STUDIES OF SILICON FERTILIZATION IN CITRUS TO ENHANCE CHILLING INJURY RESISTANCE

By

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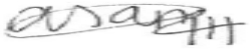
DECLARATION

I, Mireille Asanzi Mvondo-She, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy in Horticultural Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: 

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Colossians 1:11
“Strengthened with all might, according to his glorious power, unto all patience and long suffering with joyfulness”

DEDICATION

To the Almighty God: The Author and Finisher of this dream

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ABSTRACT

Several studies focusing on graminaceous crops such as rice, sorghum and wheat have provided evidence that Si fertilization can reduce the impact of (cold) stress by improving plant growth and yield under cool conditions. The uptake mechanisms have been extensively studied in graminaceous crops to grasp the underlying stress mitigation. Numerous studies in high-Si accumulators found physiological and biochemical adaptation processes were enhanced by Si application in crops stressed by cold conditions. Meanwhile, there is relatively little information on the impact of Si fertilization on low-Si accumulator crops (dicots) in respect of physiological and biochemical adaptation to stress, including citrus, which is classified as a chilling-sensitive species. The semi-arid citrus production region of Vaalharts (Northern Cape Province) is prone to frost throughout the year and particularly around the period of flowering induction during August and September. Cold stress is known to reduce the photosynthetic activities, and eventually yield and quality in citrus. Earlier studies in citrus have determined that Si fertilization improved fruit quality and growth. They also demonstrated that Si fertilization reduced the impact of cold stress, accompanied by an improvement in dry matter production. However, the underlying mechanisms were not investigated. The objectives of this study were: 1) To validate the method for Si analysis in citrus plants; 2) To determine factors that influenced Si uptake in citrus and its uptake

mechanism; 3) To establish the impact of Si fertilization and cold stress on the photosynthetic and photochemical efficiency of citrus plants; 4) To determine the impact of Si fertilization and cold stress on fruit quality and yield of citrus plants; 5) To determine the impact of Si fertilization and cold stress on the levels of key sugars, and proline in citrus trees.

There are many analytical methods to determine Si levels in plants. Some of these procedures have not proved to be reliable for horticultural crops because these crops contain low levels of Si. Furthermore, there is limited information on the validation of these methods for most horticultural crops. The results of the method validation showed that the ICP-OES analytic method was fast and sensitive, with a detection limit five times lower than a colorimetric analysis.

To establish the impact of Si fertilization on citrus under cold stress, it was important to determine the factors that influenced Si uptake and the maximum absorption rate in citrus. The experiments conducted showed that Si uptake in citrus increased significantly ($P< 0.05$) with the duration of application, leaf age and concentration applied, with 1000 mg L^{-1} being the optimum concentration tested. Si fertilization onto the roots of citrus trees was shown to be the best method of application compared to foliar application. The season influenced Si uptake, during the summer months Si uptake was significantly higher ($P< 0.05$) than during the winter months. Dry matter production was significantly increased by Si application.

After establishing the uptake of Si in citrus, it was important to see where Si was deposited in specific tissues to understand the uptake mechanism. Electron microscopy studies suggested that Si accumulates in the adaxial epidermal cell regions of leaves and constitutes a double Si layer. The levels of Si in citrus leaves increased from young leaves<mature leaves<roots, which confirmed that Si was taken up largely in the roots and transferred into the xylem flow and then moved into the leaves on a consistent basis. Hence with age, Si levels built up in

citrus leaves. Winter uptake of Si was close to zero, whereas summer uptake was substantially higher, which suggested that the uptake and transfer of Si into tissues was an active process depending on the prevailing temperature conditions and the physiological activity of the roots.

The investigation of the impact of Si fertilization on the physiological adaptations of citrus to cold stress suggested that an improvement in photochemical efficiency and water use efficiency occurred in both citrus cultivars (Delta and Nules). The photosynthetic rate in Nules clementine trees improved significantly ($P< 0.05$) during prolonged conditions of cold stress. A significant reduction ($P< 0.05$) in transpiration and stomatal conductance was found in Si-treated plants.

Si fertilization of citrus trees made them more cold stress tolerant via physiological factors as well as improvements in the osmotic balance of their sugar (sucrose and fructose) and proline content, resulting in improved membrane rigidity during cold stress. Both factors impacted on water use efficiency via osmoregulation processes.

In a greenhouse trial, the improvement in photosynthetic rate and water use efficiency of citrus trees fertilized with Si and subjected to cold stress during the flowering induction stage did not correlate with fruit yield and fruit quality.

Overall, the current study provided substantial information on the response of citrus to Si fertilization under both normal and lower temperature conditions. The present study also identified crucial parameters in citrus adaptation to cold stress that may be useful in improving the tolerance of citrus crops to abiotic stress and particularly in frost-prone production areas in South Africa, or similar environments.

CHAPTER 1

THESIS INTRODUCTION

1.1. Background of the study

Silicon (Si) is the second most abundant element in the earth's crust after oxygen. These two elements are combined in silicates and aluminosilicates of rocks, clays and soil minerals (Birchall, 1995). Silicon is absorbed by plants as monosilicic acid $Si(OH)_4$ (Epstein, 1999) and is found in the interstitial water of soils at concentrations of up to 28 mg L^{-1}. The Si content of plants varies considerably. Even among genotypes of the same species, great differences were observed, more so for silicon than other elements (Epstein, 1999). Silicon is also the only element that does not damage plants when applied in excess (Ma & Takahashi, 2002). The variation in Si content among plant species has been attributed mainly to the differences in the mechanisms of Si uptake and transport (Epstein, 1994, Liang *et al.*, 2005, Ma & Yamaji, 2006). In general, graminaceous species accumulate much more Si in their tissues than other species, while most dicotyledonous plants were believed to take up Si passively, and dicotyledons such as tomatoes were considered to be Si excluders (Mitani & Ma, 2005, Ma & Yamaji, 2006). Meanwhile, dicotyledonous such as soybean and dry beans have demonstrated highest Si uptake in the leaves compared to the roots (Kidane, 2008, Arsenault-Labrecque *et al.*, 2012). Earlier definition of Si mode of uptake was based on Si content in plants and the uptake rates of Si relative to water, Takahashi *et al.* (1990) defined three modes of Si uptake (active, passive and rejective) corresponding respectively to Si accumulator, intermediate-type and excluder plants (Takahashi *et al.*, 1990, Mitani & Ma, 2005). However, the above definition by Takahashi and his team was conducted by quantifying Si solely on leaves parts, previously classified excluders such as tomato have demonstrated to take up Si substantial in their roots (Heine, 2005, Tubana *et al.*, 2016).

