nitrate (LAN) containing 28% N every three months during the experimental period. Pests were controlled as needed with 350 g.L^{-1} imidacloprid (chloronicotinyl) and applied as recommended by the manufacturer. For each of the different experiments, trees

Table 3.1: Soil analysis of the growing medium used for potted 'Midknight' trees.

Bulk density	kg.m^{-3}	1489.0
pH (H$_2$O)		6.9000
CEC	cmol$_c$.kg^{-1}	6.0000
P		155.00
K		53.000
Ca		844.00
Mg		182.00
Na		34.000
Zn	mg.kg^{-1}	3.8000
Mn		0.5400
Fe		4.7000
Cu		*BDL
B		0.0900
Mo		0.0015
Ca/Mg		2.8000

*BDL: Below detection limit

were selected from the available 60 trees.

Micronutrient concentrations in leaves were determined by physical extraction of leaf sap (Mason & Phillis, 1939) or by leaf digestion (Ascher *et al*., 2009). Results from the leaf sap extraction and microwave assisted digestion (MAD) preparation methods were compared to determine the most suitable method for single citrus leaf analysis for micronutrient concentration.

3.1   Experiments

3.1.1 Comparison between leaf sap measurements and microwave assisted digestion method

It was necessary to determine whether the MAD method for wet acid digestion and analysis of plant material, as recommended by the FSSA (2003) and used widely as a standard method (Ascher *et al*., 2009; Castro *et al*., 2009; Kopsell *et al*., 2005; Hartz *et al*., 2007), would be the most suitable technique to determine if foliar applied micronutrients had entered the leaves. Results from leaf analyses using the MAD method and leaf sap extraction were compared. A hydraulic leaf sap press device (Figure 3.1) was used in this study to extract leaf sap that is present in the transport vessels and cell solutions of the leaf.

The experiment consisted of a Mn, B and control treatment at concentrations and formulations given in Table 3.4. The control, Mn and B treatments were applied and repeated on a second set of trees, so that each treatment was replicated eight times. The treated leaves and the leaves directly above and below the treated leaves were separately sampled 48 h after application. One half of the citrus leaves sampled for

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each treatment were pressed with the sap press to extract the leaf sap and the other half of the sampled leaves were analysed with the MAD method.

Table 3.2: Formulations and elemental concentrations of foliar applied elements for the comparison between sap press measurements and the MAD method.

Element	Element concentration g.L^{-1}	Formulations of element applied
Mn	0.5490	$MnSO_4$ / EDTA / AA
B	0.3497	H_3BO_3

For the determination of micronutrient concentration in plant sap, sap was collected from the citrus leaves immediately after sampling, washing and rinsing by inserting a single leaf in a hydraulic press (Figure 3.1). The release valve was then tightened and the handle pushed down to increase the hydraulic pressure until sap was forced from the leaf. Leaf sap was collected in a vial and then diluted to a standard volume of 10 ml. The diluted leaf sap samples were filtered through Econofilt AHS (Ashless Hardened Slow) Flow filter paper and analysed by ICP-AES (inductively coupled plasma – atomic emission spectrometry) to determine micronutrient concentrations.

Preparation of leaf samples with the MAD method was done by washing the sampled leaves with 10% acetone and then rinsing them with distilled water. Excessive water from the leaf surface was removed by pressing the leaves lightly with soft tissue paper before drying the leaves at 65 °C for 24 h. Dried samples were digested with HNO_3 and deionised H_2O in a microwave Multiwave 3000 (Anton Paar GmbH, Graz, Austria) at 1400 W. Digested samples were made up to a standard volume, filtered through Econofilt AHS (Ashless Hardened Slow) Flow filter paper and finally analysed by ICP-AES to determine micronutrient concentrations.
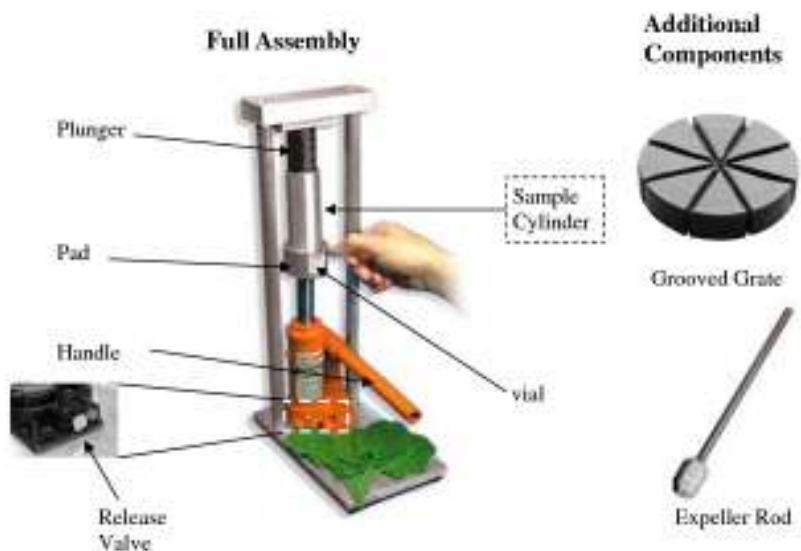
Figure 3.1: Hydraulic leaf sap press with individual components (Spectrum technologies, 2013).

3.1.2 Time of sampling

The influence of contact time between the micronutrient and citrus leaves on uptake was determined by applying different micronutrient formulations (Table 3.2), at the FSSA (2003) and AA manufacturer's recommended concentration for foliar sprays, to the leaves and sampling then at different time intervals - 12, 24, 48, 96 and 192 h. The treatments consisted of the elemental formulation and concentration given in Table 3.2. $MnSO_4$ and $Na_2MoO_4.2H_2O$ were combined in one solution, while all other treatments were applied as separate solutions. The Zn elemental concentrations used were much lower than recommended by the FSSA (2003) and AA manufacturer, because the low solubility of ZnO was brought into consideration, so that all Zn treatments contained the same amount of Zn in solution. All AA products used were specified as 'amino acid chelates' by the manufacturer. Samples were dried and digested as discussed in section 3.3.

Table 3.3: Concentrations and formulations of elements used to determine time of sampling.

Element	Element concentration (g.L^{-1})	Formulations of element applied
Zn	0.001	$ZnSO_4$ / ZnO / EDTA / AA
Cu	0.281	$CuSO_4$ / EDTA / AA
Fe	0.408	$FeSO_4$ / EDTA / AA
Mn	0.549	$MnSO_4$ / EDTA / AA
B	0.350	H_3BO_3
Mo	0.050	$Na_2MoO_4.2H_2O$

3.1.3 Maximum element uptake within the concentration range of the applied element

For each of the elements (Mn, Cu, Zn, Mo, Fe, and B) and each of their formulations (inorganic, AA and EDTA) a maximum concentration was determined for the concentration range used. This was done by applying five different concentrations, as multiples of the FSSA (2003) and the AA manufacturer's recommended concentrations for foliar sprays (FSSA, 2003), of each element and their formulations (Table 3.3). Solutions were prepared as described in section 3.2.1 except that the $Na_2MoO_4.2H_2O$ solution was prepared separate from the $MnSO_4$ solution and the Zn concentration was increased to concentrations recommended by FSSA (2003) and the AA manufacturer (Table 3.3). Since the elemental concentration for Zn exceeded the solubility of ZnO, the containers of ZnO solutions were shaken directly before the leaf was immersed into that specific solution to ensure a homogenous suspension. All AA products used were specified as 'amino acid chelates' by the manufacturer.

Treated leaves and the leaves directly above and below it were sampled and the results from the experiment on time of sampling were used as guideline for the time of sampling of the different treatments. The Cu treatments were sampled at 24 h,

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Table 3.4: Formulations and elemental concentrations of the elements used to determine application concentration.

Element	Formulations of element applied	Element concentration (g.L^{-1})				
		0.5	1	2	4	8
Zn	$ZnSO_4$ / ZnO / EDTA / AA	0.449	0.897	1.794	3.588	7.176
Cu	$CuSO_4$ / EDTA / AA	0.141	0.281	0.563	1.126	2.251
Fe	$FeSO_4$ / EDTA / AA	0.204	0.408	0.816	1.632	3.264
Mn	$MnSO_4$ / EDTA / AA	0.275	0.549	1.098	2.196	4.392
B	H_3BO_3	0.175	0.350	0.699	1.399	2.798
Mo	$Na_2MoO_4.2H_2O$	0.025	0.050	0.100	0.200	0.400

Mo and B at 48 h, Zn and Mn at 96 h, and the Fe treatment was sampled at 192 h. Leaves were dried, digested and analysed as discussed in section 3.3.

3.2   Treatment application and experimental layout

All treatments were applied in a split-split plot design within a completely randomised block design and each treatment was replicated four times (in the comparison between MAD and sap press extraction, treatments were replicated 8 times). Since trees were selected from a larger group and a split-split plot design was used, interaction was greatly reduced, and considered negligible. One micronutrient element was applied per tree but, to accommodate the different formulations (where applicable), one branch per formulation was used. A maximum of four branches on each tree were marked for the application of different formulations of the same element and a control treatment. One healthy young, fully expanded leaf from each labelled branch was selected and submersed into a solution containing the specific formulated element and removed immediately. Foliar applications were conducted in early mornings and late afternoons under greenhouse conditions during October and

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February (southern hemisphere). Since all foliar applications were conducted during the warmer months, in the same season, the possibility of biased foliar uptake due to different seasonal environmental conditions and nutrient cycling in the tree, as reported by Bondada *et al*., 2001, were minimized

Care was taken to ensure that the entire leaf surface was covered with the solution and that no dripping occurred on the rest of the tree or growing medium.

All solutions applied to leaves were made with deionised water and contained 1 ml pH buffer (Allbuff, Tsunami Plant Protection (PTY) Ltd, Heidelberg, RSA) and 0.05 ml surfactant (Breakthru S240, Evonik Goldschmidt, GmbH, Essen, Germany) per litre of solution. The elemental concentrations and formulations used in the solutions are given under the relevant sections. Since B and Mo are not available in EDTA or AA formulations, only the inorganic formulation of these elements were evaluated, as only specific formulations were sought after. A control treatment was also applied where only distilled water, surfactant and buffer at the aforementioned rates were used.

Directly after harvest, the citrus leaves were washed with 10% acetone and rinsed with distilled water. Excessive water from the leaf surface was removed by pressing the leaves lightly with soft tissue paper. Washing of samples is recommended to ensure surface decontamination of applied solutes, according to Labanauskas (1968). Treated leaves and the leaves on the same shoot that were directly above (from here on referred to as 'above') and below (from here on referred to as 'below') the treated leaves, were sampled at a specified time after application. Petioles were sampled as part of the leaf. Leaves of the same approximate age were selected, to avoid the possible influence of leaf age on solute penetration.

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3.3   Scanning electron microscopy

Samples for electron microscopy study were collected from commercial citrus orchards in the Groblersdal area on 25 April 2012 during late morning and early midday (Table 3.5). The trees were sprayed with a mixture of Cryptogran (biological insecticide for the control of false codling moth larvae on citrus), molasses (sticker) and Wetcit (surfactant) a few weeks before the samples were taken.

Table 3.5:   Information of trees from which samples were taken at Schoeman Boerdery Moosrivier (25° 01' 41'' S 29° 22' 08'' E) on 25/04/2012 for comparative leaf surface studies.

Sample reference	Cultivar	Type of citrus	Rootstock	Plant date	Leaf age
25/4/1a	'Bahianinha'	Navel	Carrizo Citrange	1994	Young
25/4/1b	'Bahianinha'	Navel	Carrizo Citrange	1994	Old
25/4/2a	'Washington'	Navel	Carrizo Citrange	2008	Young
25/4/2b	'Washington'	Navel	Carrizo Citrange	2008	Old
25/4/3a	'Midknight'	Valencia	Carrizo Citrange	2005	Young
25/4/3b	'Midknight'	Valencia	Carrizo Citrange	2005	Old
25/4/4a	'Satsuma'	Mandarin	Carrizo Citrange	2005	Young
25/4/4b	'Satsuma'	Mandarin	Carrizo Citrange	2005	Old
25/4/5a	'Genoa'	Lemon	Carrizo Citrange	2010	Young
25/4/5b	'Genoa'	Lemon	Carrizo Citrange	2010	Old
25/4/6a	'Star Ruby'	Grapefruit	Swingle Citrange	2003	Young
25/4/6b	'Star Ruby'	Grapefruit	Swingle Citrange	2003	Old

Young (light green with a soft texture) and mature (dark green with thickened cell walls) sun-exposed leaves were harvested and segments of the leaf (Figure 3.2) were removed for the electron microscope study.
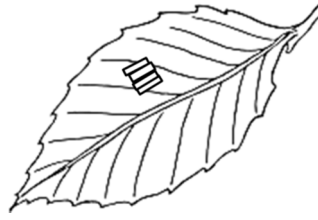
Figure 3.2: Position where leaf segments were removed for SEM analysis.

Three to four leaf pieces (1 mm x 4 mm) of three leaves were dissected from the middle region of the lamina (Figure 3.2) and fixed for at least 4 h in a 100 mM phosphate buffer pH 7, containing 3% (v/v) glutaraldehyde at 5 °C. Samples were then post-fixed for 4 h in 2% (w/v) osmium tetroxide in phosphate buffer before dehydration in an ethanol series and held in 95% ethanol for 2 h. Tissue segments were then freeze-dried, pieces of the leaf material were fractured and fixed on SEM stubs with double-sided tape, coated with gold in a sputter coater and examined and photographed with an Ultrahigh resolution Field Emission SEM (JEOL model 6000F) with a Gatan Digital Micrograph imaging system using an accelerating voltage of 15 kV (Bondada *et al*., 2006).

3.4 Statistical analysis

Statistical analysis of data was performed separately for each element and each experiment, where applicable, with SAS 9.3 program (SAS Institute, 2002) with the General linear model (GLM) using Duncan's Multiple Range Test at a probability level of α=0.05.

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Chapter 4 Results and discussion

4.1 Introduction

When applying foliar fertilisation to citrus leaves, plant response to the applied product is determined by, among other factors, the concentration of the applied product. Determining the optimal concentration range for a particular element and crop is therefore important and may aid to reduce the environmental impact, minimise costs and maximise the benefits of foliar fertilisation. To be able to determine the optimum concentration for the plant, it is necessary to determine how long after application the applied product is still present in the leaves to improve sampling methodology. When foliar products are applied at too high concentrations it may result in damage to the leaves (Fageria *et al*., 2009) or at concentrations lower than optimal which can lead to plant demands not being met (Fageria *et al*., 2002). The efficacy of foliar fertilisation can be assessed by the rates of nutrient penetration and availability in the leaf tissue and these aspects should be considered when conducting foliar fertilisation studies (Weinbaum, 1988).

According to Fernandez & Eichert (2009), the response from foliar sprays is variable, and experiments have not been highly reproducible. Many factors influence a growing plant's ability to absorb nutrients. These include plant, soil and climate factors and also the interactions that exist between them (Fageria *et al*., 2009). More variability in foliar application is introduced with variable leaf surface characteristics such as wettability that may be influenced by leaf age, leaf position on the plant and leaf surface topography, which may differ for individual leaves and

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plants (Fernandez & Eichert, 2009). Reduced wettability may result in less solute retention on the leaf surface. Although care was taken to select leaves of the same age and appearance in the present study, variability did exist between leaf ages and sizes due to the large number of experimental units. This may increase variability according to Jordan & Brodribb (2007) in the results from the present study. Cracks in the wax layer of the leaves may also be non-uniformly distributed and aid in higher variability of foliar uptake of applied solutes (Fernandez & Eichert, 2009). Yield responses as a result of foliar sprays also tend to be highly variable (Fageria *et al*., 2009). Although high variation was apparent in the results, the number of samples and time constraints did not allow inconclusive experiments to be repeated.

4.2 Comparison between the concentration of elements measured in plant sap and those determined with the microwave assisted digestion (MAD) method

The Mn and B content of citrus leaves treated with different Mn formulations (SO_4^{2-}, EDTA and AA) and H_3BO_3 were determined with the MAD method (FSSA, 2003) and compared with the nutrient concentration of plant sap that was collected by applying pressure to citrus leaves (Mason & Phillis, 1939). Results from the leaf analysis methods are given in Table 4.1.

Mn and B concentrations in the leaves determined with the MAD procedure were significantly higher than those measured with the plant sap extraction technique, since all concentrations in the plant sap samples were lower than the detection limit.

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Table 4.1: Comparison between the Mn and B concentration in citrus leaves determined with the MAD method and sap from citrus leaves, treated with different formulations of Mn and B[1].

	Above leaves		Treated leaves		Below leaves	
Cation	MAD $(mg.kg^{-1})$	Sap press $(mg.L^{-1})$	MAD $(mg.kg^{-1})$	Sap press $(mg.L^{-1})$	MAD $(mg.kg^{-1})$	Sap press $(mg.L^{-1})$
$MnSO_4$	5.46^d (30.03)	*BDL^e	11.58^b (24.29)	BDL^e	4.64^d (32.27)	BDL^e
Mn-EDTA	5.23^d (71.80)	BDL^e	9.15^{bc} (73.43)	BDL^e	7.19^{cd} (56.09)	BDL^e
Mn AA	7.06^{cd} (24.43)	BDL^e	17.44^a (28.20)	0.01^e	4.84^d (21.00)	BDL^e
Control	7.55^{cd} (30.45)	BDL^e	6.13^{cd} (25.71)	BDL^e	5.70^{cd} (38.38)	BDL^e
Anion						
H_3BO_3	16.36^b (40.80)	BDL^d	26.73^a (16.11)	BDL^d	10.98^c (31.27)	BDL^d
Control	9.22^c (6.19)	BDL^d	9.43^c (11.37)	BDL^d	8.89^c (16.21)	BDL^d

*BDL= Below detection limit

[1]Statistical analysis is independent for Mn and B. Means followed by the same letter are not significantly different at α=0.05. Coefficient of variation is indicated as percentage in brackets.

The highest Mn and B concentrations, using the MAD technique, were measured for the treated leaves for the different formulations. The Mn concentration in the treated leaves for the Mn AA and $MnSO_4$ formulations was significantly higher than the Mn concentration of the control, while the Mn-EDTA treatment was not significantly higher than the control. Results from MAD samples indicated the H_3BO_3 treatment was significantly different from the control treatment except for leaves below the treated leaves. Above and below leaves were included to ensure that the preparation methods would be suitable for analysis of above and below leaves. Higher concentrations of Mn and B were detected in the treated leaves than above

and below leaves, indicating that concentrations of Mn and B in treated leaves were increased with Mn and B application respectively.

Analysis from the sap press extraction did not yield satisfactorily results. Although plant sap press extraction and analysis has been an accepted practice for a considerable time (Newton *et al*., 1926; Mason & Phillis, 1939; Ferguson, 1980; Netting *et al*., 2012), the lack of reasonable data from the sap press in this study may be ascribed to sampling size and dilution when extracting the samples. Leaf sap could not readily be extracted from single citrus leaves with a hydraulic press (less than 1 ml). In order for ICP-AES analysis to determine the elemental concentrations in the pressed sap samples, deionised water was added to make the samples up to a standard volume of 10 ml, the minimum volume required for ICP analysis. The concentrations of B and Mn in the sap press samples were therefore diluted substantially. The B detection limit for ICP-AES is reported as 10 $\mu g.L^{-1}$ (Van de Wiel, 2003) which converts to 0.67 $mg.kg^{-1}$ for the sample size and dilution used for the MAD method and 0.01 $mg.L^{-1}$ for the leaf sap.

From these results it can be deducted that for the analysis of single citrus leaves prepared by MAD and plant sap press, the MAD seemed to be the more sensitive sample extraction method. Leaves were treated individually on the trees, and not an entire tree, so that sample units were limited. The size of the trees and volume of leaves to process also necessitated smaller sample sizes. This ensured that contamination of untreated leaves or between different treatments could be avoided. Single leaves as sample size enabled the detection of applied solutes movement to the leaf directly above and below the treated leaf. However, this does not imply that extraction via plant sap press is less accurate than MAD method, but that MAD method is more suitable when smaller sample sizes are used.

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4.3 The uptake of leaf applied micronutrients at different time intervals

4.3.1 Manganese

Due to the large standard deviation for the results (Figure 4.1), which is not uncommon for foliar sprays (Fernandez & Eichert, 2009), no significant differences in the Mn concentration of the treated leaves between i) time of sampling for each formulations and ii) type of formulation for each time of sampling were found (Figure 4.1), except for $MnSO_4$ at the 192 h time of sampling. However, the following observations can be made. Little variation was observed among the formulations at each time of sampling and the average leaf Mn concentration for treated leaves of the different formulations was therefore used in the remainder of the study as the most suitable time for sampling leaves for the Mn treatments. The averages of the 24 h and 96 h were both high, but the average was the highest for the 96 h time of sampling. These findings are supported by results from Bukovac & Wittwer (1957), where 75% of total Mn uptake was achieved at 96 h after application. Although no significant differences was found between the type of formulations it was observed that the $MnSO_4$ treatment resulted in the highest leaf Mn concentrations over the measuring period, which is in accordance with Ozaki (1955) who concluded that $MnSO_4$ cured Mn deficiency more efficiently than chelated Mn.