Silicon uptake mode is more classified based on the Si content in the range of 5 g kg^{-1} or more in higher accumulator primarily monocots, meanwhile in dicots Si content is less than 5 g kg^{-1} classifying them as low Si accumulator (Ma & Takahashi, 2002, Liang *et al.*, 2006, Tubana *et al.*, 2016).

However, the physiological role of Si in plants is still not well understood. Silicon is an essential element for diatoms and other members of the yellow brown and golden algae as it plays a role in cell division and DNA replication whose cell walls are silicified and which serve as a mechanical protection against grazers (Hamm *et al.*, 2003, Shrestha & Hildebrand, 2015). According to a widely accepted definition for essentiality Arnon & Stout (1939), Si was not initially defined as an essential element for the higher plants (vascular plants). However, according to a proposed modification of this definition by Epstein & Bloom (2005) , Si was reclassified as an essential element for higher plants (Liang *et al.*, 2007). It's occurrence in plants is in amounts equivalent to certain macronutrients such as calcium, magnesium and potassium (Epstein, 1999, Raven, 2003). Studies showed that Si enhances the yields of many crops, especially when these are grown under conditions of abiotic and biotic stress (Epstein, 1999, Marodin *et al.*, 2014, Dorairaj *et al.*, 2017).

Silicon fertilization was found to enhance disease resistance (Fawe *et al.*, 1998, Heine *et al.*, 2006, Marschner, 2012, Sakr, 2016), alleviate toxicity of heavy metal (Treder & Cieslinski, 2005), alleviate salt stress (Romero-Aranda *et al.*, 2006, Gunes *et al.*, 2007, Zhu & Gong, 2014, Wang *et al.*, 2015), alleviate drought stress (Matoh *et al.*, 1991, Gong *et al.*, 2003, Wang *et al.*, 2015) and alleviate cold stress (Zhu *et al.*, 2006, Liang *et al.*, 2007, Habibi, 2015, 2016). Therefore, the integration of Si in the fertilization program of crops is common in crop production systems worldwide (Tubana *et al.*, 2016).

1.2. Problem statement

Climate change may result in an increased frequency of spring frost events (Habibi, 2016). This would affect citrus production because cold stress usually causes a reduction in the yield and quality of citrus crops (Arpaia, 1994, Ladaniya, 2008).

Barthel *et al.* (2014) observed that a sudden decrease in temperature from 25 to 10^{o}C could result in a delayed plant carbon transport and increased carbon loss via the respiration process in preference to involvement in growth and storage. Citrus (*Citrus sp.* L.) is an important fruit crop, grown commercially in more than 100 countries globally (FAO, 2015). It is a major horticultural crop in South Africa, and 61% of production is exported (Exporters, 2019). There is potential for South Africa to increase its production areas (Exporters, 2019). To achieve such a goal, production of citrus would need to be adapted to the environments of the new production areas. The Northern Cape region is a relatively new citrus production area that may experience frost spells during the year, especially in late winter (Schulze & Schütte, 2016), which would coincide with the period of flower induction. Such frost events would impact on the fruit quality and yield of the subsequent harvest.

The optimum temperature for citrus leaf photosynthesis ranges between 25 to 30^{o}C (Ribeiro *et al.*, 2004, Gao *et al.*, 2006). Under cold stress, chilling and freezing injuries on citrus would limit crop production and yield by reducing stomatal conductance (Ribeiro & Machado, 2007). This would impair photosynthetic activity due to the reduction in CO_2 availability to Rubisco (Machado *et al.*, 2002, Medina *et al.*, 2002). Consequently, flower induction would be negatively affected, which would lead to reduction in fruit yield and quality (Ribeiro & Machado, 2007). This would impact on revenue of citrus crops since the higher rate of acceptance in the market is dependent on the quality of fresh fruit (Ladaniya, 2008).

Cold sensitivity has been observed in plants from tropical, subtropical and temperate climate origins (Lyons, 1973). Exposure to temperatures in the range of 0-20^{o}C has been identified as having an influence on plant growth and production in cold sensitive plants (Ruelland & Collin, 2012). Which resulted in a breakdown of many physiological processes such as photosynthesis and respiration (Levitt, 1980).

Two theories of chilling injury have been identified in plants: (1) the first theory developed by Lyons (1973) proposed that cold stress induced cell phase changes from liquid to crystal phase, which led to membrane damage via solute leakage, as the membrane lost flexibility; (2) The second theory hypothesized that a sudden increase in K^+ ion was the primary cause of damage. This led to stomata opening and a significant increase in transpiration, which exceeded water uptake from the roots, thus leaf wilt was one of the first indices of cold stress in many plant species (Minorsky, 1989, Liang *et al.*, 2009). A reduction in photosynthetic efficiency was also linked to the damage sustained by chloroplast thylakoids during cold stress (Levitt, 1980).

Mitigation of chilling injury using osmoprotectants was linked to increased production of osmolytes, such as sugars and proline, which influence osmotic adjustments during cold stress (Shetty, 2004, Yamaguchi-Shinozaki & Shinozaki, 2006, Ashraf & Foolad, 2007). Previous research demonstrated the acquisition of cold tolerance to be linked to the production of sucrose, raffinose and sugar alcohols (Kaplan & Guy, 2005, Ruelland & Collin, 2012).

Proline is an amino acid with antioxidant properties linked to plant response to cold stress (Shetty, 2004, Ashraf & Foolad, 2007) and combats cold stress by decreasing water potential, thus preventing excessive cell desiccation (Ruelland & Collin, 2012). It may also assist in proteins stabilization (Ruelland *et al.*, 2009).

Si influences in physiological responses during cold-stressed conditions have been investigated. In several studies, photosynthetic rate and water-use efficiency were significantly inhibited under cold stress, but this effect was reversed through Si application (Zhu *et al.*, 2006, Liang *et al.*, 2008, Zuccarini, 2008, Maghsoudi *et al.*, 2015). The protective role of Si in cucumber (*Cucumis sativus* cv. J*inchun* 4) plants exposed to cold stress has been linked to the increase in antioxidant activity (Liu *et al.*, 2009). Habibi (2016) studied the impact of Si fertilization on cold stressed maize (*Zea mays L.*) plants and found increased levels of photosystem II (PSII) efficiency in Si-treated plants compared to the untreated Si plants. The mechanism proposed was the involvement of protective pigments, particularly carotenoids and anthocyanins, in the protection of PSII. Silicon application had also been known to stimulate the over-production of sugar under chilling conditions, which reduced the water potential of cells, leading to protection from cellular desiccation in plant tissues (Kidane, 2008, Ruelland & Collin, 2012). Silicon nutrition has shown to significantly alter the soluble sugar and proline contents in plants under abiotic stress conditions (Yin *et al.*, 2013, Sattar *et al.*, 2017). Silicon fertilization resulted in reduced levels of proline in banana (*Musa acuminata*) plants subjected to low temperature stress (Kidane, 2008). In another study conducted by Liang *et al.* (2008), it was reported that proline levels were significantly higher in plants under cold stress conditions than under normal growing conditions. However, when subjected to Si fertilization there was a significant reduction in proline levels. Si dissolved in the cytoplasm showed the potential to regulate or reduce the levels of endogenous plant hormones in stress conditions, whereas Si involvement in stress tolerance via osmolyte compounds needed further investigation (Zhu & Gong, 2014).

Silicon fertilization on citrus was first conducted in Russia 80 years ago. It was found that Si fertilisation accelerated growth of citrus by 30-80%, fruit maturation by two to four weeks

and also increased the amount of fruit (Taranovskaia, 1939). It was also found that Si fertiliser increased the frost tolerance of lemon trees (*Citrus limon*) (Taranovskaia, 1940). In greenhouse experiments on optimization of Si fertilization, one-year-old and two-year-old orange trees (*Citrus sinensis*) showed increased fresh weight during a six-month period (Wutscher, 1989). In a study on the influence of Si fertilisers on citrus in Florida, it was concluded that there was a relationship between Si fertilization and the health of the treated citrus tree (Matichenkov *et al.*, 1999). Silicon in citrus leaves increased with maturity and biotic stress, and the weight of shoots and roots of grapefruit (*Citrus paradisi*) seedlings increased from 20% to 60%, with improved root systems in terms of lateral branching (Matichenkov *et al.*, 1999).

Insect damage and infections from plant diseases resulted in increased Si content in citrus leaves (Matichenkov *et al.*, 1999). A follow up study on the effect of Si fertilisers on citrus in Florida confirmed the relationship between soil and leaf Si content, and tree vigour of Valencia orange (Matichenkov *et al.*, 2001). Silicon application also improved the mean weights of both plants and roots when grown under aluminium toxicity, salt and cold stress conditions (Matichenkov *et al.*, 2001). In another study conducted on citrus, preharvest Si application improved the control of *Penicillium digitatum* (postharvest disease) in Valencia (Abraham, 2010)

Overall, previous citrus studies demonstrated a positive role of Si in increased tolerance of abiotic and biotic stress conditions. However, the underlying mechanism involved, and uptake mechanism of Si were not fully investigated.

1.3. Objectives

The main objective of this study was to determine the influence of Si uptake on cold-stressed citrus trees and their yields.

This study also sought to understand the effect of Si uptake and chilling injury on citrus photosynthetic and fluorescence activities and to determine cultivar tolerance to stress. Subsequently, the impact of Si application on fruit quality and yield was assessed. Sugar and proline accumulation patterns were used to assess stress adaptation mechanisms.

The specific objectives of this study were to:

- Validate a method for Si analysis in citrus plants.
- Determine the factors that influence Si uptake in citrus (age, concentration applied, volume applied, frequency of application, method of application, season, and Si formulations).
- Investigate the Si uptake mechanism in citrus.
- Measure photosynthesis and photosystem II efficiencies of citrus plants in cold-stressed trees, and Si uptake.
- Determine fruit quality and yield of citrus plants in relation to Si uptake and cold stress.
- Determine physiological activities (sugar and proline) of citrus trees in relation to chilling injury and Si uptake.

1.4. Research approach and thesis outline

A general introduction outlining the scope of the study and the motivation for the study is firstly presented. This is followed by a literature review on Si uptake and chilling injury in citrus plants. Chilling injury in citrus plants is explored after which Si uptake in higher plants is reviewed with emphasis on uptake mechanisms, transport involved and beneficial effect in abiotic stress (Chapter 2).

Various trials were conducted to optimize Si quantification in citrus plants. Pot trials were conducted to establish Si optimum quantification in horticulture crops. Two methods of Si extraction (microwave assisted digestion and colorimetric analysis) were validated and compared using ICP-OES analytical method (Chapter 3).

Pot trials were conducted to determine Si uptake and dry matter production in two citrus cultivars (Delta and Nules) with respect to timing of Si application, leaf age, concentration applied, method of application, and Si formulations to determine the optimum Si application rate (Chapter 4).

Pot trials and electron microscopy trials were conducted to investigate Si deposition in citrus plants. This enabled the determination of Si uptake mechanism within the plant (Chapter 5).

Pot trials were conducted to determine the exogenous Si application effect on chilling injury tolerance and photosynthetic performance of citrus. This study investigated the impact of Si fertilization on the photosynthetic and PSII efficiencies of citrus trees (Chapter 6).

Field experiments were conducted at the Saam commercial farm (Northern Cape), which is a frost prone area. Experiments investigated fruit quality and yield in cooler conditions among citrus plants subjected to Si fertilization (Chapter 7).

Pot trials were conducted to investigate the effects of exogenous Si applications on the physiological responses of citrus cultivars under cold stress conditions. The physiological parameters investigated were proline and sugars (Chapter 8).

Finally, significant findings were summarised, and future perspectives were given (Chapter 9).

CHAPTER 2

LITERATURE REVIEW

2.1. Silicon in soil and plants, and its uptake mechanisms

2.1.1. Silicon available in soils

Silicon (Si) is the second most abundant element in the earth's crust after oxygen. These two elements are combined in silicon dioxide (sand) and the aluminosilicates of rocks, clays and soil minerals (Birchall, 1995). Therefore, most Si found in soils is unavailable to plants (Richmond & Sussman, 2003). Plant-available Si was found in the soil solutions in an undissociated form as monosilicic acid ($Si(OH)_4$) in a concentration range of 90-150 mg L^{-1} in soils with a pH lower than 8 (Jones & Handreck, 1967, Epstein, 1999, Ma & Yamaji, 2006). The availability of labile Si in soil solutions was reduced considerably by increasing pH, organic complexes, the presence of aluminium, iron and phosphate ions, temperature, adsorption/dissolution reaction and soil moisture (Jones & Handreck, 1965, Epstein, 1999, Haynes, 2014). Soil type and weathering processes also contributed to reduced plant available Si (Haynes, 2014). Organic rich soils (histosols) contained little Si in their native state because they are highly weathered, leached, and low in base saturation. They typical of tropical soils (Epstein, 1999, Debona *et al.*, 2017). Further, soil rich in quartz sand [silica (SiO2)] rich soils (e.g., sandy entisols) were high in insoluble Si but very low in plant-available Si (Debona *et al.*, 2017). The low levels of plant-available Si under the above mentioned limiting factors warranted silicon fertilization in crops that benefit from silicon uptake in order to improve quality and yield under abiotic and biotic stress conditions (Epstein, 1999, Ma & Takahashi, 2002, Tubana *et al.*, 2016).