4.3.2 Zinc

For all treatments, except ZnO, the highest leaf Zn concentrations were detected 96 h after Zn application (Figure 4.2), where the highest Zn concentrations in the leaves

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Figure 4.1: Mn concentration of treated 'Midknight' leaves for different Mn formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

were measured for the Zn AA treatment, followed by $ZnSO_4$ and Zn-EDTA for the different times of sampling. ZnO treatments had the lowest leaf Zn concentrations, due to the low solubility that resulted in limited uptake of ZnO, since only a compound in a dissolved state is able to penetrate the leaf surface (Singh *et al*., 2013). Modaihsh (1997) found that uptake and translocation of a commercial available Zn-EDTA in wheat grain were lower than that of $ZnSO_4$ while Barber & Lee (1974) also found that $ZnSO_4$ was absorbed by the leaves and roots at higher rates than Zn-EDTA, which may have been due to permeation restriction of Zn-EDTA into to the free space.

4.3.3 Copper

In general, the variation in results is high (Figure 4.3), although this has also been reported by Fernandez & Eichert (2009) and Weinbaum (1988) to occur in foliar application studies and especially with Cu (Bukovac & Wittwer, 1957). The $CuSO_4$

Figure 4.2: Zn concentration of treated 'Midknight' leaves for different Zn formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

formulation resulted in the highest leaf Cu concentrations for the different times of sampling, while the Cu-EDTA formulation had the lowest Cu concentrations in the treated leaves at 12 h, 24 h, 96 h, and 48 h (significant) and 192 h (non-significant). Cu concentrations of leaves that were treated with Cu AA remained rather constant (between 2.9-5.8 mg.kg^{-1}) and did not differ from the concentration of the control over the experimental period. In contrast to a statement made by Mengel (2002) that $CuSO_4$ have caused leaf scorching on many occasions, $CuSO_4$ did not cause any visible leaf scorching in this trial. Although treatments were not significantly different over the experimental period, large variations existed among the different formulations, especially $CuSO_4$ at each time of sampling and literature was therefore used to determine a suitable time of sampling to be used in the remainder of the study. Chamel & Gambonnet (1979) reported that more than 90% of leaf applied Cu

was detected inside leaves 24 h after application, and leaves were sampled at 24 h in the following investigations.
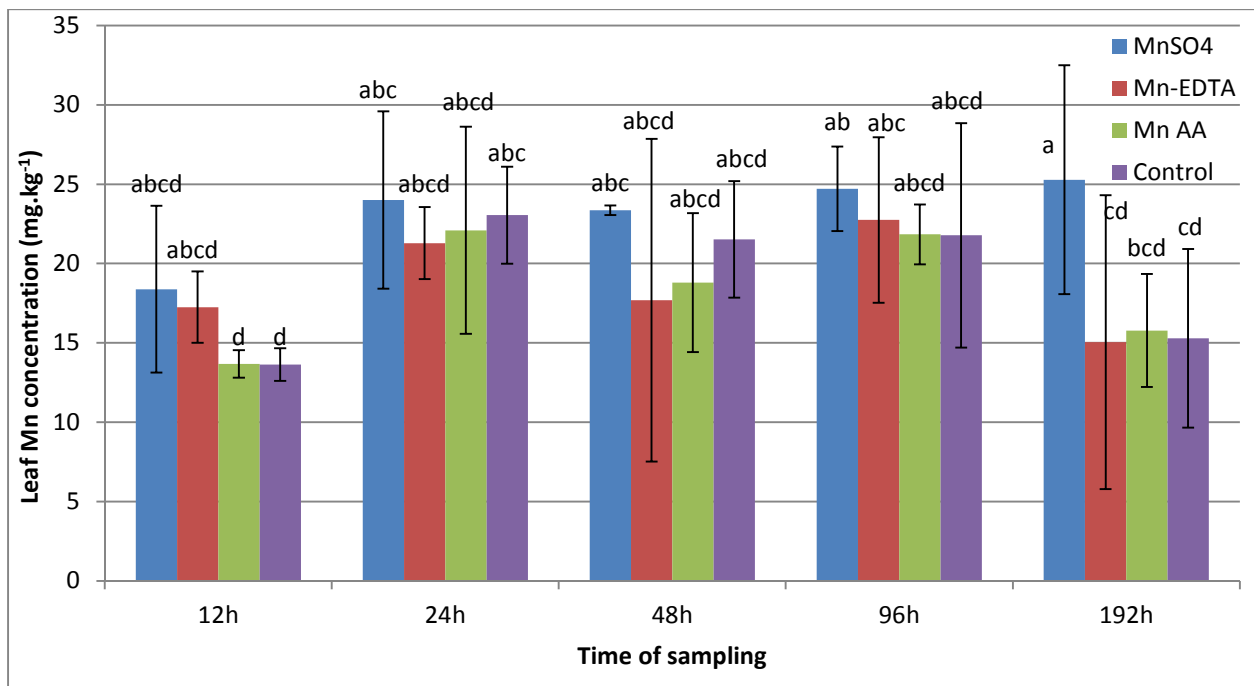

Figure 4.3: Cu concentration of treated 'Midknight' leaves for different Cu formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.3.4 Iron

Surprisingly the control treatments had higher leaf Fe concentrations than some of the treatments. However, the variation in the Fe concentration of the leaves of the control was high. A clear trend regarding a concentration increase with time could not be distinguished for Fe concentrations of the leaves sampled at different times (Figure 4.4). The highest Fe concentrations were detected for $FeSO_4$, Fe-EDTA and Fe AA at the 192 h time of sampling, and Fe samples were taken at 192 h in the remainder of the study. This is in agreement with findings reported by Midwest

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Laboratories (1994) stating that 10-20 days are required for 50% uptake of foliar applied Fe, although the formulation was not specified. The lowest Fe concentrations in the treated leaves were found 48 h after Fe application while the highest Fe concentration in the leaves was measured for the FeSO$_4$ formulation and the lowest for the Fe-EDTA formulation over the majority of the sampling period. Basiouny & Biggs (1971), who studied foliar penetration of different Fe compounds through *Citrus aurantium* leaves, concluded that synthetic chelates penetrated and translocated faster than inorganic Fe compounds.
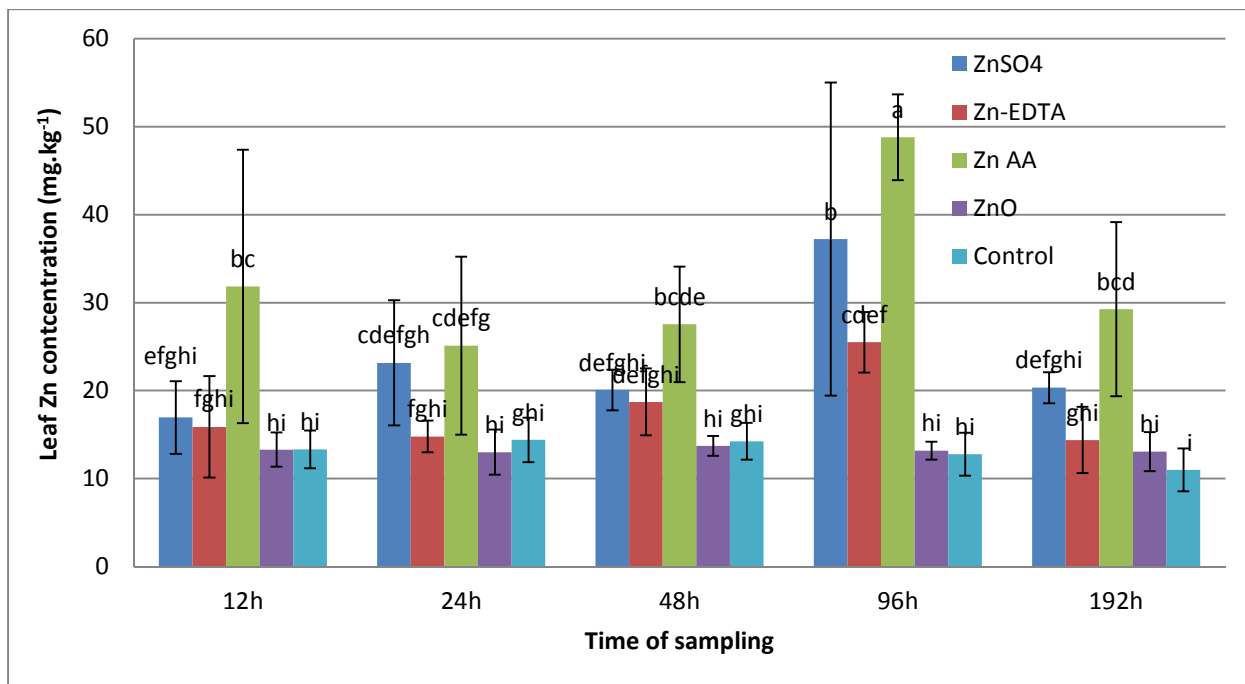

Figure 4.4: Fe concentration of treated 'Midknight' leaves for different Fe formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.3.5 Boron

The B concentration in the treated leaves was significantly higher at the 48 h time of sampling compared to any other time, and decreased thereafter. This may be due to the mobility of B in the apoplast (Mengel, 2002) that follows the transpiration stream (Brown & Shelp, 1997). B is absorbed into the treated leaf until a maximum B concentration is reached at 48 h and then translocated from that leaf via the transpiration stream to other parts of the tree. Even though B may not be mobile in the phloem, the xylem still translocates B readily (Neumann, 1988). Hanson (1991) found that foliar applied [10]B isotope was exported rapidly to other tissues with the most rapid movement occurring within three days after application on cherry trees. The highest B concentrations in the treated leaves were detected at the 48 h and the lowest at 192 h time interval. 48 h after treatment application was consequently applied as the time of leaf sampling in the remainder of the study. The control treatment had significantly lower B concentrations than the B treatment for the different times of sampling.

4.3.6 Molybdenum

No significant differences were detected between the treatment and control over time. A large standard deviation in the Mo concentration of the leaves had a negative impact on the quality of this data. Products were combined on the basis of Mulder's chart (Mulder, 1953) from which information on S was not included. The magnitude of the measured Mo concentrations was very low. After obtaining unexpected results, an additional lengthy in-depth study revealed that the low Mo concentrations may have been due to an antagonistic interaction between the Mo
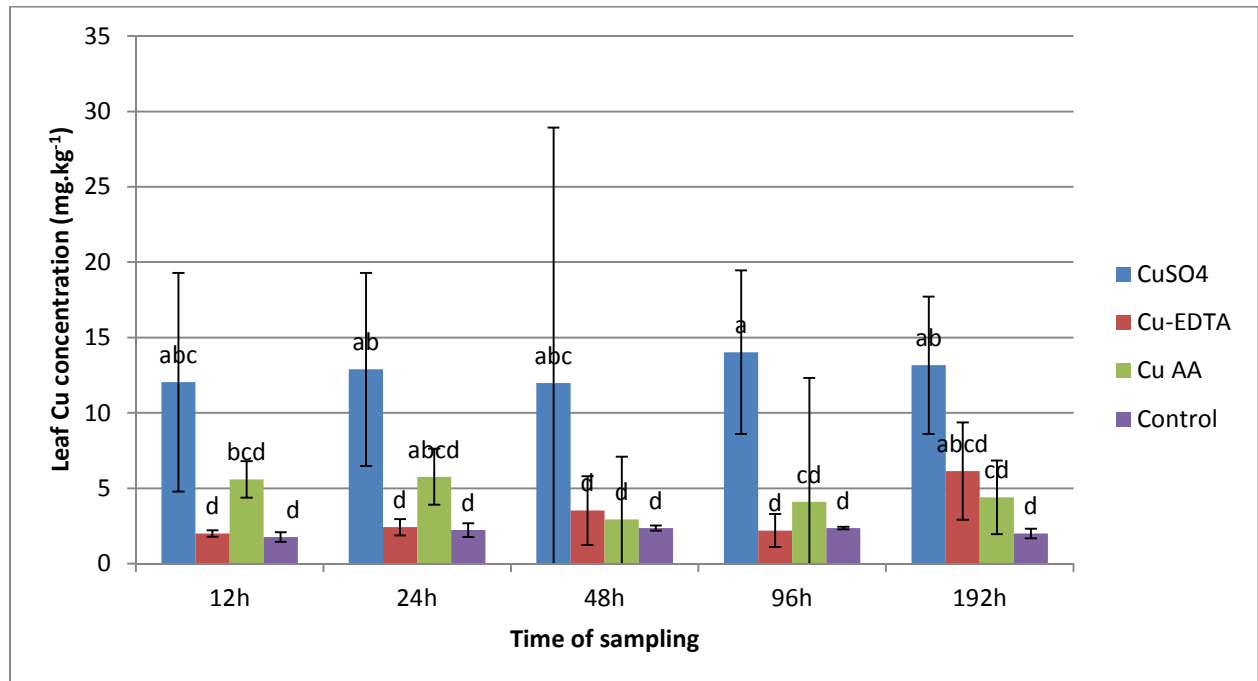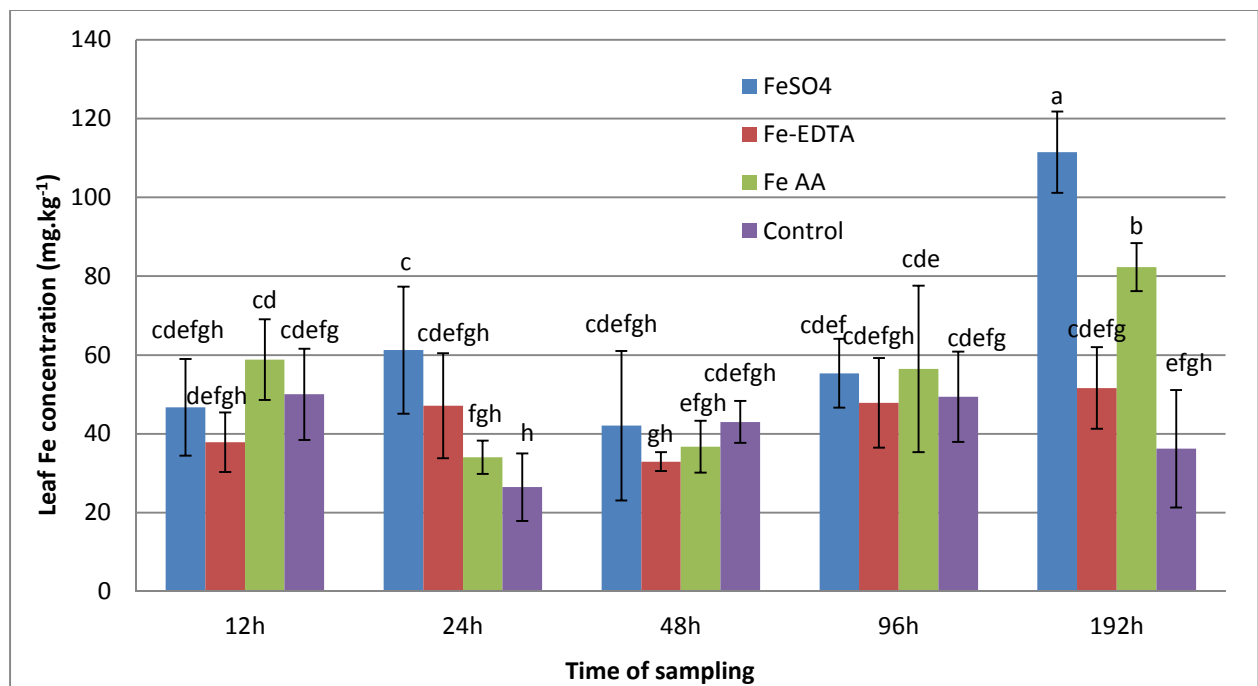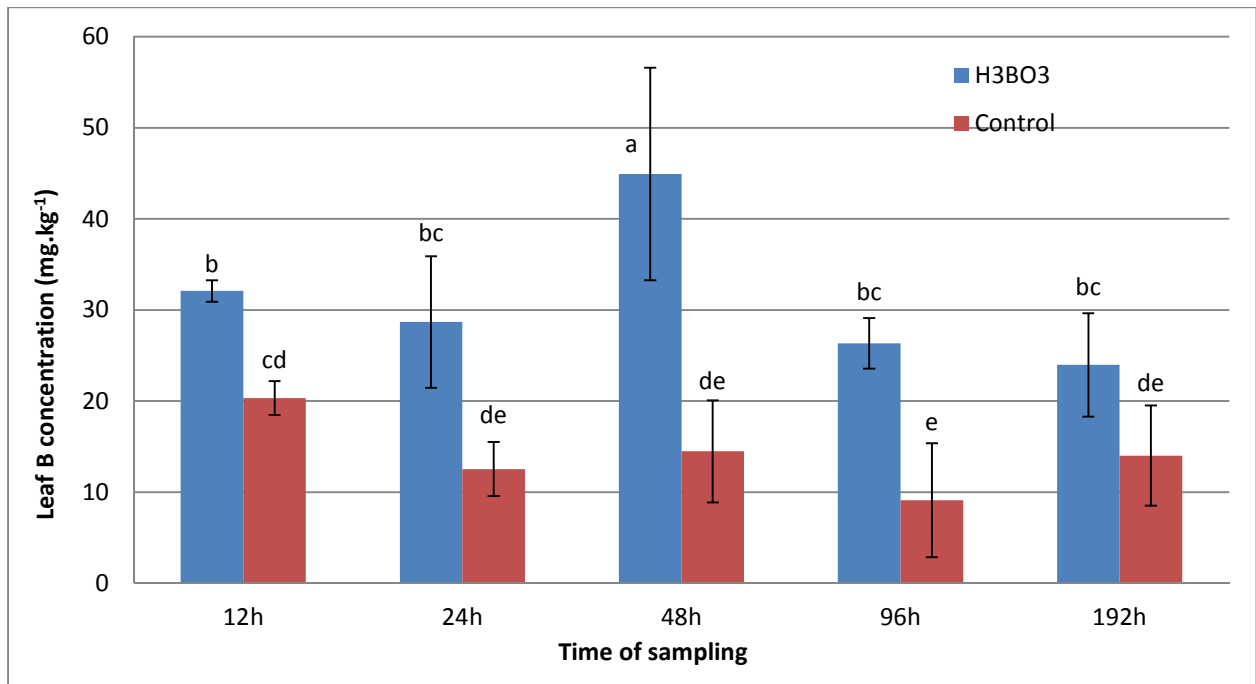
Figure 4.5: B concentration of treated 'Midknight' leaves for different B formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

and SO_4^{2-} applied in the same solution (see Section 3.1.1) (Stout *et al.*, 1951). A decrease in uptake of MoO_4^{2-} occurs in the presence of SO_4^{2-} and has been reported by various authors (Sims *et al.,* 1979; Marschner, 1995; Chatterjee *et al.,* 1992). The MoO_4^{2-} anion behaves much like the SO_4^{2-} anion, and MoO_4^{2-} is said to be transported into the plant via an unspecific anion transporter, probably using the same transport system as SO_4^{2-} (Marschner, 1995). Cole *et al*., (1993) found that SO_4^{2-} was required at concentrations three orders of magnitudes higher (on a mole basis) than MoO_4^{2-} to inhibit MoO_4^{2-} uptake in Cyanobacteria. In the present study, the solution used contained 121.3 times more SO_4^{2-} (0.0097 mol) than MoO_4^{2-} (0.000080 mol) and an antagonistic effect can thus be assumed to occur. However, the SO_4^{2-} concentration in the applied solution remained the same for all times of leaf sampling and is therefore assumed to have a constant and consistent antagonistic

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effect on the concentration of MoO_4^{2-} absorbed by the leaves. Williams *et al*., (2004) reported maximum uptake within 24 h of foliar applied MoO_4 to 'Merlot' grapevine.