2.1.2. Silicon uptake in higher plants

Silicon dioxide concentration (SiO_2) in plant tissues typically ranged from 0.1-10.0% on a dry mass basis, but both higher and lower values were encountered (Epstein, 1999, Richmond & Sussman, 2003, Ma *et al.*, 2011). This wide variation in Si concentration in plant tissues was attributed mainly to differences in the methods of Si uptake, transport and deposition (Epstein, 1994, Ma & Yamaji, 2015). Comparison of these values with other elements such as Ca (0.1-0.6%) and S (0.1-1.5%) showed that Si is one of the major constituents of the soil solution in contact with plant roots (Epstein, 1999). Even genotypes within a species may have had different Si tissue concentrations, as found in barley, (*Hordeum vulgare* L.*)*, grown in nutrient solutions (Nable *et al.*, 1990).

Three different modes of Si uptake were proposed: active, passive, and rejective uptake (Jones & Handreck, 1967, Takahashi *et al.*, 1990, Mitani *et al.*, 2005). Plants with an active mode of uptake took up Si faster than water, which resulted in a depletion of Si in the uptake solution. Plants with a passive mode of uptake took Si up at a similar rate as water uptake; thus, there were no significant changes in the concentration of Si in solution. By contrast, plants with a rejective mode of uptake tended to exclude Si, as demonstrated by the increasing concentration of Si in the solution. The active uptake mechanism of Si involved at least two processes: the radial transport of Si from the external solution, and transport from the cortical cells into the xylem. In rice (*Oryza sp.* L.*)* it has been demonstrated that radial transport of Si is mediated by a transporter protein (Ma *et al.*, 2004, Yamaji *et al.*, 2008, Yamaji *et al.*, 2015).

There are two general mechanisms for Si uptake and transport: active and passive. Both uptake mechanisms were reported to co-exist with their relative contribution being dependent upon the plant species, soil temperatures and external Si concentration (Liang *et al.*, 2005, Mitani & Ma, 2005).

The monocotyledonous rice and maize (*Zea mays L.*) plants were reported to have Si levels in their tissues in the order of 5% or higher (dry mass basis), and were labelled Si active accumulators (Epstein, 1999, Ma & Takahashi, 2002, Tubana *et al.,* 2016). In contrast, the dicotyledonous crops, were reported to take up less than 5% of their dry matter as silicon and considered to be passive accumulators (Liang *et al.,* 2006, Tubana *et al.,* 2016). In the uptake study performed on tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*) and rice, the plants were exposed to similar Si concentrations and growing conditions. The rice uptake was significantly higher than cucumber and tomato (Mitani & Ma, 2005). The significant lower Si uptake of cucumber and tomato than rice might be explained by a lower density of the transporter proteins to transport Si from the external solution into the cortical cells, and inefficient or absent transporter proteins to transport Si from the cortical cells into the xylem, which led to their classification as Si non-accumulators (Mitani & Ma, 2005). In other experiments conducted on cucumber, rice, banana (*Musa acuminata* Colla) and forage crops, Si uptake increased as the external supply of Si increased (Adatia & Besford, 1986, Ma & Takahashi, 2002, Henriet *et al.*, 2006, de Melo *et al.*, 2010).

In banana, sunflower (*Helianthus annuus*) and wax gourd (*Benincasa hispida*) both Si uptake mechanisms took take place, depending on the Si concentration present in the soil solution. Si transport occurred passively with high Si concentrations in the soil solution, but switched to active transport when concentrations were low (Henriet *et al.*, 2006, Liang *et al.*, 2006).

Following the uptake by the roots, Si was shown to be translocated to the shoots via the xylem. Chemically, the concentration of Si in the xylem sap in rice and wheat (*Triticum aestivum*) was usually higher than 56 mg L^{-1}, the major form of Si in their xylem was monomeric silicic acid (Figure 2.1) (Mitani & Ma, 2005). However, $Si(OH)_4$ polymerized to form silica gel ($SiO_2.nH_2O$) when the concentration of $Si(OH)_4$ exceeded 200 mg L^{-1} (Mitani *et al*., 2005). The process of Si polymerization converted silicic acid to colloidal silicic acid,

which then precipitated out as silica gel (Ma & Takahashi, 2002). If the silicic acid concentration in the xylem sap exceeded this concentration, it started to polymerize *in vitro* (Mitani & Ma, 2005). In the shoots, silicic acid dissolved in the cytoplasm of plants was concentrated through loss of water via transpiration, which then triggered polymerization of the silicic acid, and resulted in its removal from any physiological role. In rice plants, more than 90% of total Si in the shoot was present in the form of silica gel, whereas the concentration of colloidal plus monomeric Si was kept below 140–230 mg Si L^{-1}. A similar pattern of uptake was observed in cucumber leaves, although the total Si concentration of cucumber was much lower than that of rice, which was explained by a lower density of transporter proteins to transport Si from the external solution into the cortical cells (Mitani & Ma, 2005). The distribution of Si in the shoot is controlled by the transpiration stream in the xylem, which is explained by the nonselective and energetically passive uptake of undissociated H_4SiO_4, which lead to the assumption that the movement of Si follows that of water (Jones & Handreck, 1965). Silicon deposit were also found in leaf trichome bases (Samuels *et al.*, 1991).

More Si accumulated in older tissues because this element was immobile within the plants once transferred from the xylem stream into tissues. In rice Si was deposited as a 2.5 mm layer in the space immediately beneath the thin (0.1 mm) cuticle layer, forming a double layered Si cuticle in rice leaf blades. (Figure 2.1) (Ma & Takahashi, 2002, Rao & Susmitha, 2017). Two types of silicified cells occurred in rice leaf blades: silica cells, and silica bodies or silica motor cells, which developed when polymerization of silicic acid occurred at high concentrations (Figure 2.1) (Ma & Takahashi, 2002, Rao & Susmitha, 2017). Dumbbell-shaped Silica cells were located on vascular bundles, whereas silica bodies are in bulliform in rice leaves cells and these silica bodies increased with leaf age (Zhang *et al.*, 2013). The

silicification of cells proceeded from silica cells to silica bodies. In addition to leaf blades, silicified cells were also observed in the epidermis and vascular tissues of the stem, leaf sheath and hull of rice plants (Ma & Takahashi, 2002).

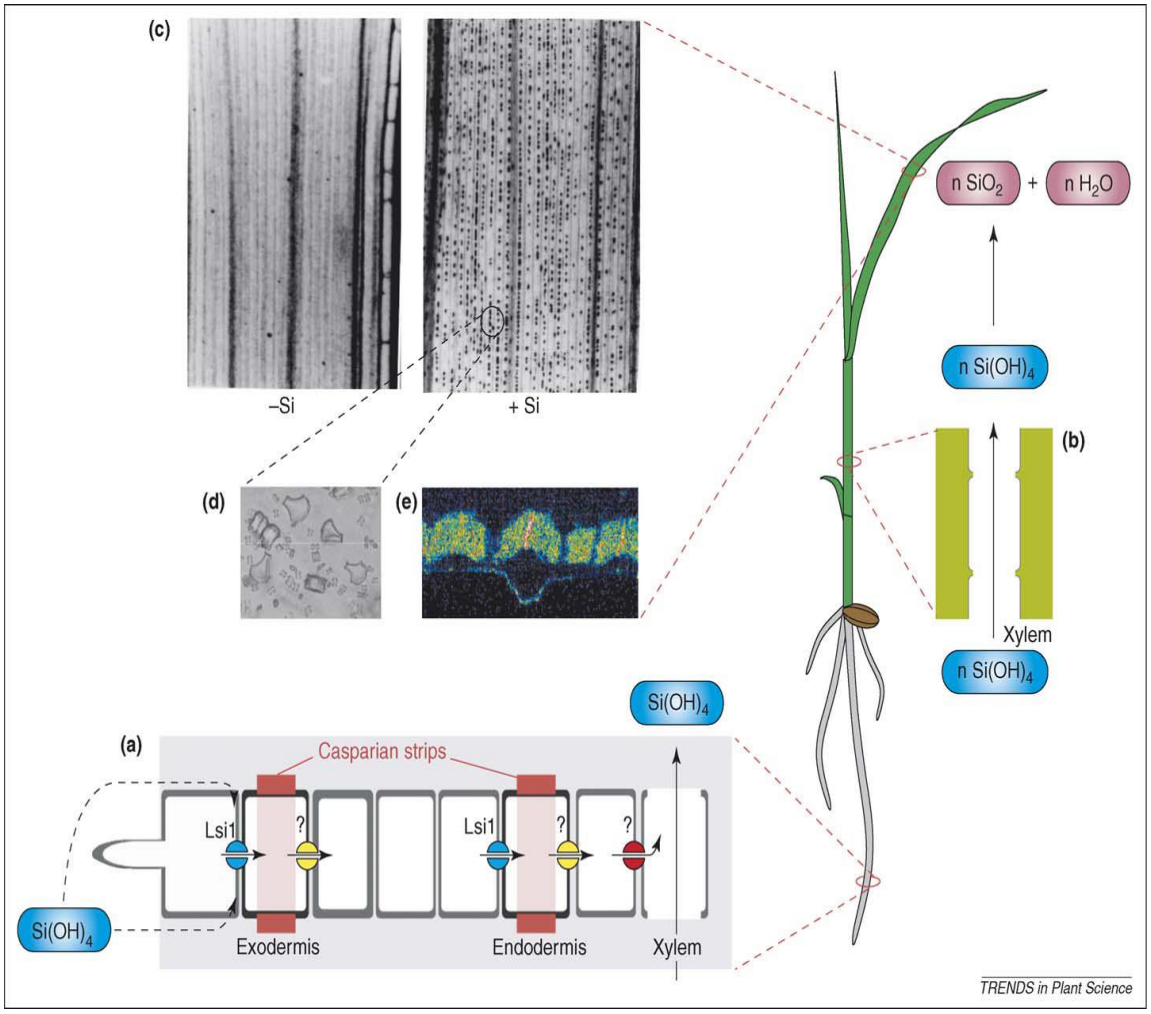

Figure 2. 1 Uptake, distribution, and accumulation of silicon (Si) in rice detected by soft X-ray and by scanning electron microscopy. Silicic acid is taken up via transporters in the (a) and then translocated to the shoot in the same form (b). In the shoot, Si is polymerized into silica and deposited in the bulliform cells (silica body) (c, d) and under the cuticle (e) (Ma & Takahashi, 2002).

In a subsequent study aiming at quantifying Si uptake and distribution in two banana cultivars, $Si(OH)_4$ was supplied in the range of 0-160 mg L^{-1}. Si uptake increased linearly with Si availability in solution. Again, two uptake mechanisms were observed. At low Si concentrations (2-80 mg L^{-1}), Si uptake was higher than the transpiration rate, implying an

active mechanism. At a high Si concentration of 160 mg L^{-1}, Si uptake occurred at the same rate as transpiration, implying a passive uptake (Henriet *et al.*, 2006). Additionally, the distribution of Si in shoot organs (pseudo-stem <petiole and midrib < young leaf < old leaf) also suggested the involvement of transpiration in Si accumulation (Henriet *et al.*, 2006).

Two patterns of Si distribution have been observed in the tissues of bamboo grass (*Sasa vetichii sp.*). Si accumulated in the fusoid and epidermal cells after leaf maturation, while Si accumulation in chlorenchyma cells decreased with maturity. This implies that Si uptake of Poaceae may be cell dependent, on transpiration and the physiological response of specific cell types (Motomura *et al.*, 2004).

2.1.3. Aquaporins and transporters involvement in Si uptake in plants

Plants aquaporins have been defined as intrinsic proteins involved in transporting water, carbon dioxide and nutrients such as boron and Si in the plant (Maurel *et al.*, 2015). Aquaporins genes are highly diverse and have been localized in many plant species maize, rice, tomato, soybean (*Glycine max sp.*) and cotton (*Gossypium hirsutum sp*.) (Maurel *et al.*, 2015). Most plant aquaporins have been observed in the endoplasmic reticulum during their biogenesis. Subsequently, they are transferred to a destination membrane. The plant plasma membrane has three subclasses of aquaporins, namely the plasma membrane intrinsic proteins (PIPs), the nodulin26-like intrinsic proteins (NIPs) and the uncategorized intrinsic proteins (XIPs). Aquaporins are also involved in the alleviation of abiotic stresses such as dissipation of ROS produced in the chloroplast during stressed condition (Maurel *et al.*, 2015).