The highest Mo concentration for Mo treated leaves was detected at 96 h and the lowest at 192 h. However, according to Bukovac & Wittwer (1957), highest concentrations of Mo were detected in leaves 48 h after treatment application and 48 h was therefore used as time of sampling in the remainder of the study.
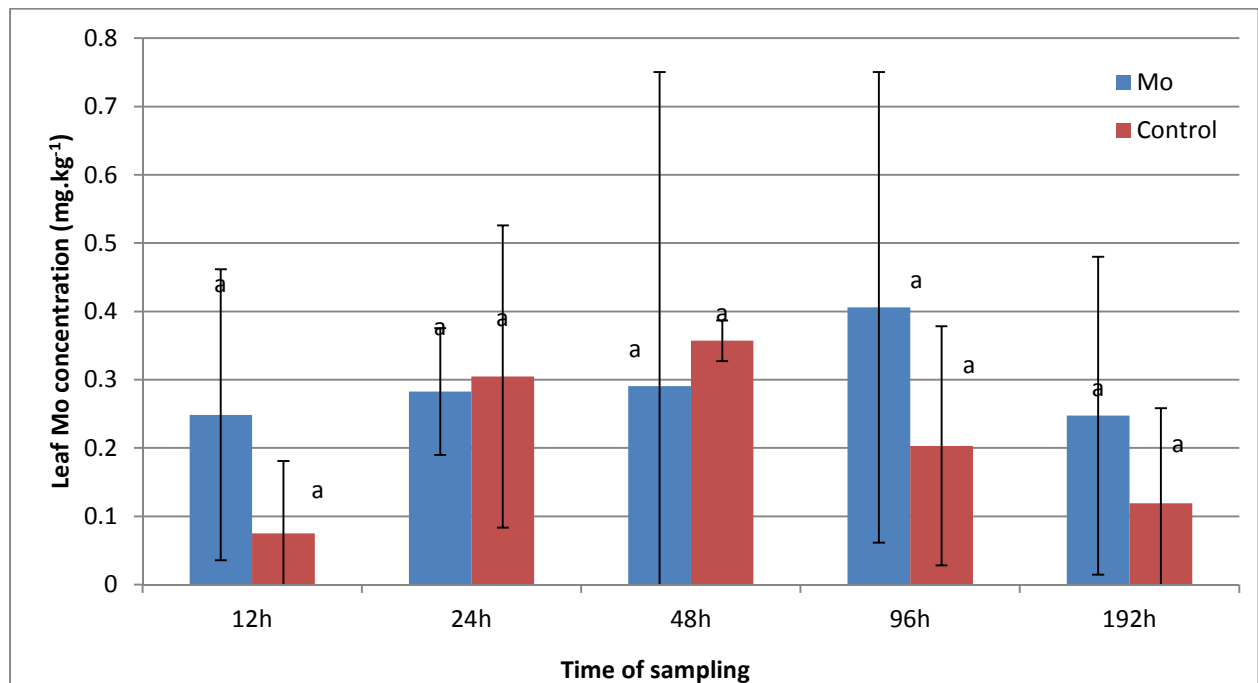
Figure 4.6: Mo concentration of treated 'Midknight' leaves for different Mo ($Na_2MoO_4.2H_2O$) formulations sampled at different time intervals. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.4 Influence of nutrient concentration and formulation on maximum leaf uptake of the applied element

To correct micronutrient deficiencies or apply them as part of production practice, the maximum leaf uptake of the most effective nutrient formulation needs to be facilitated per foliar application. The molecule size, mobility and compound solubility of formulations are some of the factors that determine foliar uptake. Fick's law states that a direct relationship exist between the concentration and uptake of the application solution (Fernandez & Eichert, 2009) and therefore a study was conducted to investigate this relationship by applying five different concentrations of each micronutrient formulation to the leaves of potted 'Midknight' trees. No visible leaf damage was observed for any of the treatments. The time of sampling for each treatment was as explained in section 4.3 and analysed separately. In this study the same pool of trees were used as for the study on the uptake of leaf applied micronutrients at different time intervals, from which the necessary amount of trees were selected randomly, (Chapter 4.3) and, as a result of possible contamination in-between treatments., initial micronutrient concentrations were higher in the leaves.

4.4.1 **Manganese**

Results (Figure 4.7) of different formulations ($MnSO_4$, Mn-EDTA and Mn AA) and concentrations of Mn applied to citrus leaves and sampled after 96 h.

In general the Mn concentrations in the leaves did not increase with application concentration. Mn concentrations were higher than the control treatments (significantly higher at 1X, 4X and 8X for $MnSO_4$, and at 1X for Mn AA) except for

Mn-EDTA at 1X. The variation of the 1X Mn-EDTA was very high and this had a negative impact on reliability, and this result is therefore not considered further. The highest Mn concentration in the treated leaves was measured for the 1X Mn AA treatment, while the lowest leaf Mn concentration was measured for the 1X Mn-EDTA treatment

$MnSO_4$ and Mn AA were not significantly different at any of the concentrations applied. The large standard deviation in the results of the different treatments does not allow for conclusive significant differences for the treatments. Papadakis *et al*., (2005) compared foliar applications of $MnSO_4$ and Mn-EDTA on uptake by leaves of 'Washington' navel trees. Both sources of Mn increased the leaf Mn concentrations when Mn concentration was increased in the applied solution, although $MnSO_4$ increased leaf Mn concentrations significantly more than the Mn-EDTA treatment. Papadakis *et al.,* (2005) found that 1.2 $g.L^{-1}$ of Mn applied to 'Washington' navel leaves resulted in an increase of leaf Mn content to 19.6 and 30 $mg.kg^{-1}$ Mn for foliar applied Mn-EDTA and $MnSO_4$ respectively. Papadakis *et al*., (2005) concluded that in 'Washington' navel trees, $MnSO_4$ would be the preferable formulation used when correcting Mn deficiency. Mn-EDTA application resulted in leaf Mn concentrations that varied between 32.18 and 42.92 $mg.kg^{-1}$ with an average of 35.08 $mg.kg^{-1}$ for all treatments. So that even when considering the large standard deviation, leaf Mn concentrations of Mn-EDTA treatments stayed relatively constant over the concentration range used and did not increase leaf Mn concentrations as markedly as the $MnSO_4$ and Mn AA treatments did (Figure 4.7).

Figure 4.7: The Mn concentration of 'Midknight' leaves treated with different Mn concentrations and formulations sampled after 96 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.4.2 Zinc

An increase in the leaf Zn concentration was recorded for all Zn treatments although not significant in all cases (Figure 4.8). The highest Zn concentrations in the leaves were found for the 8X ZnO treatment while lowest concentrations were found for the 1X Zn-EDTA treatment (Figure 4.8). Although not as pronounced as ZnO, an increase in the Zn leaf concentration for the Zn AA and $ZnSO_4$ treatments was observed with an increase in application concentration (Figure 4.8).

The high leaf Zn concentration measured for the ZnO formulation was surprising because, due to the low solubility of ZnO, it was expected that very low amounts Zn will be absorbed (Fageria *et al*., 2009). Therefore, it is speculated that ZnO residues may have remained on the leaf surface, even after washing and rinsing with water and acetone. Because ZnO is practically insoluble, little ZnO would have dissolved

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in the water and acetone during the wash and rinse procedure and residues remained on the leaf surface. Difficulty in removing applied soluble residual Zn from citrus leaves has also been reported by Wallihan & Heymann-Herschberg (1956), when using various leaf washing techniques, such as acid detergent solutions and 10% sodium ethylene di-amine tetra acetic acid (Na-EDTA) adjusted to pH 5, although they applied soluble $ZnCl_2$ to the leaves. The high Zn concentrations at the 8X treatment exceeded 200 mg.kg^{-1}, which is more than the upper limit for Zn in citrus (FSSA, 2003). Visible toxicity symptoms were expected on the treated leaves if ZnO was indeed absorbed by the leaf, and since no visible toxicity symptoms were detected, the high Zn concentrations from the ZnO treatment is regarded as unabsorbed residual Zn on the leaf surface.

Zn-EDTA application resulted in the lowest leaf Zn concentration for all treatments

and did not differ significantly over the concentration range or from the control. The low variation of leaf Zn concentration were found for all Zn-EDTA concentrations applied - between 18.76 and 33.17 mg.kg^{-1} - also indicate that Zn-EDTA does not enter the plant as readily as the other formulations used. According to Boaretto *et al*., (2002) foliar applied Zn-EDTA sources of Zn did not result in higher leaf concentrations of Zn than inorganic Zn when applied to grapes and vegetable crops. This may be due to the larger molecular sizes of Zn-EDTA (Midwest Laboratories, 1994).

4.4.3 Copper

The $CuSO_4$ treatment increased the Cu content in the treated leaves as the applied
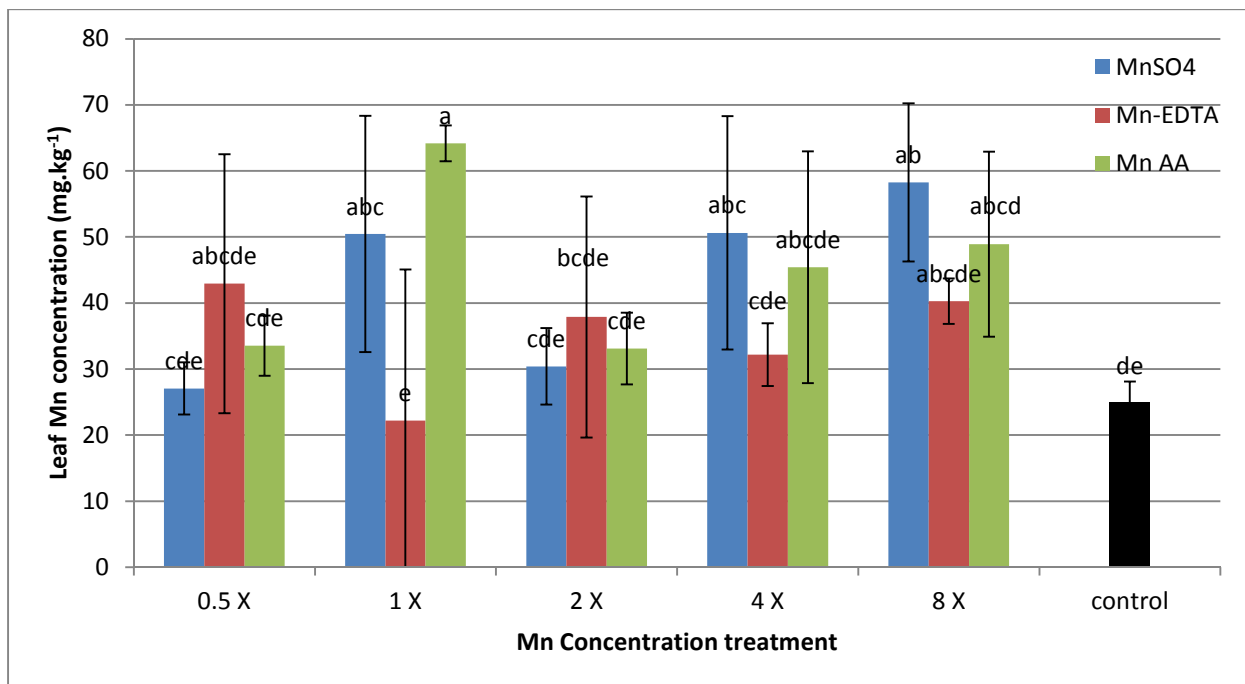
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Figure 4.8: The Zn concentration of 'Midknight' leaves treated with different Zn concentrations and formulations sampled after 96 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

Cu concentration increased until the leaf concentration reached a maximum at 2X (Figure 4.9). Leaf Cu concentrations then remained relatively constant, with no significant differences, as applied Cu concentrations increased. This correlates with a study reported by Chamel (1988), where uptake of Cu by plant leaves increased with increase in Cu concentration of the $CuSO_4$ application solution in the treated leaves and are in accordance with recommendations made by Fageria *et al*., (2002) that $CuSO_4$ applications should be made at 1-2 g.L^{-1} (or 0.1-0.2% $CuSO_4$). Leaf Cu concentrations increased as applied Cu concentration of Cu AA treatments increased. Cu AA treatments reached a maximum leaf Cu concentration at 8X, which resulted in it being the formulation, after $CuSO_4$, with the highest leaf Cu content. Higher concentrations may have been absorbed by leaves if Cu concentrations higher than 8X were applied to leaves. At 0.5X, 1X and 2X Cu AA

treatments had the lowest Cu concentrations of applied Cu. Cu-EDTA treatments increased leaf Cu concentrations as the applied concentrations increased, until it reached a maximum at 4X and stayed relatively constant at the higher 8X concentration. The maximum Cu concentration of Cu-EDTA was the lowest when compared to maximum Cu concentrations of $CuSO_4$ and Cu AA. This correlates with findings reported by Modaihsh (1997), that Cu-EDTA did not supply as much Cu to leaves of wheat (*Triticum aestivum* L., cv. 'Yecora rojo') as foliar applications of $CuSO_4$. Although Cu concentrations in leaves treated with $CuSO_4$ at all concentrations applied and Cu AA applied at 8X, were high (according to Table 2.2), no toxicity symptoms were observed on leaves. Cu concentrations are considered toxic in citrus leaves when higher than 40 mg.kg^{-1} (FSSA, 2003).
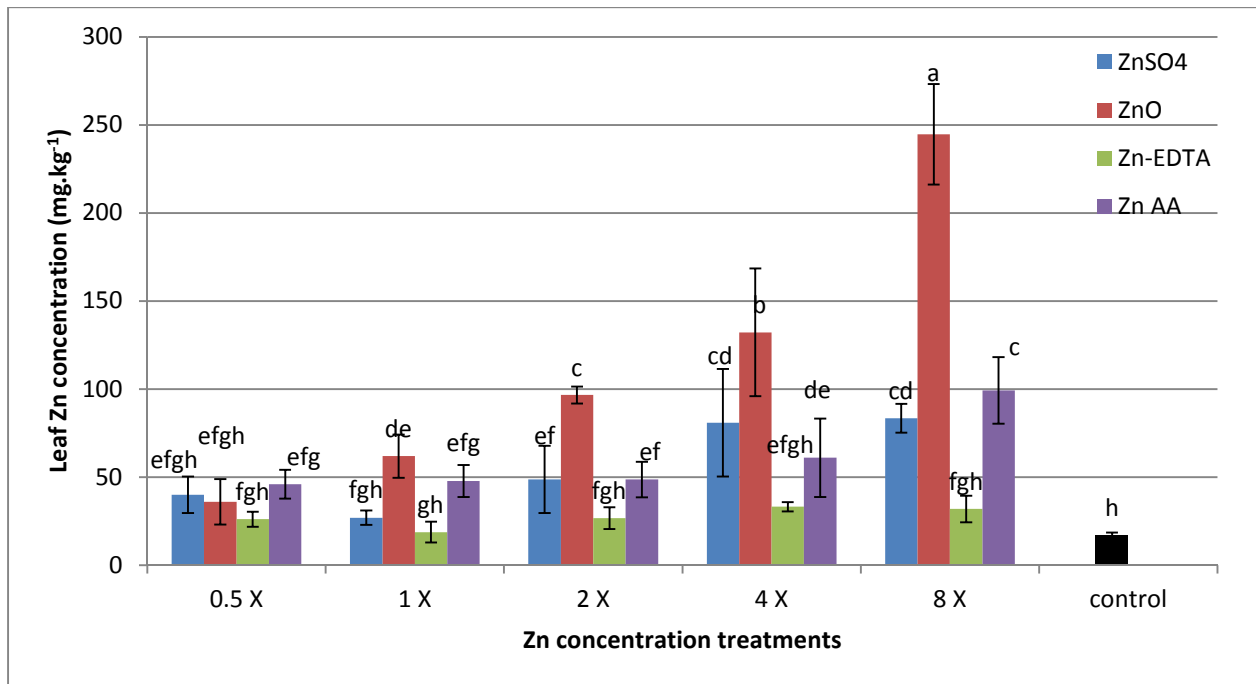

Figure 4.9: The Cu concentration of 'Midknight' leaves treated with different Cu concentrations and formulations sampled after 24 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.4.4 Iron

$FeSO_4$ treatments increased Fe concentration in the treated leaves as the applied Fe concentration increased, until it reached a maximum and significantly higher Fe concentration, at 4X (Figure 4.10) but which were lower at 8X. Leaf Fe concentrations of Fe AA were relatively constant for the 0.5X, 1X and 2X treatments with no significant differences between them. At 4X, the Fe AA treatments resulted in a significant maximum of 148.79 $mg.kg^{-1}$, and declined slightly thereafter. Fe-EDTA treatments resulted in the lowest leaf Fe levels at all applied Fe concentrations except 1X and did not follow a clear trend, although maximum Fe concentrations were found at 8X. Except for Fe-EDTA, the highest overall concentrations were found when Fe was applied at 4X, while the lowest concentrations were achieved by applying 0.5X the recommended concentration of $FeSO_4$ and Fe-EDTA only. This is in accordance with findings from Alvarez-Fernandez *et al.,* (2004) that $FeSO_4$ was more effective in curing leaf chlorosis than Fe-DTPA when sprayed at four times the concentration of Fe-DTPA. Reports from Abadia *et al*., (2002) suggest that Fe^{2+} salts are generally as effective as Fe^{3+} chelates as a foliar application to increase leaf chlorophyll content. Schönherr *et al*., (2005) found that penetration rates of chelated Fe decreased as the Fe-EDTA concentration increased. They proposed that one should rather apply low concentrations to foliage since high concentrations tend to penetrate slower and will be subject to photo-degradation.

4.4.5 Boron

Results (Figure 4.11) of different concentrations of B (H_3BO_3) applied to citrus leaves

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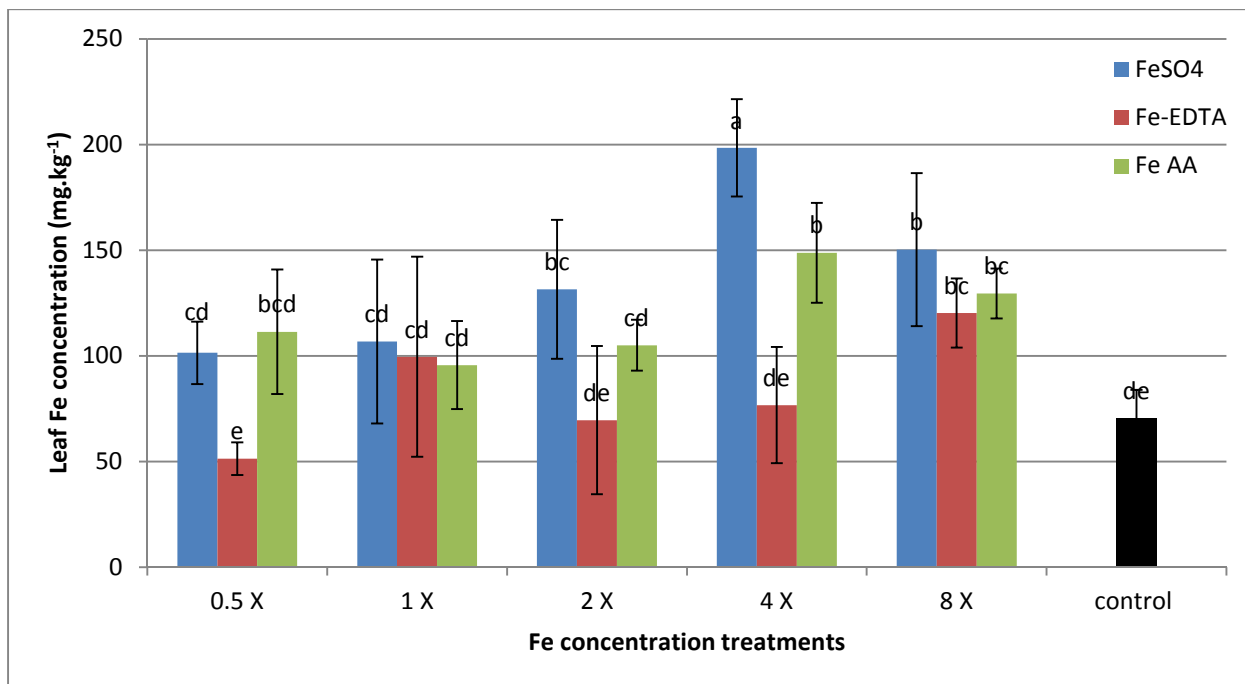
Figure 4.10: The Fe concentration of 'Midknight' leaves treated with different Fe concentrations and formulations sampled after 192 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

and sampled after 48 h.