Silicon uptake in rice plants is influenced by four transporter genes Lsi1, Lsi2, Lsi3 and Lsi6, as illustrated in Figure 2.2 (Ma & Yamaji, 2006, Ma *et al.*, 2011, Ma & Yamaji, 2015, Yamaji *et al.*, 2015, Yan *et al.*, 2018). The Si influx transporter Lsi1 is mainly expressed in

the main roots and lateral roots but not in root hairs, and it assists with Si radial transport (Ma & Yamaji, 2006, Ma *et al.*, 2011, Yan *et al.*, 2018). Homologs of Lsi1 have also been reported in barley (*Hordeum vulgare* L), pumpkin (*Cucurbita maxima* L.), maize and wheat (Chiba *et al.*, 2009, Mitani *et al.*, 2009, Mitani *et al.*, 2011, Montpetit *et al.*, 2012). Aquaporins also mediate Si transport by co-regulation with Si transporters in maize (Mitani *et al.*, 2009, Liu *et al.*, 2014).

In Lsi1, the NIP2 isomer is expressed on the endofacial side of root endodermal cells and mediates passive cellular transport of Si to shoots (Ma & Yamaji, 2006, Mitani *et al.*, 2009, Yamaji & Ma, 2009). Another study showed that the transporter protein is localized on the plasma membrane at the site of Casparian strips. The selective permeability of Casparian strips prevents apoplastic movement into the root stele, hence transporter proteins are needed to reach the stele for relocation of Si to take place from the roots to the shoot (Mitani *et al.*, 2005).

The anion transporter Lsi2 has only been found in the endodermis of maize, rice and barley roots during the vegetative growth stage (Ma *et al.*, 2011, Ma & Yamaji, 2015). Homologs of the Lsi2 transporter have been reported in pumpkin, where it is expressed in both shoots and roots, and facilitates radial transport of Si (Mitani *et al.*, 2009, Mitani *et al.*, 2011, Yamaji *et al.*, 2015). The transporter Lsi3, located at nodes, is involved in the inter-vascular transfer of Si in rice across apoplastic barriers (Yan *et al.*, 2018). In a previous study, the introduction of Lsi3 into a rice mutant resulted in a significant increase in Si uptake (Yamaji *et al.*, 2015).

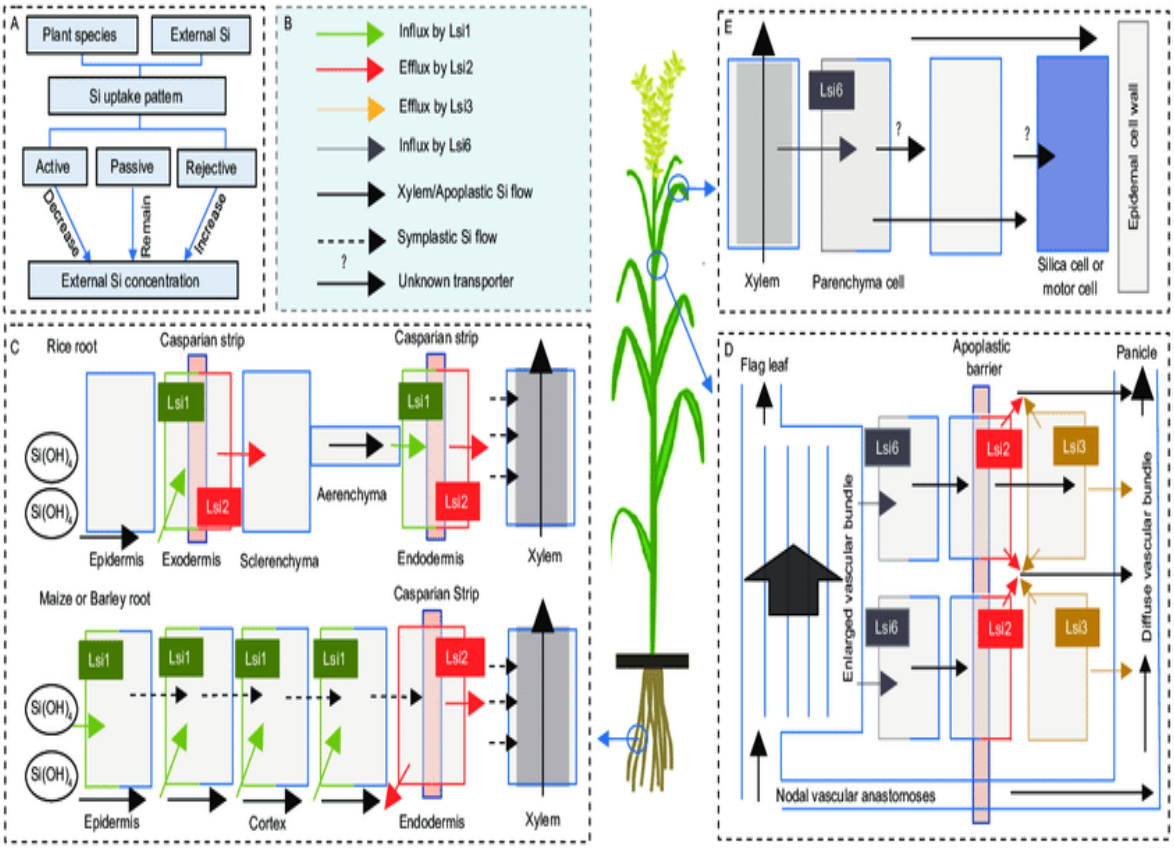

Figure 2. 2 A schematic model of Si transporters in plants (Yan *et al.*, 2018). A: Three uptake mechanism found in plants. B: arrows with different colors showing different Si movement in plants in C-E. C: Two possible Si uptake and transporters Lsi1 and Lsi2 involvement in radial transport pathways in the root. D: possible Si transport pathway and distribution model in the node. E: Possible Si unloading and deposition pathway in leaf.

The transporter Lsi6 is a homolog of the Si influx transporter Lsi1 in rice that is found in barley and maize (Ma & Yamaji, 2006, Yamaji *et al.*, 2008, Yamaji *et al.*, 2012). It is a plasma membrane protein localized in xylem cells of leaf sheaths and blades (Yamaji *et al.*, 2008). This implies that Lsi6 plays a role in the unloading of Si from xylem to leaf tissues (Mitani *et al.*, 2008, Yamaji *et al.*, 2015).

When continuously supplied with Si , a down regulation of the Lsi1 and Lsi2 transporter gene expression takes place in rice, while in other species such as maize and barley, the transporters are unaffected by the concentration of the Si supply (Chiba *et al.*, 2009, Mitani *et al.*, 2009, Mitani-Ueno *et al.*, 2016).

2.1.4. Growth and development responses of plants to silicon fertilization

Silicon has been identified in several reports as a growth promoter in plants. In many instances, the growth stimulation was due to the protection that Si provided plants against abiotic and biotic stresses.

Plants for which Si has been shown to be essential fail to grow normally when the element is deficient, as per the definition of 'essentiality' formulated by Arnon & Stout (1939). However, many species for which Si has not been shown to be essential, may grow better when provided with Si fertilization, whether grown in in hydroponics or soil. Hence, Si has been recognized as a beneficial element (Epstein, 1999).

In well-controlled experiments in which cucumber plants were grown in recirculating nutrient solutions containing SiO_2 at a low (10 mg L^{-1}) or high (100 mg L^{-1}) concentrations, Adatia & Besford (1986) observed a number of positive effects of the high-Si treatment on the growth of the plants, such as a greater leaf thickness, greater dry weight per unit area of leaf, a small but significant increase in root fresh and dry weight, and a reduction in the numbers of leaves wilting on stressed plants.

Si applications have also been found to enhanced the growth and development grapefruit (*Citrus paradisi*) (Matichenkov *et al.*, 1999), and cotton *(Li et al., 1989).*

In the experiments of Ma & Takahashi (1993), the growth of rice was enhanced by the addition of $Si(OH)_4$ to the nutrient solution at 100 mg L^{-1}, an effect possibly related to the calcium concentration of the shoot tissue, which was reduced by the Si treatment. However, not all instances in which Si promoted growth can be attributed to its effect in moderating or alleviating nutrient imbalances (Marodin *et al.*, 2014, Cuong *et al.*, 2017). The beneficial role of Si has been recognized in other experiments conducted in rice in which insufficient Si

has resulted in yield loss and increased disease pressure (Ma & Takahashi, 2002, Tamai & Ma, 2003).

In a greenhouse experiment plants that were not treated with Si fertilizer were reduced in growth and fruit size in both a non-accumulator plant, tomato Miyake & Takahashi (1978), and in an accumulator plant, cucumber Miyake & Takahashi (1983). All these reports were from experiments conducted on plants grown in hydroponic systems. However, in respect to plant growth, field experiments provide the most striking evidence for the role of Si. From a plant physiological point of view this evidence is not as precise as that from hydroponic experiments, because of indirect effects of Si on the plants brought about by interactions of Si with various soil constituents (Epstein, 1994, 1999).

In at least two major crops, rice, and sugarcane (*Saccharum officinarum sp.*), application of silicon fertilizers has become a standard commercial practice in Brazil, Australia, South Africa, Japan, and India. Experiments with soil-grown plants therefore cannot be overlooked. Early observations on Si applications in fields were noted in a review by Jones & Handreck (1967). The organic soils (Histosols) of the Florida Everglades, like many such soils, are low in plant-available Si. Applications of calcium silicate slag can raise the yield of sugar from sugarcane in Florida (Elawad *et al.*, 1982). The sugar content increases were large, in the order of 50% to over 100%. Although many soil properties are affected by the application of Si materials, these and other such field experiments nevertheless suggest that the growth-promoting effects of these applications were largely due to the increased Si content of the plants (Elawad *et al.*, 1982, Crooks & Prentice, 2017, Cuong *et al.*, 2017). Indications are that for optimum yields in sugarcane, leaf Si concentrations has to be in excess of 1% (dry mass basis) (Epstein, 1994). The highly leached upland soils (Ultisols) of the humid tropics tend to have low levels of minerals, and their Si content is often low. Applications of Si increased the

yield of rice grown in such soils (Yamauchi & Winslow, 1989). The effect was apparently direct and not due to the increase in soil pH caused by the Si application. Different rice genotypes responded differentially to Si application (Winslow, 1992). The *Indica* group of genotypes was more responsive to Si applications than were the *Japonicas*, both in terms of yield and in terms of the total amount of Si in the shoots. The Si application nearly doubled the yield of rice over a two-year period (mean for all eight genotypes in the study). The higher yields due to Si application were correlated with higher flag leaf Si concentrations (Ma & Takahashi, 2002).

In a subsequent study on Si fertilization conducted in rice by Detmann *et al.* (2013), a low-Si rice mutant Lsi1 and 'Oachikara' cultivars were drenched with solutions of 0 and 200 mg L^{-1} soluble Si. It was observed that all Si-treated plants showed an increase in yield and nitrogen use efficiency compared to the control plants. The increase in yield was explained by the improvement in photosynthesis linked to the efficiency in the mesophyll conductance. In an experiment conducted in strawberry (*Fragaria ananassa sp.*), foliar and root applied Si increased fruit production, values of titratable acidity, chlorophyll and anthocyanins content (Silva *et al.*, 2013). Applications of the Si to roots are more effective than foliar-Si application (Silva *et al.*, 2013). In an experiment on tomato, Si application improved commercial fruit production and quality, consequently increased the economic return (Marodin *et al.*, 2014). A later study by Dorairaj *et al.* (2017) to evaluate the effect of Si fertilization on rice showed that there was an increased number of tillers, spikelets per panicle and weight per panicle in Si treated plants. Additionally, Si treated plants were more resistant to lodging. The authors speculated that the mechanism behind lodging resistance was the cell silicification of rice leaves, which provides rigidity to the cell wall, hence improving the ability to develop a firm plant. A more plausible theory is that silicic acid in the cytoplasm

enabled the rice plants to develop cell walls with enhanced rigidity (Raven, 1983). This enabled the Si treated plants to achieve and maintain an erect habit, conducive to high levels of light interception.

Other indices of plant performance are also greater in high-Si plants. For instance, when compared with the leaves of low-Si plants, the lower leaves of the high-Si plants are darker green and better positioned for light interception, and their senescence is retarded. Rafi *et al.* (1997) found that leaves of high-Si wheat plants had 50% more total chlorophyll and 50% more ribulose-1,5-bisphosphate carboxylase. This implies that, even when there is no effect of Si on the overall growth of plants, specific aspects of growth or development may be positively affected when the Si is supplied in large amounts. Furthermore, the difference between high-Si and low-Si plant were quantified by Rafi *et al.* (1997) using X ray microanalysis which discovered the deposition of Si in trichomes of awns in silicon treated plants but was absent in silicon depleted plants. The silicon deposited in trichomes makes extremely sharp needles that probably evolved to discourage herbivores from eating the spiky awns. Similarly, the trichomes on the leaves and fruit of cucurbits have a high Si content if adequate Si is available.