Since the integrity of samples from the 4X treatment was compromised by an unplanned spillage during MAD preparation, the 4X results are omitted but the remainder of the B treatments was not affected. The B concentrations in leaves increased as the applied B concentration increased until it reached a maximum at 2X and stayed relatively constant thereafter. Highest B concentrations were achieved at 2X and 8X while the lowest were observed at 0.5X, with 0.5X and 1X not significantly different from the control. Higher applied B concentrations could result in higher leaf B concentrations, since, in the current study, B concentrations in the leaves were low (≤ 80 mg.kg^{-1}) according to Mattos *et al*., (2012).
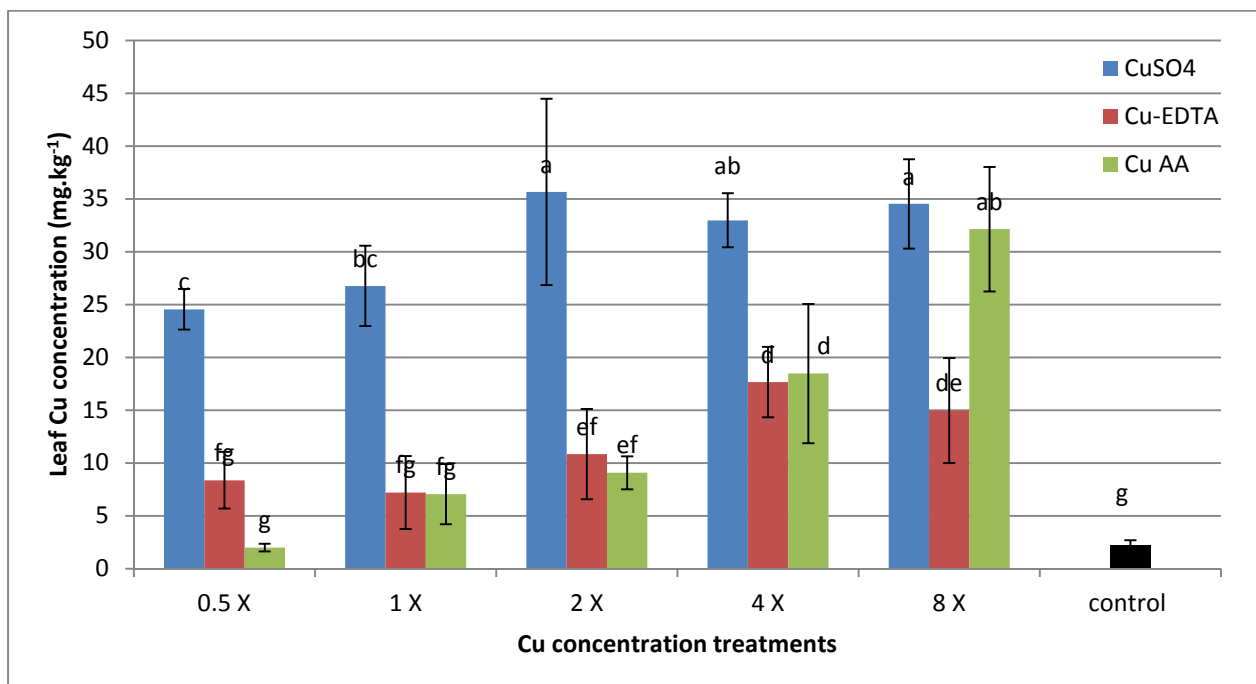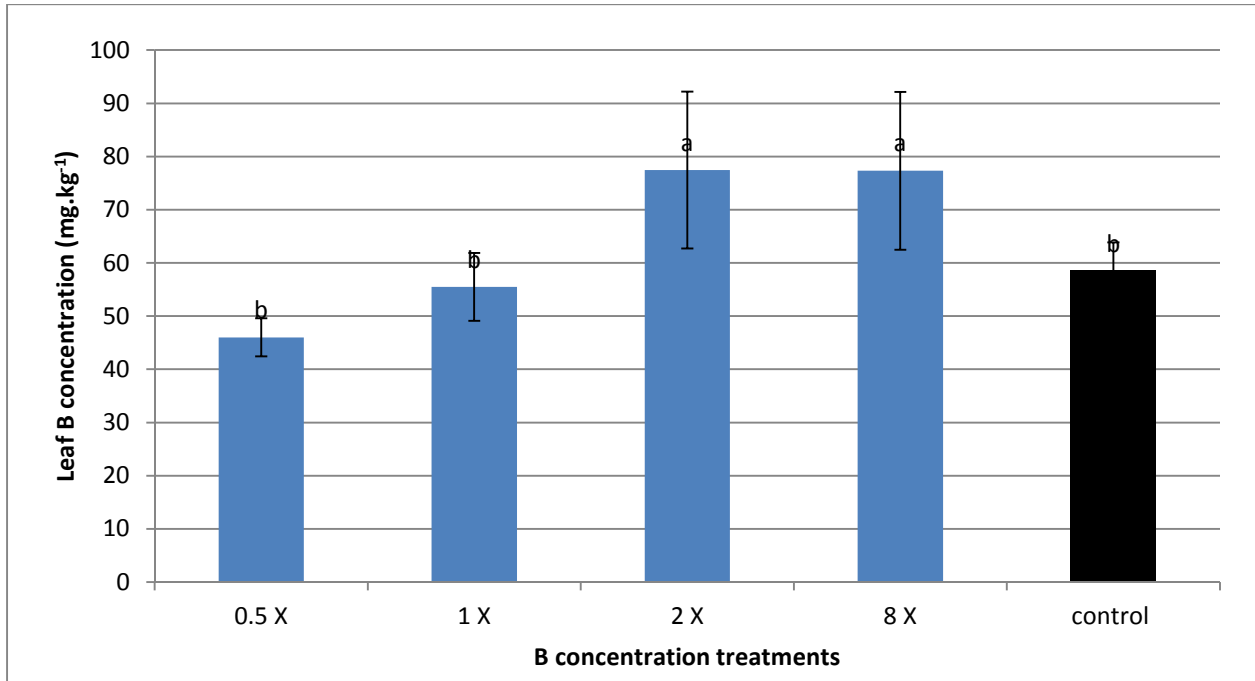
Figure 4.11: The B concentration of 'Midknight' leaves treated with different H_3BO_3 concentrations sampled after 48 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at α=0.05].

4.4.6 Molybdenum

Mo content of the treated leaves increased with increase in Mo concentration of the applied solution. Mo concentrations in the leaves treated with 8X concentration was significantly higher than all other treatments while the lowest Mo concentration in leaves were found for the 0.5X and 1X treatment that was similar to the control (Figure 4.12). The Mo concentration in the 8X solution is equivalent to 0.40 g.L^{-1}, which is in accordance with the recommendation of Stewart & Leonard (1952) that 0.45 g.L^{-1} $Na_2MoO_4.2H_2O$ is the optimum concentration for foliar sprays on citrus trees.

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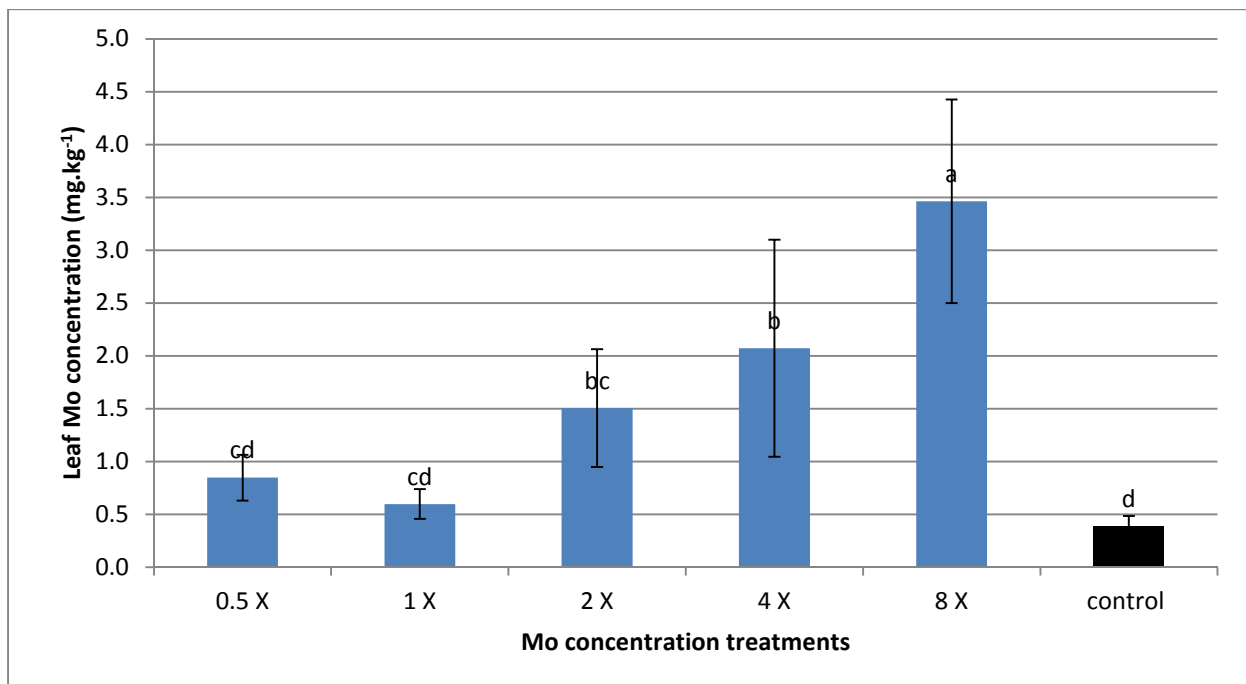
Figure 4.12: The Mo concentration of 'Midknight' leaves treated with different Mo ($Na_2MoO_4.2H_2O$) concentrations sampled after 48 h. [Error bars represent standard deviation, treatments with the same letter/s do not differ significantly at $\alpha=0.05$].

4.5 Nutrient movement to the leaves above and below the treated leaves

A main consideration regarding the effectiveness of a foliar application is the rate at which the applied nutrients are translocated to non-treated plant parts (Weinbaum, 1988). Translocation mainly occurs via the phloem and is important in redistribution of the applied elements, to meet plant demands and according to Bukovac & Wittwer (1957) great differences exist between elements with regards to rate and the quantity that is translocated.

Leaves were treated separately with differently formulated micronutrients and at different concentrations. Where different concentrations were applied, the elemental concentrations in solutions applied are as reported in Table 3.3 for the respective nutrients. For leaves sampled at different times after treatment application,

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elemental concentrations applied to leaves are given in Table 3.2 for the respective nutrients. The leaves above and below the treated leaves were sampled, respectively, at different times and for the different concentrations used. Nutrient concentrations of the above and below leaves are compared against nutrient concentrations of the treated leaves.

4.5.1 Manganese

In Figure 4.13 the Mn concentrations of the leaves above and below the treated leaves are shown. Results are given for different Mn formulations, time of sampling and application concentrations.

Regardless of the time of sampling or formulation the Mn content of the leaves above and below the treated leaves followed the concentration of the treated leaves in most cases (Figure 4.13). Therefore, if the Mn concentration of the treated leaves increases then the Mn content of the leaves above and below the treated leaves also increased, which imply that Mn was translocated to these leaves. In the majority of treatments the leaves above the treated leaves had higher Mn concentrations than the corresponding below leaves (Figure 4.13). This indicates that transport of Mn occurs more readily upwards in the plant than downwards which may be explained by the low phloem mobility of Mn, so that downward transport in the plant is less than upward transport (Fageria *et al*., 2009).

Figure 4.13: Mn concentration in treated 'Midknight' leaves and above and below leaves sampled at different time intervals after treatment with $MnSO_4$ (A), Mn-EDTA (B) and Mn AA (C) and treated with different Mn concentrations of $MnSO_4$ (D), Mn-EDTA (E) and Mn AA (F). [Error bars represent standard deviation].

4.5.2 Zinc

In Figure 4.14 and Figure 4.15 the Zn concentrations of the leaves above and below the treated leaves are shown. Results are given for different Zn formulations, time of sampling (Figure 4.14) and application concentrations (Figure 4.15).

Although the Zn concentration of the leaves treated with $ZnSO_4$ (Figure 4.14 A) and Zn AA (Figure 4.14 D) increased for the different times of sampling up to 96 h, the concentration of Zn did not increase accordingly in the leaves above or below the treated leaves. The Zn concentration in leaves treated with ZnO and Zn-EDTA was not considerably higher than Zn concentrations in the leaves above and below the treated leaves. Above and below leaves did not have high variation throughout the period of sampling with Zn concentrations ranges between 6.16-16.279 mg.kg^{-1} and 8.446-21.385 mg.kg^{-1} respectively.

Treated leaves of the $ZnSO_4$, Zn AA and ZnO treatments displayed significantly higher Zn concentrations compared to the leaves sampled above and below the treated leaves at all concentrations applied (Figure 4.15 A, B and C). The Zn concentrations in the corresponding leaves above and below the treated $ZnSO_4$, ZnO and Zn-EDTA leaves did not increase either.

This indicates that higher concentrations in treated leaves may have been due to Zn residues that are difficult to remove and remained on the leaf surface after washing. Leaves above and below the Zn-EDTA treated leaves had significantly lower Zn concentrations than the treated leaves at all concentrations except 1X. Poor translocation of Zn has been reported, where less than 7% of the applied Zn from chelated and inorganic Zn sources is translocated from treated plant parts (Ferrandon & Chamel; 1988; Boaretto *et al*., 2002).

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Figure 4.14: Zn concentration in treated leaves and above and below leaves sampled at different time intervals after treatment with ZnSO$_4$ (A), ZnO (B), Zn-EDTA (C) and Zn AA (D). [Error bars represent standard deviation].
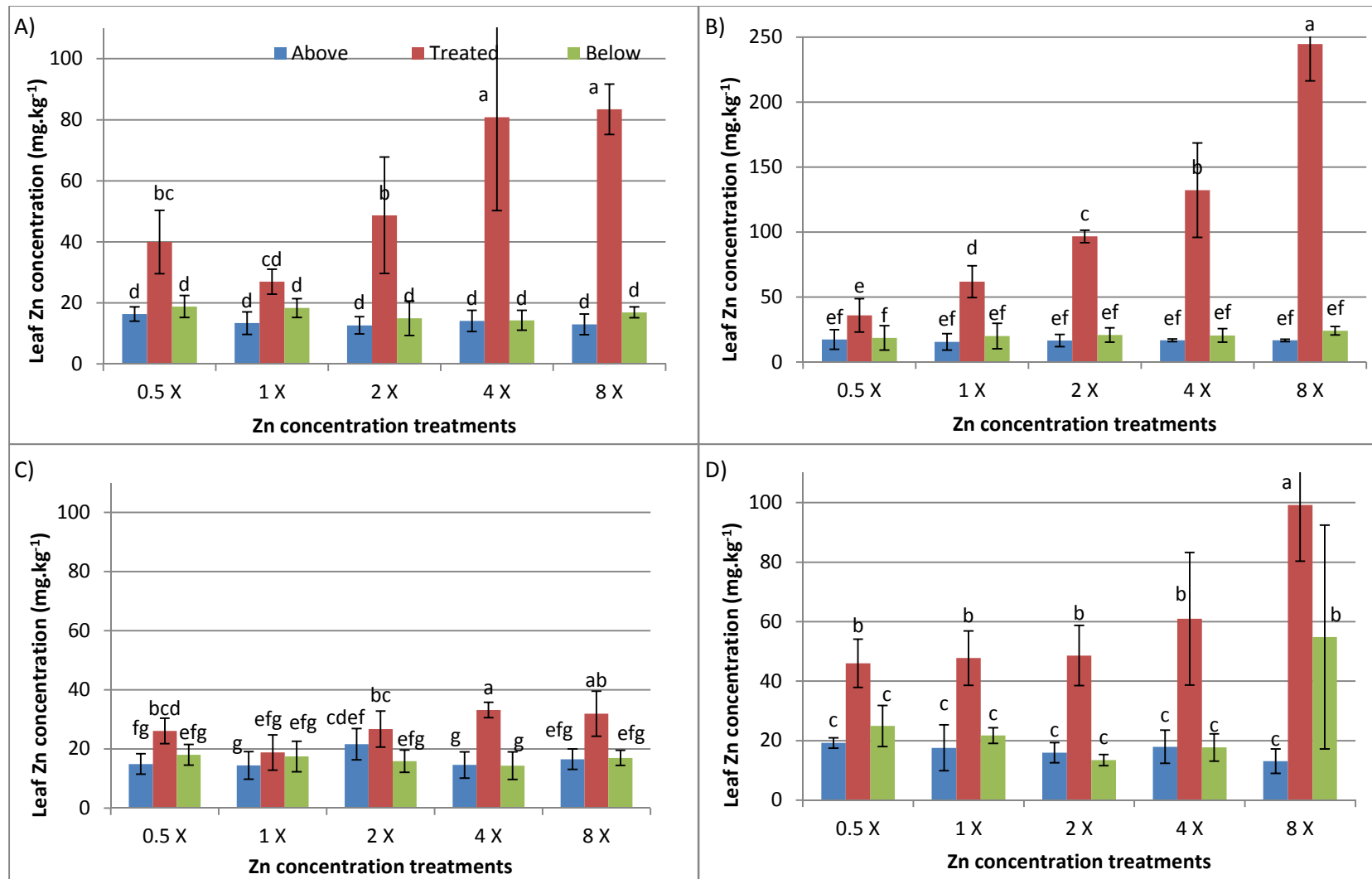
Figure 4.15: Zn concentration in treated leaves and above and below leaves treated with different Zn concentrations of $ZnSO_4$ (A), ZnO (B), Zn-EDTA (C) and Zn AA (D). [Error bars represent standard deviation].

4.5.3 Copper

In Figure 4.16 the Cu concentrations of the leaves above and below the treated leaves are shown. Results are given for different Cu formulations, time of sampling and application concentrations.

For all treatments, treated leaves show elevated concentrations compared to above and below leaves, except for Cu-EDTA sampled at different times after treatment application. Little or no Cu translocation could be detected, since Cu concentrations in the leaves above and below the treated leaves did not increase with increased Cu concentration in the treated leaves for either the different times of sampling or different concentrations applied. These findings are in accordance with reports from Mengel (2002) that Cu is relatively immobile in the phloem, so that rapid translocation to leaves above and below treated leaves is not expected.

4.5.4 Iron

In Figure 4.17 the Fe concentrations of the leaves above and below the treated leaves are shown for different Fe formulations, time of sampling and application concentrations.

For most of the treatments the Fe concentration of the treated leaves showed elevated Fe concentrations in some instances when compared to above and below leaves. When Fe was applied at different concentrations, higher Fe concentrations were found in leaves above and below the treated leaves for all treatments (Figure 4.17 D, E and F) than when applied at one concentration and sampled at different times (Figure 4.17 A, B and C). These findings are in contrast to a statement by
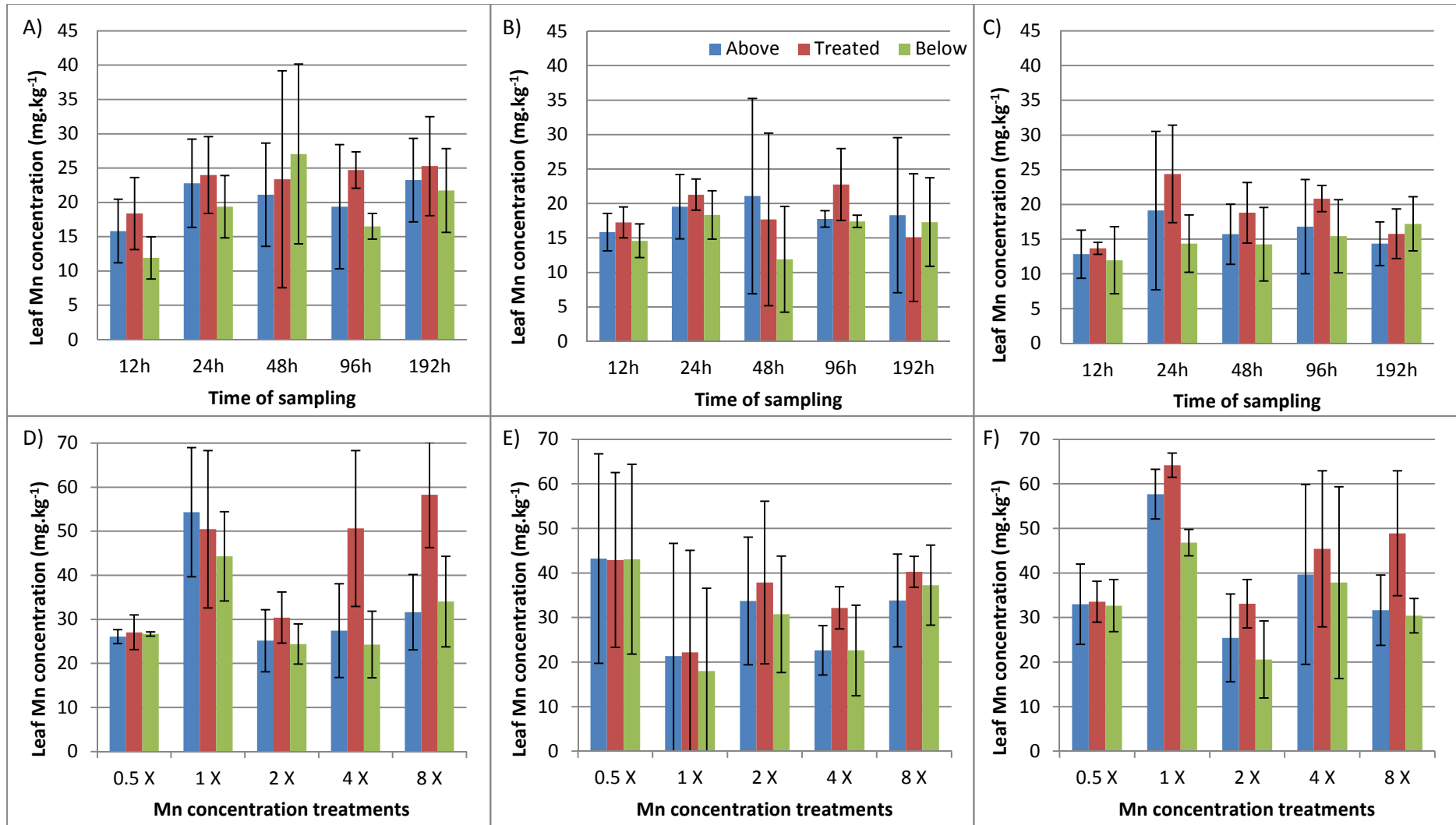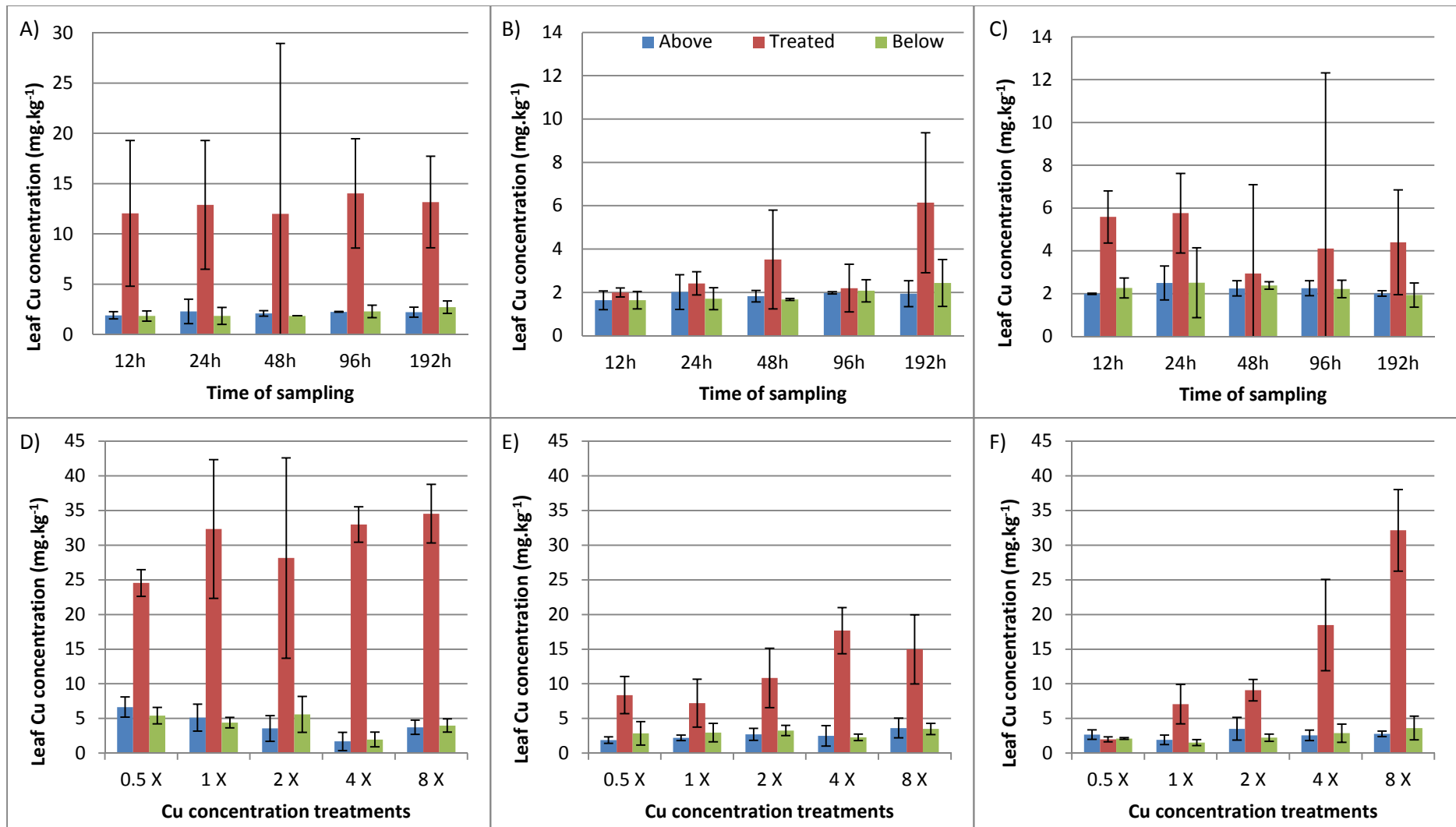
Figure 4.16: Cu concentration in treated leaves and above and below leaves sampled at different time intervals after treatment with $CuSO_4$ (A), Cu-EDTA (B) and Cu AA (C) and treated with different Cu concentrations of $CuSO_4$ (D), Cu-EDTA (E) and Cu AA (F). [Error bars represent standard deviation].

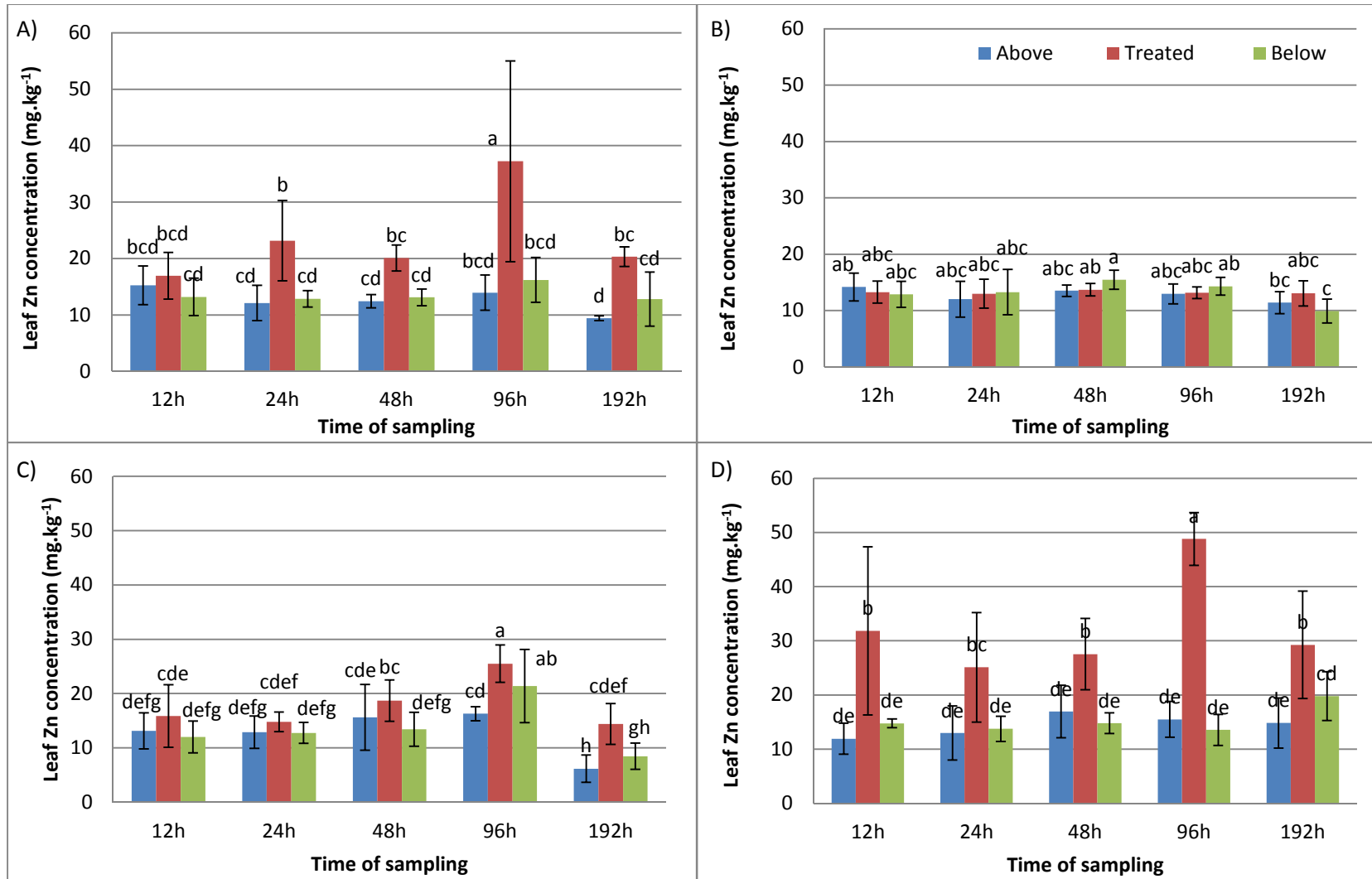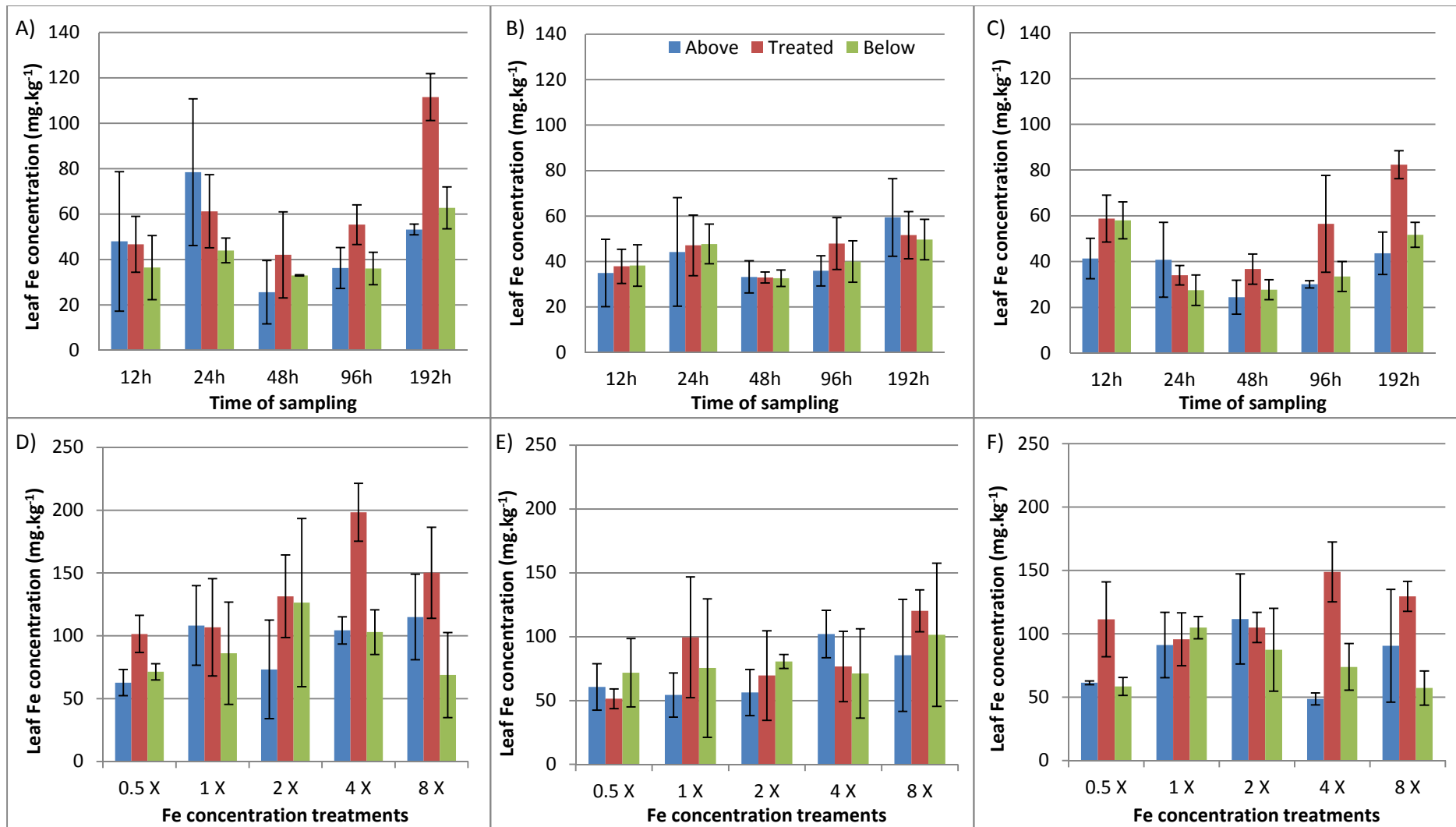**Figure 4.17:** Fe concentration in treated leaves and above and below leaves sampled at different time intervals after treatment with $FeSO_4$ (A), Fe-EDTA (B) and Fe AA (C) and treated with different Fe concentrations of $FeSO_4$ (D), Fe-EDTA (E) and Fe AA (F). [Error bars represent standard deviation].

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Wittwer *et al*., (1967) that organic chelating agents are able to enhance micronutrient transport when applied foliarly, but that Fe uptake is retarded greatly compared to inorganic Fe sources.

4.5.5 Molybdenum

Mo concentrations in leaves above and below the treated leaves did not increase as Mo concentrations increased in treated leaves. Mo concentrations in the above and below leaves did not have large variation relative to each other when Mo was applied at different concentrations. Although Mo is considered a phloem-mobile nutrient, as reported by Mengel (2002), translocation of Mo from treated leaves to above and below leaves did not occur (Figure 1.19 B). This may be due to leaf Mo concentrations in the above and below leaves that are considered normal Mo concentrations (between 0.05-3.00 mg.kg^{-1}) in citrus plants, according to FSSA (2003). Mo may also be stored inside the leaves (Stout & Meagher, 1948).

4.5.6 Boron

Results from the treated leaves and leaves above and below the B treated leaves are presented in Figure 4.18. Regardless of the time of sampling, application concentration or formulation, the B content of the leaves above and below the treated leaves followed the B concentration in the treated leaves. The maximum concentrations of B in the above and below leaves (79.21 mg.kg^{-1} and 61.09 mg.kg^{-1} respectively) were measured
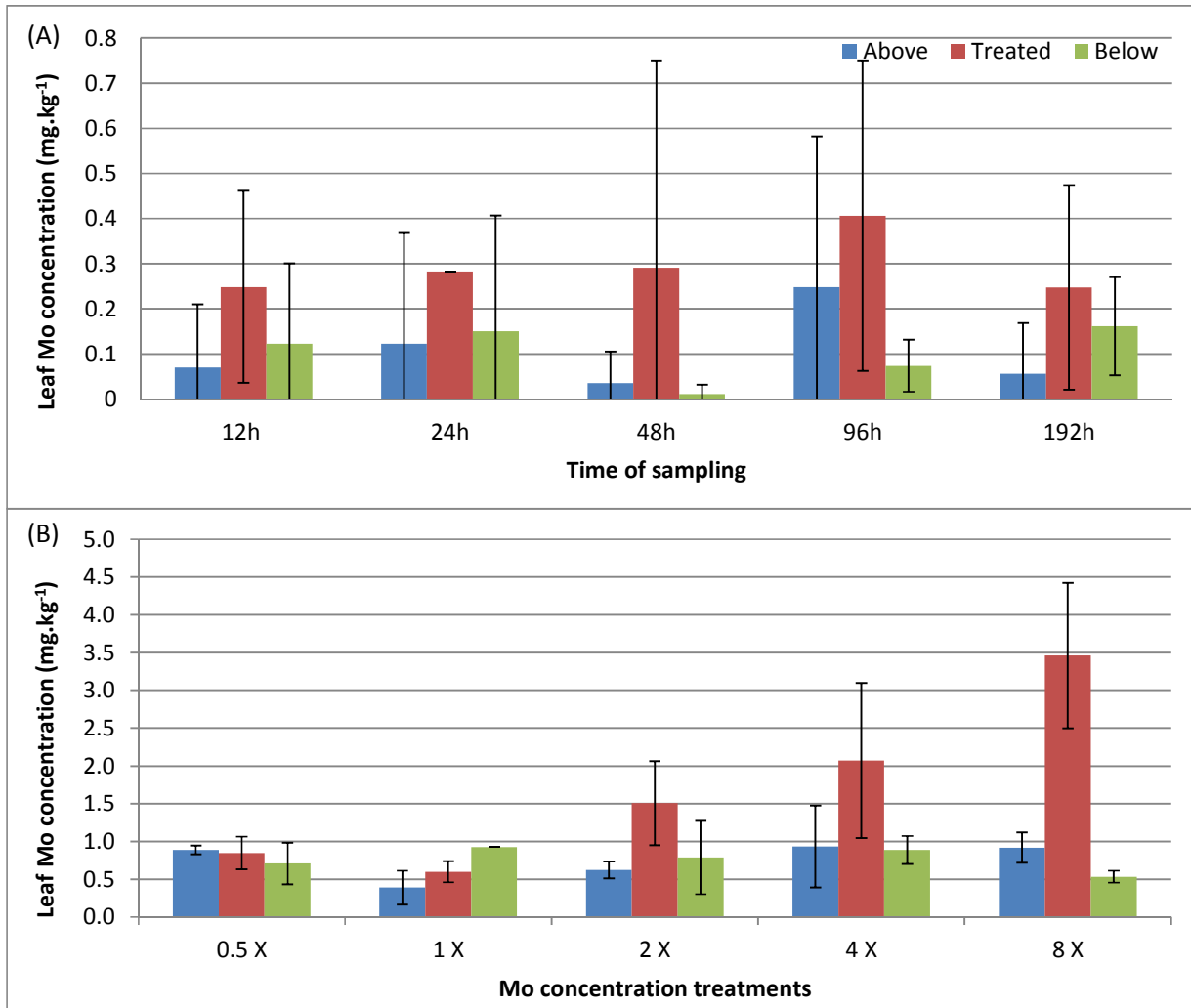
Figure 4.18: Mo concentration in treated leaves and above and below leaves sampled at different times after treatment (A) and treated with different Mo concentrations (B). [Error bars represent standard deviation].

at 48 h, while the lowest B concentrations (40.45 mg.kg^{-1} and 34.81 mg.kg^{-1} respectively) were measured at the 96 h after B application (Figure 4.18 A). The above leaves had consistently higher concentrations of B than the corresponding below leaves for all measurements (Figure 4.18 A and B). Similar results were found by Srivastava & Singh, (2005a) who concluded that the translocation of B in lemon trees take place via the transpiration stream in an upward direction.

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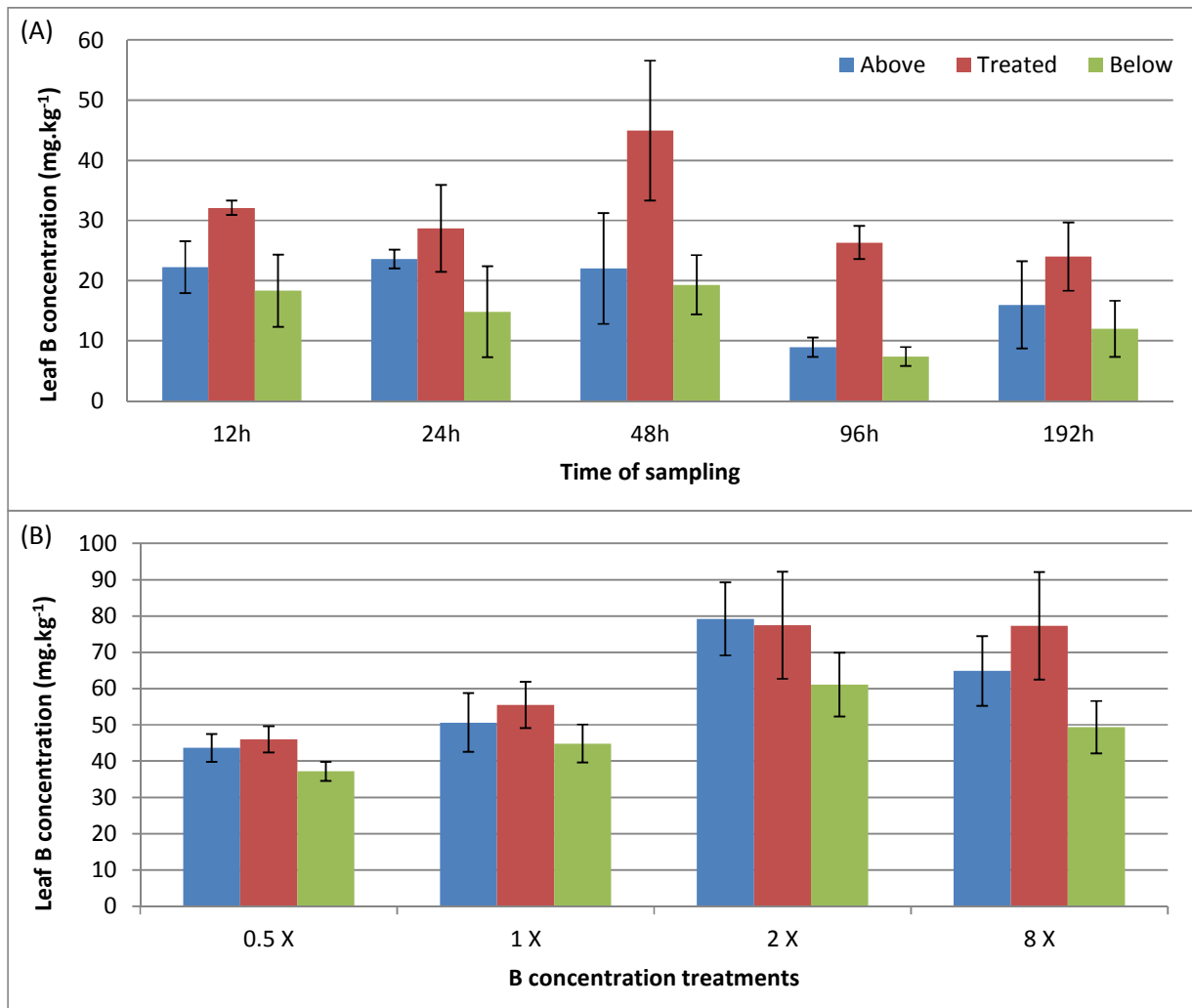
Figure 4.19: B concentration in treated leaves and above and below leaves sampled at different time intervals after treatment (A) and treated with different B concentrations (B). [Error bars represent standard deviation].

4.6   Summary

The application of most products applied to leaves resulted in higher concentrations of that nutrient in the treated leaves.

The highest average Mn concentrations were found in treated leaves 96 h after treatment. 1X Mn AA increased leaf Mn concentrations to the highest Mn concentration

(64.19 mg.kg^{-1}) and 1X Mn-EDTA increased Mn concentrations to the lowest concentration measured (22.183 mg.kg^{-1}) in treated leaves. Mn concentrations in the above and below leaves followed the concentrations of treated leaves. Above leaves had higher concentrations than below leaves, regardless of time of sampling or concentration applied. Considering the optimum Mn concentration in citrus leaves (Table 2.2), Mn concentrations in leaves were low (<34 mg.kg^{-1}) for treatments sampled at different times and at optimum (35-50 mg.kg^{-1}) for most treatments applied at different concentrations.

The application of Zn to leaves, when sampled at different times after treatment, resulted in elevated Zn concentrations, especially for the $ZnSO_4$ and Zn AA treatments. Highest Zn concentrations were found in treated leaves after 96 h. The 8X ZnO treatment resulted in the highest Zn concentration in treated leaves, but were high (>100 mg.kg^{-1}) according to Table 2.2. Lowest Zn concentrations in leaves treated with different Zn concentrations were found for 1X Zn-EDTA and are considered low (Table 2.2). Most leaves treated with different Zn concentrations had optimum Zn concentrations (Table 2.2). Zn concentrations in above and below leaves did not increase as Zn concentrations increased in treated leaves.

Cu treated leaves had highest Cu concentrations at 192 h after sampling. However, due to high variation and non-significant results, 24 h was used as time of sampling (Chamel & Gambonnet, 1979). Cu concentrations in leaves increased to a maximum of 35.65 mg.kg^{-1} when treated with 8X $CuSO_4$. Lowest Cu concentrations were found in leaves treated with 0.5X Cu AA. Cu concentrations in leaves above and below the treated leaves did not follow the Cu concentration of treated leaves. Leaf Cu

concentrations were high (Table 2.2) for leaves treated with different $CuSO_4$ concentrations and were optimal for most leaves treated with different Cu concentrations.

Leaves that were treated with Fe had highest Fe concentrations 192 h after treatment. Maximum Fe concentrations (198.39 mg.kg^{-1}) were measured in leaves that received the 4X $FeSO_4$ treatment, while lowest concentrations (51.37 mg.kg^{-1}) were found in leaves treated with 0.5X Fe-EDTA. Fe concentrations in above and below leaves did not follow Fe concentrations in the treated leaves. The majority of Fe concentrations in leaves treated with different concentrations of Fe was within the optimal range for citrus leaves (Table 2.2).

Leaves treated with H_3BO_3 had highest B concentrations at 48 h. Leaves treated with 2X and 8X H_3BO_3 had maximum B concentrations (77.46 mg.kg^{-1} and 77.31 mg.kg^{-1}, respectively) although these concentrations are low according to Table 2.2. The B concentrations in above and below leaves followed the B concentrations in the treated leaves and the above leaves had consistently higher B concentrations than below leaves. All H_3BO_3 treatments resulted in leaf B concentrations that are considered low for citrus leaves (Table 2.2).

When leaves were treated with $Na_2MoO_4.2H_2O$, highest Mo concentrations were found in leaves after 192 h although no significant differences were found. Bukovac & Wittwer (1957) however reported 48 h as the optimal time of sampling.   Highest Mo concentrations (3.46 mg.kg^{-1}) were found in leaves treated with 8X Mo, while lowest Mo concentrations (0.60 mg.kg^{-1}) were found in leaves treated with 1X Mo.   Mo

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concentrations in above and below leaves did not follow those of treated leaves. Leaf

Mo concentrations at 4X and 8X are considered optimal, while all other leaves have low

Mo concentrations (Table 2.2).

Chapter 5 Leaf anatomy of different citrus species

5.1 Introduction

In any horticultural crop, including citrus, information on the properties of leaf surfaces is important for the foliar applications of leaf nutrients, growth regulators, fungicides and pesticides. The life span of a citrus leaf can vary between nine and 24 months. Leaf growth usually occurs during the shoot growth flushes in autumn and spring, when it will expand in lamina length and width for up to 130 days. The leaves have a soft texture and appear light green during this period. As the leaf matures, it becomes dark green and thickening of the cuticle and veins occur (Scott *et al.*, 1948). Younger leaves have a much lower diffusion resistance (3.4 s.cm^{-1}) than older leaves (7.2 s.cm^{-1}) (Albrigo, 1977), so that foliar applied products are absorbed more readily by young leaves (Mattos *et al.*, 2012). According to Bondada *et al.*, (2006) and Bukovac & Petracek (1993), epicuticular waxes, together with cuticle thickening, decrease the penetration of leaf applied solutes.

For the investigation in the leaf anatomy of different citrus species, scanning electron microscope images were taken of the adaxial and abaxial surfaces and of fractured leaf surfaces from young and mature leaves.

5.2 Adaxial and abaxial leaf surfaces

Baker & Procopiou (1975) studied the composition of intracuticular waxes in leaves and fruits of different citrus cultivars. They found that orange (*Citrus sinensis* 'Frost' Valencia) leaves (80 cm^2) had the highest cuticular membrane weight (316 µg.cm^{-2}) and the largest cuticular wax content (68 µg.cm^{-2}). Smaller (52 cm^2) 'Clementine' (*Citrus reticulata* Blanco cv. 'Clementine') leaves had a cuticular membrane weight of 310 µg.cm^{-2} and a cuticular wax content of 34 µg.cm^{-2} (Baker & Procopiou, 1975). The cuticular waxes consist of a high percentage fatty acids. The amount of fatty acids in cuticular wax varies among the different *Citrus* cultivars e.g. the fatty acids constituted 1.6% of the total surface waxes in lemons and 19.8% in Valencias (Baker *et al*., 1975). The results from these studies indicate that the wax composition and quantity on the leaf surfaces of the different species may differ to very large degrees.

All leaves contained elliptically shaped stomata only on the abaxial surfaces, regardless of leaf age, and citrus leaves are therefore hypostomatous (Turrell, 1947). Although the number of stomata on the leaves was not recorded in this study, Hirano, (1931) reported that leaves of grapefruit and lemons had greater stomatal densities than navels (lowest density) and Valencia (second lowest density) and that precipitation during leaf development may influence the density of stomata.

5.2.1 'Bahianinha' navel leaves

In Figure 5.1 images of young and mature 'Bahianinha' leaves are shown. While the adaxial surface (Figure 5.1 A and B) is relatively smooth underneath the epicuticular

wax, some grooves are formed on the abaxial leaf surface (Figure 5.1 C and D) over the anticlinal walls of the underlying epidermal cells. Small clusters of wax that are lighter in colour than the rest of the leaf surface are visible on the adaxial and abaxial leaf surfaces for the larger magnification of the young 'Bahianinha' leaves (Figure 5.1 A and C). An amount of flakiness is visible in especially Figure 5.1 A but also in C, as lighter-coloured particles, although impurities may also contribute to its appearance.

Images are presented of the adaxial (Figure 5.1 E and F) and abaxial (Figure 5.1 G and H) surfaces of a mature 'Bahianinha' navel leaf. Grooves over the anticlinal epidermal cell walls are visible on the abaxial leaf surface, while the adaxial surface is relatively smooth. The mature 'Bahianinha' leaf's epicuticular wax had a rougher texture and seemed more weathered than the young leaves with some globular individual wax aggregates (Figure 5.1 E, F, G, and H), that, together with wax platelets, forms a layer of wax over the leaf surface. Young leaves show a smooth surface with less dense wax deposits compared to the older leaves.

5.2.2 'Washington' navel leaves

The surfaces from young and mature 'Washington' navel leaves are shown in Figure 5.2. On the abaxial side of the young 'Washington' navels leaves (Figure 5.2 C and D) an uneven surface with a wrinkled appearance and groove formation over the anticlinal cell walls is visible. The wrinkled appearance may be artifacts formed as a result of the drying procedure, but has been reported by Leece (1976) to occur in air- and freeze-dried specimens and may therefore be present *in vivo* as well.

Grooves are visible on the abaxial surface of the mature leaf. A closely packed

Figure 5.1: The adaxial (A and B) and abaxial (C and D) surface of a young, and the adaxial and (E and F) abaxial (G and H) surface of a mature 'Bahianinha' navel leaf. A, C, E and G are at 5000X and B, D, F and H at 700X magnification. The arrow indicates groove formation.

Figure 5.2: The adaxial (A and B) and abaxial (C and D) surface of a young, and the adaxial and (E and F) abaxial (G and H) surface of a mature 'Washington' navel leaf. A, C, E and G are at 5000X and B, D, F and H at 100X magnification.

arrangement of thin wax plates can be seen (Figure 5.2 E and F) on the adaxial surface of the mature leaf, contrasting with the wax platelets on the abaxial leaf surfaces (Figure 5.2 C, D, G and H). Wax plates on the abaxial leaf surface are arranged less dense. Mature leaves had denser wax deposits on both abaxial and adaxial surfaces compared to the younger leaves (Figure 5.2 E, F, G and H).

5.2.3 'Midknight' leaves

In Figure 5.3 images of young and mature 'Midknight' leaves are shown. Slight undulations, or groove formation, can be seen in Figure 5.3 (C, F and G) in 'Midknight' leaves, and is reported by Turrell (1947) to be caused by anticlinal epidermal cell walls under the epicuticular wax. Small platelets of wax can be seen on the surface of the adaxial surface of the young (Figure 5.3 A and B) and mature (Figure 5.3 E and F) 'Midknight' leaves. Many individual wax aggregates are visible on the surface of the young 'Midknight' leaves. The same wax structure is visible on the abaxial surface of the young leaves (Figure 5.3 C and D), but as a continuous sheet, in contrast to the platelets on the adaxial surface. The surfaces of the young leaves underlying the surface wax have an amorphous appearance, whereas the surfaces of the mature leaves are weathered. This agrees with Leece (1976) who found that adaxial surface of Valencia leaves have an amorphous wax layer with a superimposed secondary structure of platelets.

Leaf surfaces of the mature 'Midknight' leaves (Figure 5.3 E and G) have a coarser texture with heavier wax deposits than the young leaves (Figure 5.3 A and C), although

Figure 5.3: The adaxial (A and B) and abaxial (C and D) surface of a young, and the adaxial and (E and F) abaxial (G and H) surface of a mature 'Midknight' leaf. A, C, E and G are at 5000X and B, D, F and H at 100X magnification.

the images are at the same magnification (5000X). This corresponds with Baker *et al*., (1975) who reported that younger 'Frost Valencia' leaves have less wax deposits than older leaves. On the abaxial surface of the mature leaf some wax platelets can be seen protruding from the matrix (Figure 5.3 G and H). Some grooves on anticlinal walls are visible underneath the epicuticular wax, on abaxial surfaces of both the young and mature leaves of 'Midknight', although not pronounced.

5.2.4 'Satsuma' mandarin leaves

The leaf surfaces of young and mature 'Satsuma' mandarin leaves are shown in Figure 5.4. Slight stomatal grooves are observed. Individual aggregate wax plates are densely packed in clusters on the young adaxial leaf surface (Figure 5.4 A and B). The abaxial leaf surface (Figure 5.4 C and D) shows less cluster arrangement of wax than the adaxial leaf surface, so that the surface is covered almost entirely by the wax layer (Figure 5.4 D and H).

The wax plate clusters on the mature 'Satsuma' mandarin leaves are more weathered (Figure 5.4 E, F, G and H) with more wax deposits than the young leaves (Baker *et al.,* 1975). On the abaxial surface of the mature 'Satsuma' mandarins a continuous sheet of wax platelets (Figure 5.4 G and H) is clearly visible.
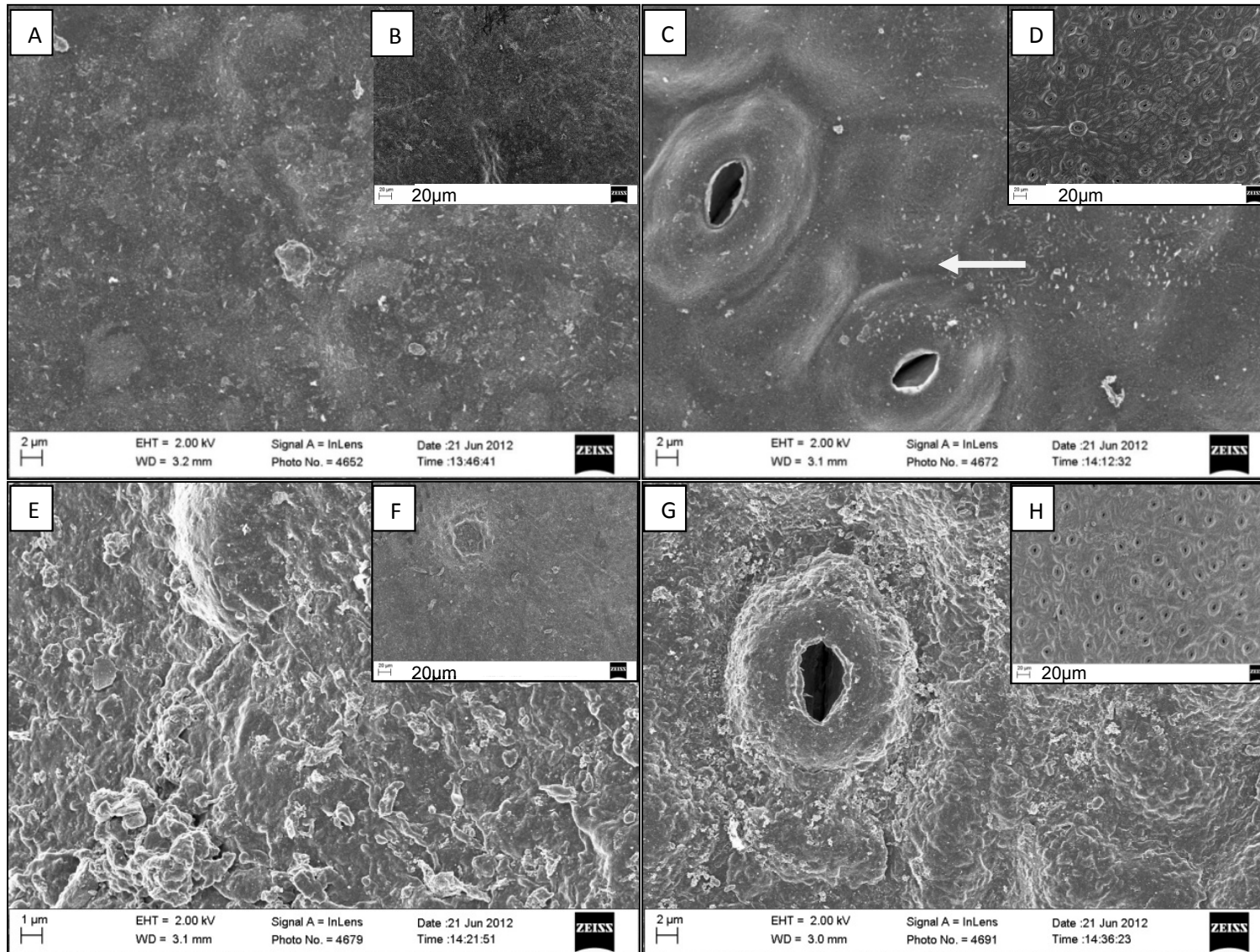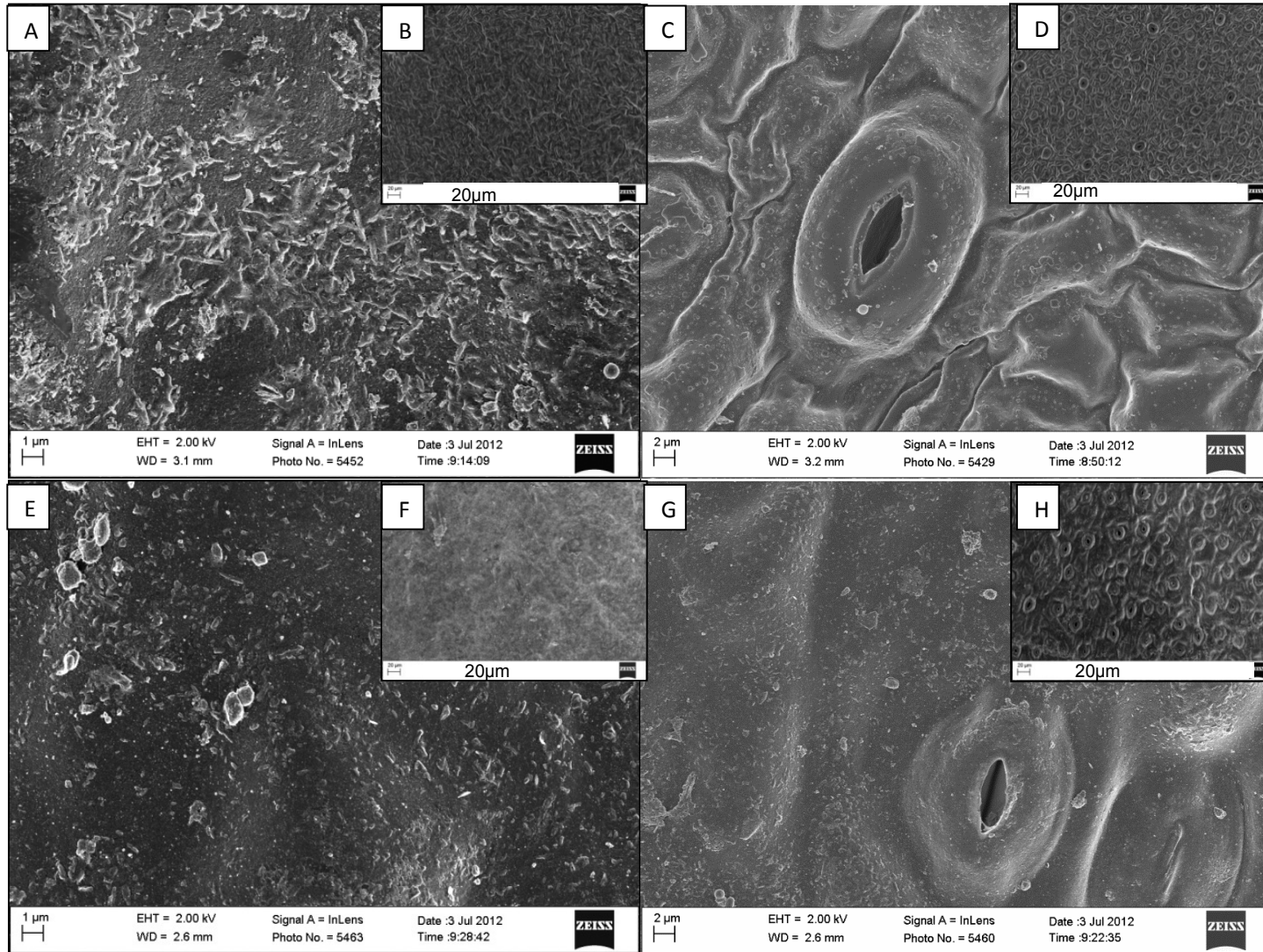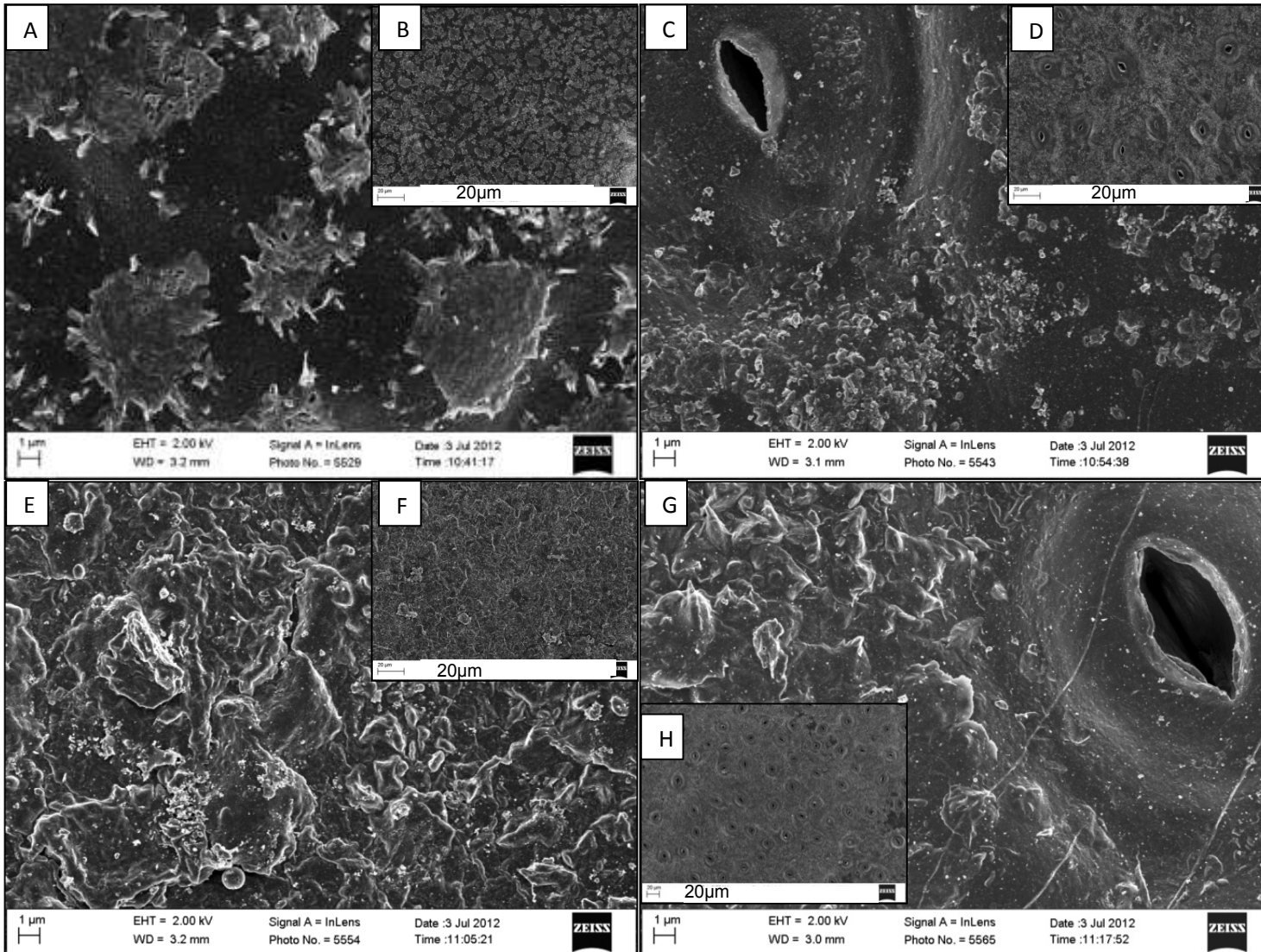
Figure 5.4: The adaxial (A and B) and abaxial (C and D) surface of a young, and adaxial and (E and F) abaxial (G and H) surface of a mature 'Satsuma' mandarin leaf. A, C, E and G are at 5000X and B, D, F and H at 100X magnification.

5.2.5 'Genoa' lemon leaves

In Figure 5.5 scanning electron microscope images are shown of young and mature 'Genoa' lemon leaves. Groove formation between adjacent epidermal cells, due to the conical surfaces of the cells, is clearly visible on the abaxial surfaces of young and mature leaves. An amorphous layer of wax can be seen covering the adaxial and abaxial surfaces of the young 'Genoa' lemon leaves (Figure 5.5 A, B, C and D), although some platelets are visible on the adaxial surface (Figure 5.5 A and B). This correlates with findings from Jeffree *et al*., (1975) that the leaf surface of *Citrus limon* 'Adamopoulou' has an amorphous wax layer, while recrystalised waxes show platelet formation.

The surfaces of mature leaves have the same amorphous wax layer with a more weathered appearance and the surface wax has expanded to form a continuous layer over the entire leaf surface (Figure 5.5 E, F, G and H). Clustering of wax can still be observed on the adaxial surface of the mature 'Genoa' lemon leaf (Figure 5.5 E and F). Some stomata are partially or completely covered with wax and may therefore not be fully functional. In the grooves that are visible on the mature 'Genoa' lemon leaf, more wax is present than on the young 'Genoa' lemon leaf. These grooves are on the outer side of the radial surfaces which aid in apoplastic translocation inside the leaf. In younger leaves the grooves may be filled with foliar applied substances which may improve leaf uptake, while more wax is present in the grooves of the older leaves that may result in less foliar applied substances in the grooves and less uptake.
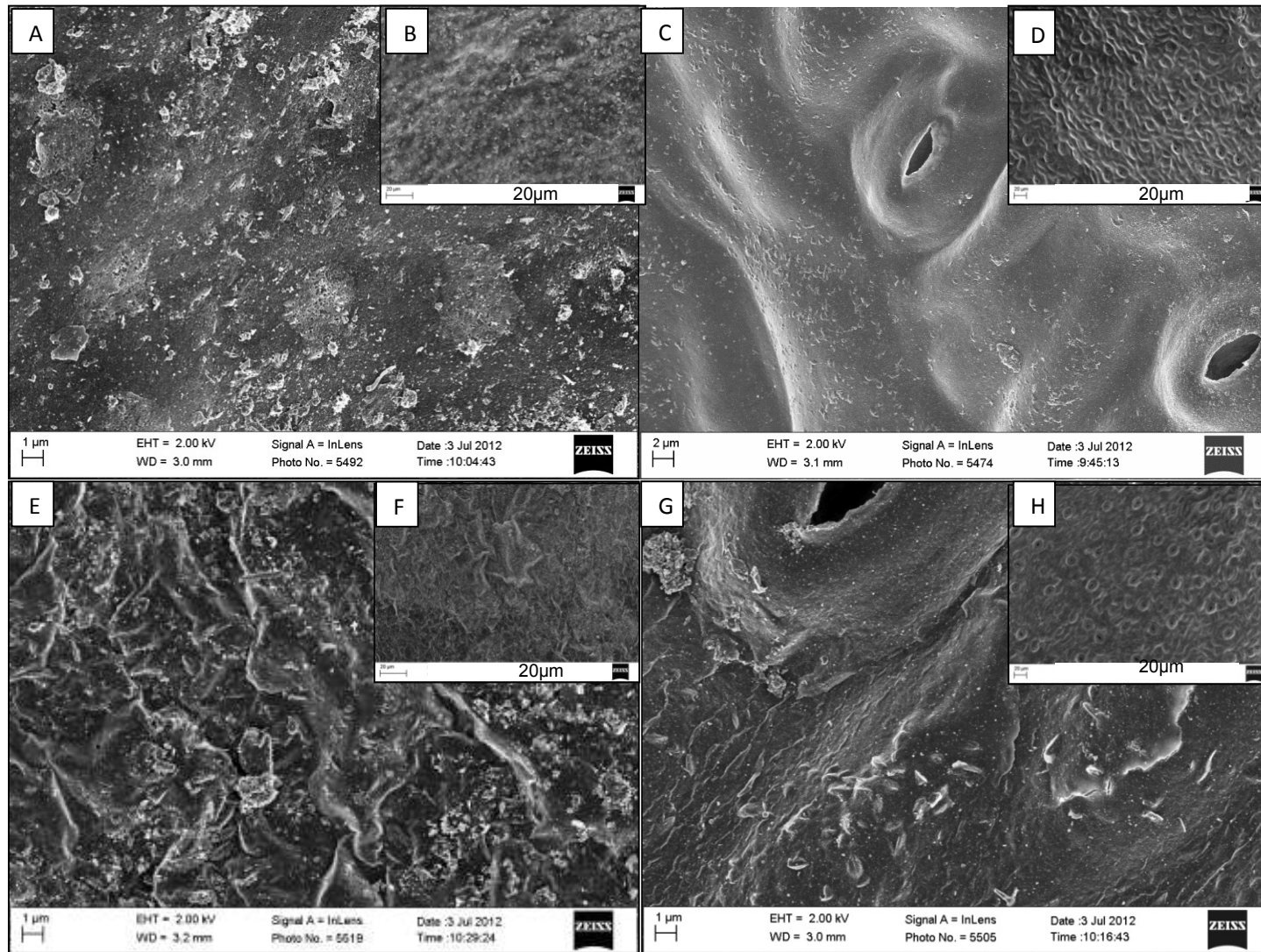
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Figure 5.5: The adaxial (A and B) and abaxial (C and D) surface of a young, and the adaxial and (E and F) abaxial (G and H) surface of a mature 'Genoa' lemon leaf. A, C, E and G are at 5000X and B, D, F and H at 100X magnification.

5.2.6 'Star Ruby' grapefruit leaves

Scanning electron microscope images are shown of the adaxial and abaxial surface of a young and mature 'Star Ruby' grapefruit leaf. The adaxial leaf surface of a young 'Star Ruby' grapefruit (Figure 5.6 A and B) has an even leaf surface with grooves due to conical surfaces of the epidermal cells (Figure 5.6 C and D). On the abaxial surface an amorphous wax layer is visible (Figure 5.6 C and D), while wax platelets can be seen on the adaxial surface of the young 'Star Ruby' grapefruit leaf (Figure 5.6 A and B). These platelets are distributed over the entire surface, and are not in a particular arrangement, such as mounds or clumps, so that a continuous layer is formed. These observations are similar to observations made of immature grapefruit leaf surfaces by Bondada *et al*., (2006).

The abaxial surfaces of the mature 'Star Ruby' leaf do not show pronounced weathering like other citrus species, although some increase in surface texture can be observed (Figure 5.6 G and H), while the same even underlying leaf surface is present as in young 'Star Ruby' grapefruit leaves. Orbovic *et al.,* (2001a) found that cuticles from mature 'Marsh' grapefruit leaves were not weathered extensively to reveal structural damage due to age and were covered with layer of epicuticular wax platelets scattered over the area (Orbovic *et al.,* 2001b). Bondada *et al*., (2006) reported an increase in epicuticular wax as platelets as the leaf matured.

5.2.7   Comparison among species

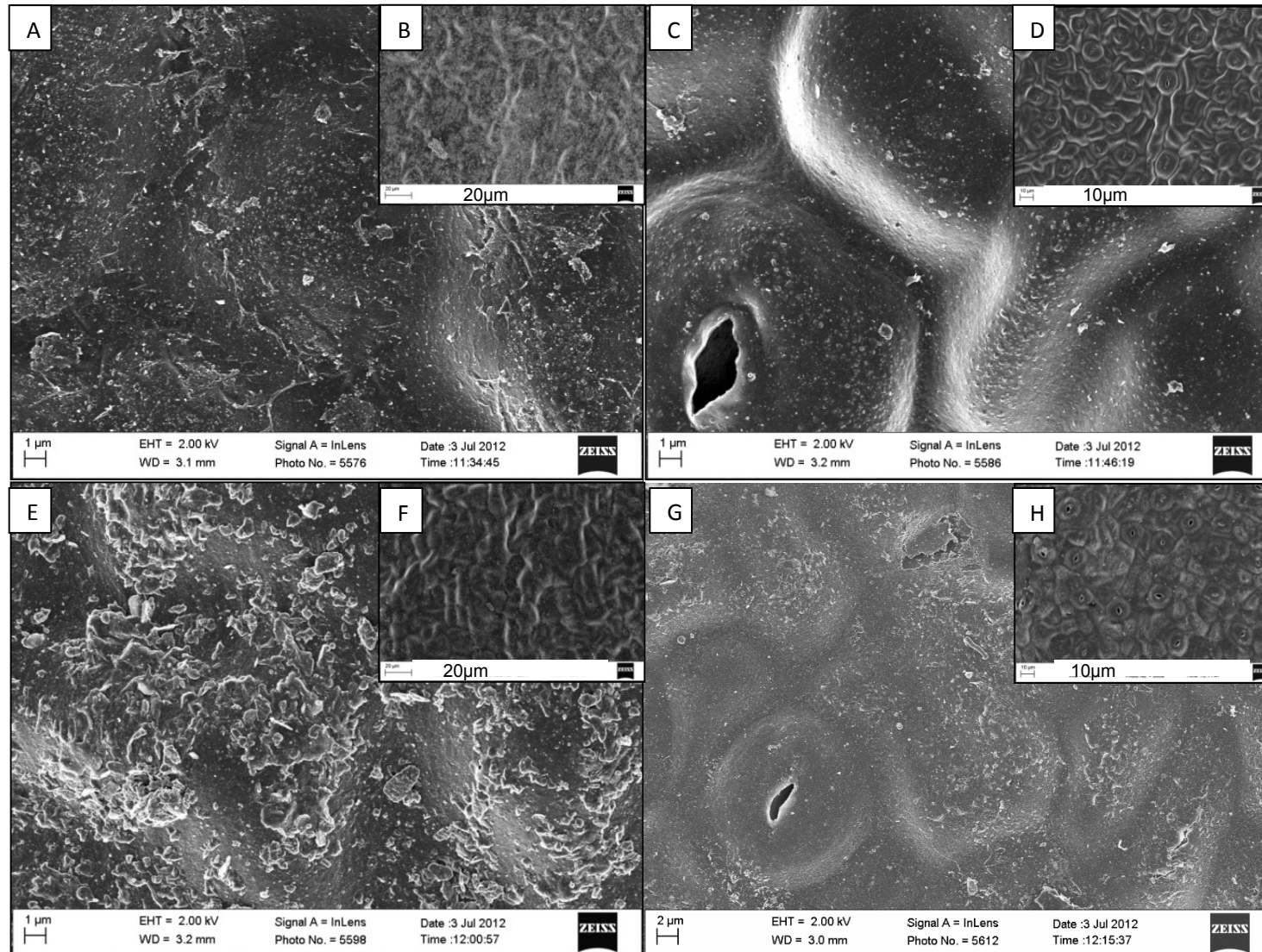Groove formation over anticlinal epidermal cell walls was visible in all species on the
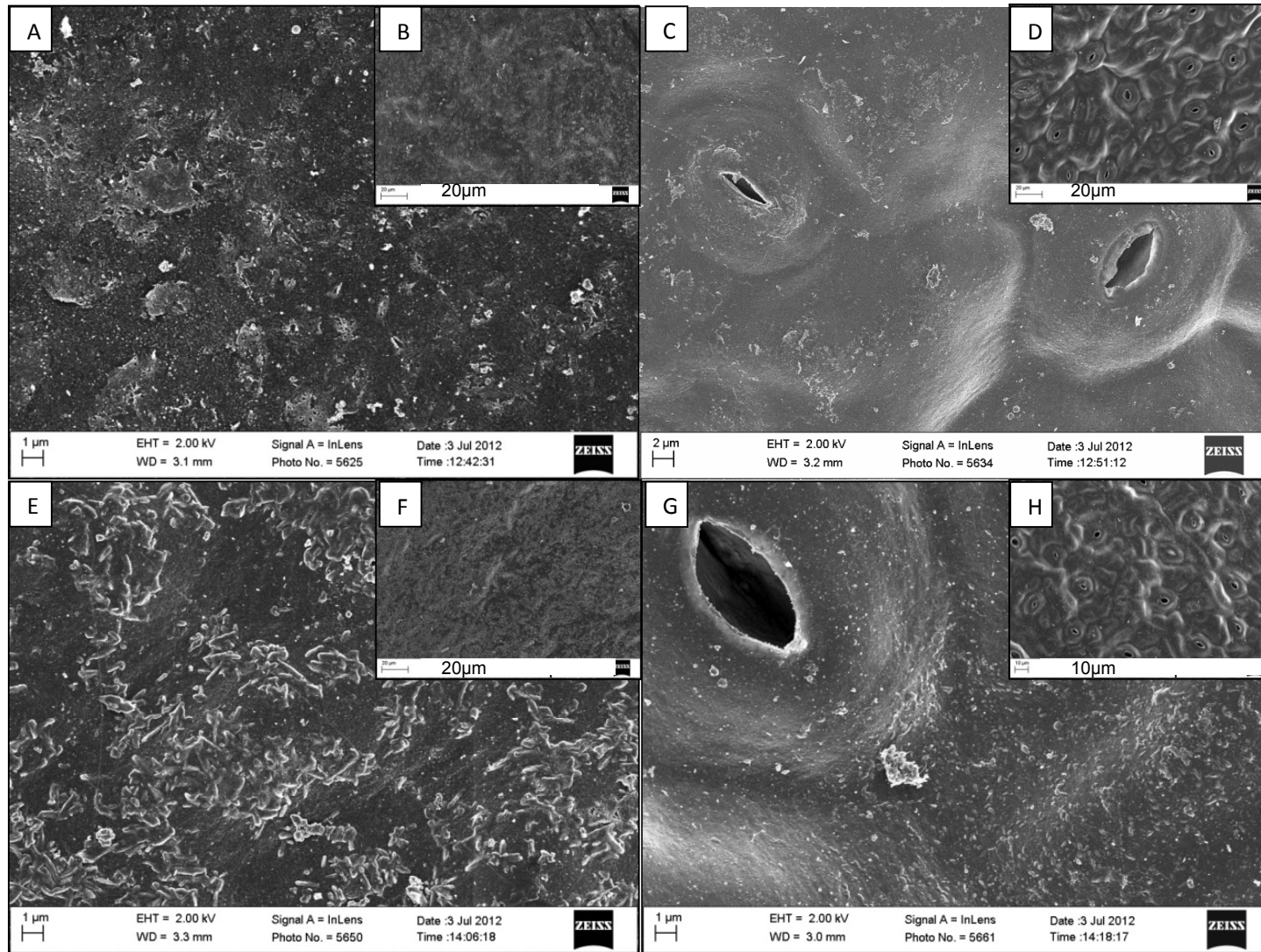
Figure 5.6: The adaxial (A and B) and abaxial (C and D) surface of a young, and the adaxial and (E and F) abaxial (G and H) surface of a mature 'Star Ruby' grapefruit leaf. A, C, E and G are at 10000X and B, D, F and H at 1500X magnification.

abaxial leaf surfaces. Some groove formation was also visible on the adaxial leaf surface of young and mature 'Genoa' lemon leaves.

Epicuticular wax deposit structures did not differ greatly between species. Wax platelets were visible on all species, in most instances together with amorphous wax layers and especially in younger leaves, individual wax aggregates or wax clusters. More epicuticular wax deposits are seen on the mature leaves, especially on the adaxial leaf surfaces of 'Bahianinha' navels (Figure 5.1 E and F), 'Midknight' (Figure 5.3 E and F) and 'Satsuma' mandarin (Figure 5.4 E and F).

The clusters formed by wax plates on the adaxial surface of 'Satsuma' mandarin leaves (Figure 5.4 A and B) are visually larger in diameter than those observed on the adaxial surfaces of the young 'Bahianinha' navel and 'Midknight' leaves (Figure 5.1 A and B, and Figure 5.3 A and B respectively) but the spaces between the clusters are also visually larger than those of the 'Midknight' and the 'Bahianinha' navels.

5.3   Cuticle thickness of different citrus species

Fracture surfaces were imaged by SEM to determine the thickness of the adaxial and abaxial cuticles for different citrus leaves. According to Peacock (2000), the primary cell wall of tobacco leaves consists of five layers of fibrils that are arranged perpendicular to the leaf surface. Similar layers were observed in the different citrus leaves, so that a clear observation could be made as to where the cuticle ended and the cell wall started, as indicated by the line in Figure 5.7.

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200 nm	EHT = 2.00 kV	Signal A = InLens	Date :24 Jul 2012	ZEISS
	WD = 3.6 mm	Photo No. = 7526	Time :14:22:49	
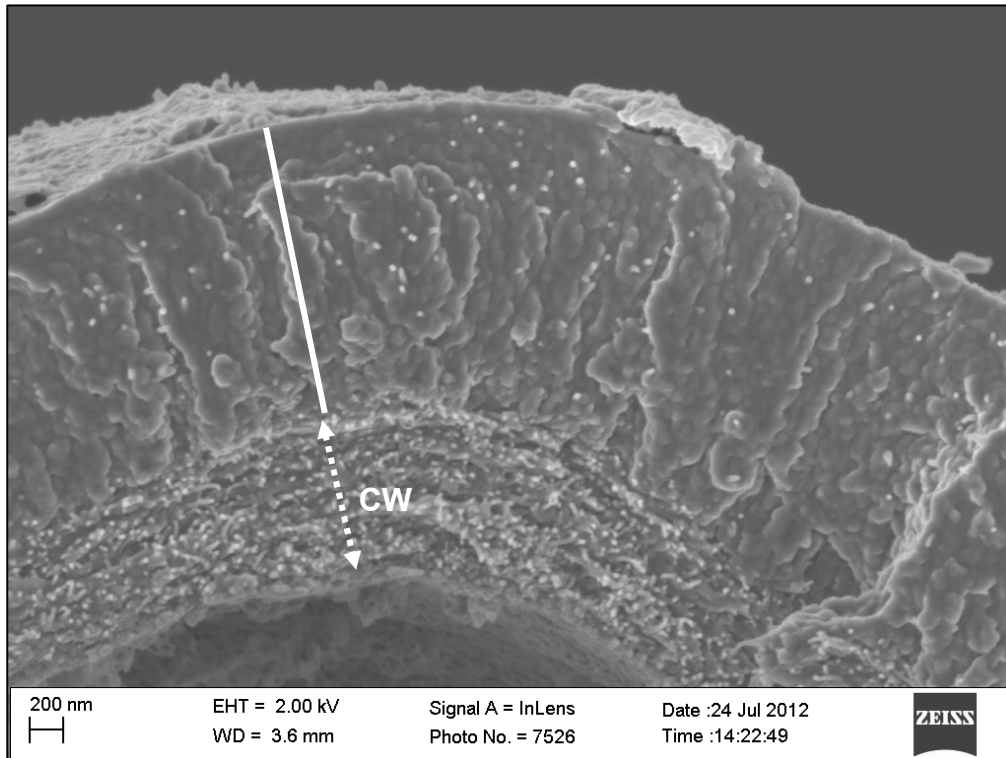
Figure 5.7: The abaxial cuticle of a mature 'Genoa' lemon leaf. The solid line indicates cuticle thickness while the broken line indicates the cell wall (CW, cell wall).

Leaf abaxial and adaxial cuticles were measured for young and mature leaves of different citrus species (Table 5.1). In young leaves the abaxial cuticle thickness ranged from 335-2496 nm and the adaxial cuticle thickness ranged from 486-2346 nm. In mature leaves the abaxial cuticle thickness ranged from 1399-2437 nm and the adaxial cuticle thickness ranged from 1700-2492 nm.

When young leaves were compared to the mature leaves it was found that the abaxial cuticle thickness of the young leaves for the 'Bahianinha' and 'Washington' navels, 'Genoa' lemon and 'Star Ruby' grapefruit was thinner than the abaxial cuticles of the mature leaves. This is in accordance with Orbovic et al., (2001a) who reported cuticles of young 'Marsh' grapefruit leaves to be thinner than those found on mature leaves. However, for the 'Midknight' and 'Satsuma' mandarins the abaxial cuticles of the young leaves was thicker than the abaxial cuticles of the mature

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Table 5.1: Cuticle thicknesses of different *Citrus* cultivars. Values given are means with ± standard error (means with the same letter do not differ significantly at α=0.05).

Specie	Leaf age	Abaxial cuticle (nm)	Adaxial cuticle (nm)
'Bahianinha' navel	Young	1845 ± 52^{efg}	2263 ± 59^{b}
	Mature	2437 ± 59^{a}	2087 ± 50^{c}
'Washington' navel	Young	335 ± 30^{l}	486 ± 59^{k}
	Mature	1695 ± 57^{fg}	2027 ± 46^{cd}
'Midknight'	Young	2035 ± 69^{cd}	1694 ± 66^{fg}
	Mature	1849 ± 43^{efg}	1780 ± 57^{efg}
'Satsuma' mandarin	Young	2496 ± 63^{a}	2346 ± 73^{ab}
	Mature	1399 ± 46^{h}	1929 ± 61de
'Genoa' lemon	Young	745 ± 50^{j}	1254 ± 57^{i}
	Mature	1923 ± 50de	1700 ± 66^{fg}
'Star Ruby' grapefruit	Young	1691 ± 59^{g}	1779 ± 55^{efg}
	Mature	1857 ± 37^{ef}	2492 ± 48^{a}

leaves (Table 5.1).

For 'Bahianinha' navels, the abaxial cuticle of the young leaf was thinner than the adaxial cuticle while the adaxial cuticle of the mature leaves was thinner than the abaxial cuticle. A large difference was observed between the cuticle thickness of the young and mature leaves of 'Washington' navels. The abaxial cuticles for both the young and mature leaves were thinner than the adaxial cuticles (Table 5.1). For 'Midknight' the abaxial cuticle of the young leaves was thicker than that of the mature leaves, but the adaxial cuticle of the young leaves was thinner than that of the mature leaves. In a study by Leece (1976) Valencia leaf cuticles were measured to have a cuticle thickness of 4.2 ± 0.1 μm for the adaxial cuticle and 3.9 ± 0.2 μm for the abaxial cuticle when cuticles were isolated enzymatically. These values are larger than those observed in this study and differences may be due to differences in specimen preparation methods and environmental conditions. The abaxial cuticle

from the young 'Satsuma' leaves was thicker than the abaxial cuticle of the mature leaves. The adaxial cuticle of the young 'Satsuma' leaves was also thicker than the adaxial cuticle of the mature leaves. For 'Genoa' lemon, the abaxial cuticle of young leaves was less than half the thickness of the abaxial cuticle of the mature leaves. The adaxial cuticle of young 'Genoa' lemon leaves was thicker than the abaxial cuticle, but still thinner than the mature 'Genoa' lemon leaf's adaxial cuticle. 'Star Ruby' grapefruit cuticles were significantly thicker on the mature leaves than the younger leaves although the adaxial and abaxial cuticles of young leaves did not differ significantly. Cuticles of the adaxial leaves were significantly thicker than those of the abaxial leaves of the mature 'Star Ruby' leaves.

'Washington' navels had the thinnest adaxial and abaxial cuticle in the young leaves while 'Genoa' lemon leaves had slightly thicker cuticles on the young leaves than the 'Washington' navels. The thickest abaxial and adaxial cuticle on young leaves was measured on 'Satsuma' mandarin, while 'Bahianinha' navels had the second thickest adaxial cuticle and 'Midknight' the second thickest abaxial cuticle on young leaves.

For the mature leaves, the thinnest abaxial cuticle was observed on 'Satsuma' mandarin, and the second thinnest on 'Washington' navels. The thinnest adaxial cuticle was measured on mature 'Genoa' lemon leaves and the second thinnest on 'Midknight' and 'Star Ruby' grapefruit leaves. Thickest and second thickest abaxial cuticles were found on mature 'Bahianinha' navel and 'Genoa' lemon leaves respectively. Mature 'Star Ruby' grapefruit leaves had the thickest adaxial cuticle and mature 'Bahianinha' navel leaves the second thickest.

Jyung & Wittwer (1964) stated that cuticle thickness may contribute to differences in uptake rates of foliar nutrients. Differences in the orientation and density of surface

wax deposits are responsible for the differential penetration rates between adaxial and abaxial leaf surfaces (Bukovac & Norris, 1966). According to Mattos *et al*., (2012), young citrus leaves have poorly developed cuticles and foliar applications should therefore be made on spring and summer flushes to facilitate better uptake and supply to plant organs. Lea-Cox & Syvertsen (1995) hypothesised that citrus leaves have higher epicuticular wax concentrations as the leaves aged, since they found that urea penetrated young citrus leaves six times more than the old leaves. This was confirmed by Bondada *et al.,* (2001) who found that younger leaves have lower cuticle weights than older leaves. Cuticles from young leaves that are thicker than cuticles from mature leaves may be due to environmental conditions that influenced the development of those leaves.

5.4   Summary

Leaf uptake rates and concentrations that were found suitable for foliar applications to 'Midknight' in Chapter 4, may give indications to those expected for other citrus species, according to their relative cuticle thickness. Although some observations were made regarding surface wax, no considerable differences were found that may affect penetration of foliar applied micronutrients relative to 'Midknight' leaves for the young and mature leaves respectively.

Differences were observed between young and mature leaves. Mature leaves had larger amounts of visible cuticular surface wax than young leaves. This was expected since cuticles are not fully developed in young leaves and cuticle thickening is in progress (Bondada *et al*., 2006). Cuticle thicknesses for the different citrus species differed among the leaf age and the abaxial and adaxial leaf surfaces.

'Bahianinha' navel, 'Satsuma' mandarin and 'Star Ruby' grapefruit leaves had thicker cuticles than 'Midknight' leaves (exceptions are abaxial cuticles of young 'Bahianinha' navel and 'Star Ruby' grapefruit leaves and abaxial cuticles of mature 'Satsuma' mandarin leaves that were thinner). 'Washington' navel and 'Genoa' lemon leaves had thinner cuticles than 'Midknight' leaves and these species are expected to absorb leaf applied products more rapidly and at higher concentrations than 'Midknight', 'Bahianinha' navel, 'Satsuma' mandarin and 'Star Ruby' grapefruit.

Chapter 6   Economic evaluation of the different formulations

Crop production costs are rising and researchers are urged to look at more cost effective ways of producing economically viable quality crops while considering the environment (Abadia *et al*., 2002; Fageria *et al.,* 2009). Prices, excluding delivery/transport fees, (in South African Rand, $1=R10.67) were obtained for the products used in this study from reputable agribusinesses that deal directly with the end users (farmers). No allowances were made for the profit margins on the products.

In Table 6.1 the applied concentration of an element, which resulted in either the highest significant concentration for that element in the leaf, or the lowest applied concentration that was not significantly different from the highest measured concentration (determined in Chapter 4.5), was regarded as the most efficient concentration. This reflects the concentration of an individual formulation that, when applied to leaves, would result in the highest concentration of the applied element in the leaf (Table 6.1, Most efficient concentration). The most efficient concentration is then multiplied by the product price to obtain the cost of a single application (Table 6.1, Price of most efficient concentration).

The application of $CuSO_4$ at 2X resulted in the same Cu leaf concentration for Cu-EDTA at 4X and Cu AA at 8X application (Table 6.1). Since the price of $CuSO_4$ is lower than that of Cu-EDTA and Cu AA, $CuSO_4$ may be the more cost effective foliar application of the Cu treatments used in this study. This same trend is seen for the Zn, Mn and Fe treatments. Inorganic formulations ($ZnSO_4$ and ZnO) of Zn at 4X and

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Zn-EDTA at 0.5X are equally effective as Zn AA at 8X. $ZnSO_4$ and ZnO are calculated as the more cost effective sources to apply foliarly. $MnSO_4$ was more cost effective at 1X than Mn-EDTA at 0.5X and Mn AA at 1X as foliar applications. Fe-EDTA proved least cost effective of the Fe sources, while $FeSO_4$ was the most effective and cost effective at 4X. Midwest Laboratories (1994) reported that $FeSO_4$ was effective as foliar sprays on grain sorghum, but was more expensive than inorganic Fe sources. Mordvedt (1991) stated that inorganic sources of fertilisers may be considered as the least costly per unit of micronutrient and that cost should be considered in conjunction with effectiveness. Chelated sources of nutrients may be more costly than non-chelated source but are not always available to farmers (Fagaria *et al*., 2009). Many companies that maintain these products in the market do so since they are by-products of other industrial processes and require costly storage environmentally friendly storage to reduce pollution and health risks (Abadia *et al*., 2002). Small quantities of these products would be used by farmers, so that more commercial or industrial uses are preferred.

Table 6.1: Price comparison of the different formulations when applied at concentrations that resulted in the highest leaf concentration of the element for that specific formulation.

Sampling time	Element	Formulation applied	Product price	Most efficient concentration		Price of most efficient concentration
			$R.g^{-1}$		$g.L^{-1}$	$R.g^{-1} \times g.L^{-1}$
24 h	Cu	$CuSO_4$	0.0054	2	2.212	0.012
		Cu-EDTA	0.0650	4	7.045	0.458
		Cu AA	0.0560[a]	8	24.000[b]	1.334[c]
96 h	Zn	$ZnSO_4$	0.0115	4	9.848	0.113
		ZnO	0.0250	4	4.466	0.112
		Zn-EDTA	0.0450	0.5	2.741	0.123
		Zn AA	0.0390[a]	8	48.000[b]	1.872[c]
96 h	Mn	$MnSO_4$	0.0105	1	1.470	0.015
		Mn-EDTA	0.0450	0.5	1.946	0.088
		Mn AA	0.0440[a]	1	5.000[b]	0.220[c]
192 h	Fe	$FeSO_4$	0.0030	4	8.120	0.024
		Fe-EDTA	0.0420	1	3.073	0.129
		Fe AA	0.0370[a]	0.5	3.000[b]	0.111[c]

a= $R.ml^{-1}$; b= $ml.L^{-1}$; c= $R.ml^{-1} \times ml.L^{-1}$.

Chapter 7   General discussion and conclusion

Foliar applications of differently formulated micronutrients were made on *Citrus sinensis* 'Midknight' under greenhouse conditions at the Experimental Farm of the University of Pretoria. To determine the most suitable sample preparation method, leaves were treated with solutions containing different formulations of Mn and one formulation of B. Treated leaves, as well as leaves directly above and below the treated leaves on the same shoot were sampled 48 h after applications were made. Leaf sap was extracted from one half of the leaves with a hydraulic press and the other half were wet-acid digested with a microwave. Microwave assisted digestion (MAD) proved to be the more suitable sample preparation method since sample size restricted the practicality of the hydraulic plant sap press. The remainder of the sample preparation in this study was done via the MAD method.

The time it takes for micronutrients to move from the leaf surface into leaf, across the cuticle membrane, is influenced by humidity (Ramsey *et al.,* 2005), cuticle thickness (Bondada *et al*., 2006), spray volumes applied (Bukovac & Petracek, 1993) and plant metabolism. Therefore, to minimize climatic variability, a time step study was conducted in a greenhouse to determine the time of sampling. This was done by dipping different citrus leaves into different micronutrient solutions and then sampling the leaves at different time intervals. The best time of sampling was taken as the time interval with the highest average concentration of the different formulations for the respective element in the leaves. For Mn and Zn the best sampling time was taken as 96 h. Due to the high standard deviation for the different treatments, the best sampling time was taken as 24 h after Cu was applied, according to

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recommendations by Chamel & Gambonnet (1979) and for Fe 192 h after the foliar application of Fe. The highest B concentration was measured 48 h after foliar applications were made and therefore 48 h was taken as the best sampling time for B. Mo was applied with $MnSO_4$ and due to possible antagonistic behavior between S and Mo the best time for sampling was not successfully determined for Mo. Therefore, according to recommendations made by Bukovac & Wittwer (1957) the suitable time for sampling was taken as 48 h in the remainder of the study. From these results it is clear that the elemental concentrations in the treated leaves increased for some of the micronutrients when contact time between the leaf surface and the applied solution increased. Therefore, the hypothesis that longer contact time between a micronutrient and the leaf surface will result in larger quantities of the micronutrients being taken up, was not successfully proven for Mn, Zn, Cu, B and Mo, but was successfully proven for Fe. Fe concentrations inside the leaf increased as the contact time of the applied solution increased.

To determine if the uptake of foliar applied nutrients will increase with an increase in the concentration of the applied nutrients, Mn, Zn, Cu and Fe, formulated as an AA chelate, EDTA chelate or inorganic salt (SO_4 or O), were applied at concentrations calculated as multiples (0.5X, 1X, 2X, 4X and 8X) of the rates recommended by the AA manufacturer. The micronutrients formulated as an inorganic salt resulted in the highest foliar uptake in the majority of the treatments in this study. For the different formulations applied, nutrient concentrations in the treated leaves increased as applied concentrations increased for Cu, Zn, B and Mo but not for Mn and Fe (nutrient concentrations increased, but not relative to applied concentrations). Highest concentrations were found in leaves when applications were made at 8X for Cu and Mo, 4X for Fe and 2X and 8X for B. Mn was the exception, as results were

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not conclusive regarding the most suitable concentration for Mn application to leaves. However, application of Mn AA at 1X resulted in the highest Mn concentration in the leaves. Although the highest Zn concentrations in the treated leaves may have resulted from the ZnO treatment, it is believed that insoluble ZnO residues may have rendered the results biased. The limited water solubility of ZnO may have resulted in the non-effective removal of the surface residues, and these results are not considered further. Therefore $ZnSO_4$ applied at 8X resulted in the highest leaf Zn concentrations.

The application of EDTA formulations of the different micronutrients increased the elemental concentrations in the treated leaves, but not as high as the inorganic or AA formulations. Nutrients applied foliarly as EDTA formulations can be recommended in instances where the use of inorganic and AA formulations is not possible, but may result in lower leaf uptake. Zn-EDTA did not increase Zn concentrations in the leaf to concentrations significantly different from those of the control and Zn-EDTA can therefore not be recommended as an effective Zn source for foliar applications. Application of Zn, Cu and Fe as AA formulations were equally effective compared to inorganic sources in increasing nutrient concentrations in treated leaves when applied at all concentrations for Zn AA, 8X for Cu AA and for Fe AA at 0.5X, 1X, 2X and 8X. The hypothesis that micronutrient uptake is concentration dependent, with the highest concentrations of each micronutrient for each formulation resulting in the highest uptake, is accepted for all micronutrients except Fe and Mn, $CuSO_4$ and Cu-EDTA.

Micronutrients translocate within trees, some to a lesser extent than others, and are essential for metabolic processes to occur in plants. A study was conducted to determine the influence of formulation, contact time and concentration on the

translocation of the applied micronutrients within the tree. The leaves directly above and below the treated leaves were also sampled in the study on the influence of the contact time and concentration of the respective foliar applied micronutrients on uptake. This was done to determine if the concentrations of applied nutrients increased as the nutrient concentrations in the treated leaves increased. For all treatments the elemental concentrations in the leaves below and above the treated leaves were lower than the treated leaves for the different contact times, concentrations and formulations. However, for Mn and B concentrations in leaves above the treated leaves were slightly higher than the elemental concentrations in the below leaves, irrespective of the concentrations in the treated leaves, indicating that Mn and B are translocated mainly in an upward direction via the xylem.

Zn, Cu and Mo concentrations in the leaves above and below the treated leaves did not increase as the applied Zn, Cu and Mo concentrations in treated leaves increased. Therefore, new leaves from growth flushes may not necessarily be supplied with these nutrients from older leaves and more than one foliar application may be necessary per season. In addition, recommendations regarding Cu applications should consider that Cu ($Cu(OH)_2$) may also be applied to plants as part of disease control measures that may result in an increased leaf Cu concentration.

Leaf uptake rates, application concentrations and translocation of the applied micronutrient formulations are some key factors that play a role in the selection of suitable micronutrient formulations for specific phenological stages and nutritional status of trees. In addition, with rising production costs, the most efficient and cost effectiveness of different types of formulations (EDTA, AA or inorganic salts) of micronutrients used as a foliar spray are important factors to consider. Therefore, the most efficient and cost effectiveness of the applied products was determined

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(Table 7.1). The sulphate formulated micronutrients were both the most cost effective and efficient at 4X or 0.5X in all cases (where applicable). $ZnSO_4$ applied at 4X will be the most efficient formulation with lowest cost to the farmer.

Table 7.1: The most cost effective and efficient formulation and nutrient concentration of foliar applied micronutrients on potted 'Midknight' trees.

Nutrient	Most efficient and cost effective	
	Formulation	Concentration
Mn	$MnSO_4$	4X
Zn	ZnO / $ZnSO_4$	4X
Cu	$CuSO_4$	0.5X
Fe	$FeSO_4$	0.5X
B	H_3BO_3	2X
Mo	$Na_2MoO_4.2H_2O$	4X

The hypothesis that AA formulated micronutrients would be the most efficient but least cost-effective could not be proven successfully, because results from this study showed that the AA formulated micronutrients were the least cost-effective (except Fe where Fe-EDTA was the least cost effective) and were less efficient than the inorganic formulations (Table 7.1).

The foliar application of different formulations, at the same elemental concentration, did not necessarily result in the same elemental concentration in the treated leaves. The sulphate formulation performed the best in this study, but choice of product is influenced by product price, product availability, ease of use, compatibility in the application mixture and antagonism between nutrients and/or products in the spray

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solution. Regardless of whether applications of micronutrients are made when nutrients are deficient or as part of planned production practices, reliable soil and leaf analyses should be the basis of recommendations made. This is applicable to recommendations made for all micronutrients applications, but especially for B and Mo. B and Mo are required by plants in low concentrations and the concentration range between deficiency and toxicity of B is very narrow. B and Mo applications may not be necessary on an annual basis, whilst Mo should not be applied in the same solution as S. This will ensure that deficiencies are corrected to adequate levels in the trees and that toxicity of nutrients will not occur so that optimal tree production is achieved. Due to the large variability in the data, sample size should be increased to more than one leaf per sample. Variability in data can be overcome by increasing the sample size and therefore improving data validity. FSSA (2003) recommends one kg of citrus leaves per sample when evaluating commercial orchards. Evaluating indicator trees, i.e. the same trees sampled every time, may also decrease variability of data when sampling in commercial orchards. Farmers may benefit from the findings by foliar application of the most suitable formulation at the most suitable concentration for each unique set of circumstances.

In addition to the nutrient formulations used in this study, other B and Mo containing products may also be applied foliarly. These include Solubor (sodium borate, $Na_2B_8O_{13}.4H_2O$), fertiliser grade borax ($Na_2B_4O_7.5H_2O$) and ammonium molybdate ((NH_4)$_6Mo_7O_{24}.2H_2O$). Applying these products may result in different leaf uptake rates and concentrations than the sources used in this study.

Leaf uptake rates and concentrations that were found suitable for foliar micronutrient applications on 'Midknight' can give indications of leaf uptake rates and concentrations expected for other citrus species when foliar applications of

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micronutrients are made, according to their cuticle thickness relative to that of 'Midknight' leaves. Leaf surfaces of the different species, especially adaxial and abaxial surface wax, were visually compared using SEM images. No considerable differences in surface wax were found that may affect penetration of foliar applied micronutrients relative to 'Midknight' leaves for the young and mature leaves separately. Differences were observed between young and mature leaves with young leaves having considerably less surface wax than mature leaves. It is advisable that foliar applications of micronutrients should therefore be made when citrus trees are in a growth flush and producing leaves at greater rates than usual. This can allow higher amounts of nutrients to enter the leaves due to lower resistance by the thinner cuticle.

Cuticle thicknesses for the different citrus species differed for the different leaf ages and between the abaxial and adaxial leaf surfaces. In most cases 'Bahianinha' navel, 'Satsuma' mandarin and 'Star Ruby' grapefruit leaves had thicker cuticles than 'Midknight' leaves, especially on the adaxial side. Exceptions were abaxial cuticles of young 'Bahianinha' navel and 'Star Ruby' grapefruit leaves, and abaxial cuticles of mature 'Satsuma' mandarin leaves. These citrus species are thus expected to absorb nutrients slower and at lower concentrations than 'Midknight'. 'Washington' navel and 'Genoa' lemon leaves had thinner cuticles than 'Midknight' leaves and these species are expected to absorb leaf applied products more rapidly and at higher concentrations than 'Midknight'. There were some exceptions, namely mature abaxial Genoa lemon and mature adaxial Washington navel leaves having thicker cuticles.

Further studies are needed on the effect of specific factors such as surfactants on cuticular permeability of different formulations to further increase permeability of

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foliar applied products and will be beneficial to the agricultural industry. The results obtained in this study may differ when such studies are conducted on different citrus types, in different seasons, as well as trees that differ in phenology. In addition, other formulations of micronutrients may also be evaluated to determine their suitability for foliar applications on *Citrus sinensis* 'Midknight' under greenhouse conditions. More research is needed on possible influences of cuticle thickness on insect damage to citrus leaves and how that may affect cuticular penetration of foliar applied products. Uptake of differently formulated foliar applied products as influenced by different citrus species, different leaf age and amount of stomata of the leaf surface may also influence uptake of foliar applied nutrients. The time of day that foliar application is made may also influence leaf uptake substantially and should be evaluated. Field trials should be conducted to evaluate the present findings under field conditions.

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