2.2. Abiotic stresses

2.2.1. Metal toxicity

Silicon-mediated alleviation of heavy metal toxicity in higher plants has been documented repeatedly. Williams & Vlamis (1957) discovered that the inclusion of Si in the hydroponic solution of barley plants mitigated Mn toxicity, by increasing the ability of leaf tissues to tolerate the absorbed Mn. Adding Si to the nutrient solution of rice grown hydroponically alleviated Mn toxicity and improved the growth of the shoots due to a reduction in the nutrient imbalance (Horiguchi, 1988). In another study conducted in cowpea (*Vigna*

unguiculata L.), it was observed that Si lowered the apoplastic Mn concentration, which suggested that Si may modify the cation-binding capacity of the cell wall (Horst *et al.*, 1999). In another experiment, the alleviation of Mn toxicity by Si in cucumber was attributed to a significant reduction in membrane lipid peroxidation caused by excess Mn and to a significant increase in antioxidants, including both enzymatic (e.g. SOD, APX, DHAR and GR) and non-enzymatic antioxidants (e.g. ascorbate and glutathione) (Shi *et al.*, 2005). The findings on the role of Si in mitigating stress in response to toxic levels of Mn have raised interest in the role of Si in the response of plants to other toxic metal ions, particularly Al (Epstein, 1999). The interaction of Al and Si has been investigated in several plant species. Crops such as sorghum (*Sorghum bicolour L.*), barley, corn (*Zea mays L.* sp. *mexicana*), and soybean showed a significant palliative effect of Si on Al toxicity. On other crops, including rice, wheat , cotton and pea (*Pisum sativum L.*), there was little alleviation of the Al toxicity (Hodson & Evans, 1995).

Adding Si to Cd and Zn contaminated soil may reduce the toxicity in maize by immobilizing these metals in soil, and this will result in an increase in biomass (da Cunha *et al.*, 2008, da Cunha & do Nascimento, 2009). The addition of Si to soil contaminated with Cd and Zn triggered a linear and significant increase in the roots and shoots dry matter yield of the maize plants. This suggests that the Cd and Zn phytotoxicity was decreased when Si was applied to the soil (da Cunha & do Nascimento, 2009). This increase in tolerance to Cd and Zn stress was attributed to the precipitation of Si in the endodermis and pericycle of the roots (da Cunha & do Nascimento, 2009). In strawberry plants, excessive uptake of Cd was prevented by using Si as a soil amendment (Treder & Cieslinski, 2005). Moreover, the amelioration of heavy metal phytotoxicity is not only due to Si action within the plant but also the decreased bioavailability of these metals in Si treated soils (Rogalla & Römheld, 2002).

Other studies also found that the effect of Si in improving boron (B) tolerance in spinach (*Spinacia oleracea*) was associated with the alleviation of oxidative damage of functional molecules, and maintenance of many physiological and biochemical processes, such as lipid peroxidation, membrane permeability and lipoxygenase (LOX) activity, and enhanced levels of antioxidant enzymes (Gunes *et al.*, 2007). The main mechanisms of Si amelioration of metal stress in plants include: (1) Co-precipitation or association of metals with Si; (2) Inhibition of metal translocation from roots to shoots; (3) Isolation of metals ions within plants; and (4) Stimulation of antioxidant systems in plants (Liang *et al.*, 2007).

2.2.2. Nitrogen toxicity

Intensive nitrogen fertilization is associated with environment pollution and reduction in plant yield. The effect of Si fertilization on the agronomic performance of rapeseed (*Brassica napus* L.) with two N fertilizer levels (60 and 160 kg ha^{-1}) was investigated under field conditions (Laîné *et al.*, 2019). This study showed that the interaction between Si fertilization and high N inputs (160 kg ha^{-1}) improved N fertilizer usage due to an increase in the efficiency of N uptake, resulting in increased biomass and yield (Laîné *et al.*, 2019). In addition, Si supply increased the micronutrient concentrations in seeds. Si supply resulted in increases in nickel (Ni) concentration, but only in seeds from plants cultivated under the lower N fertilization regime (60 kg N ha^{-1}). Under both levels of N fertilization (60 or 160 kg N ha^{-1}) there was an increase in iron (Fe) and cobalt (Co) concentrations in the seeds. This indicated the potential for combining N and Si fertilizers to promote the mineral nutritional quality in rapeseed (Laîné *et al.*, 2019).

2.2.3. Water stress

Silicon fertilization has been to shown to mitigate the effects of water stress, which impact on photosynthetic efficiency, antioxidant activities, plant growth and yield in rice (Matoh *et al.*, 1991), wheat (Gong *et al.*, 2003, Gong *et al.*, 2005), maize (Ma *et al.*, 2004, Gao *et al.*, 2005, 2006), sorghum (Hattori *et al.*, 2005, Liu *et al.*, 2014), sunflower (Gunes *et al.*, 2008) and soybean (Shen *et al.*, 2010). The mechanism in water stress alleviation in rice and maize involved the improvement in leaf water potential and photosynthetic activities through a reduction in transpiration loss through the cuticle in rice (Matoh *et al.*, 1991) and stomata in maize (Gao *et al.*, 2006). Gong *et al.* (2005) demonstrated improved growth of wheat under water stress because of Si treatment. The primary mechanism was the increase in antioxidant activities, which resulted in a reduction in free radical production. The observed improvement in photosynthetic activity concurred with other studies (Matoh *et al.*, 1991, Liu *et al.*, 2014). In soybean the photosynthetic rate of drought stressed plants improved in Si treated plants due to a reduction in membrane damage, and an improvement in chlorophyll content and relative water leaf content (Shen *et al.*, 2010). In wheat under water stress, the leaves of Si-treated plants maintained stable water content and water potential relative to the untreated plants (Gong & Chen, 2012). In a similar study on water stressed, sorghum plants fertilized with Si retained their photosynthetic rate, stomatal conductance, and leaf water content. However, the transpiration rate was higher in Si-treated seedlings than in untreated seedlings (Liu *et al.*, 2014). Pereira *et al.* (2013) demonstrated that Si applications to water-stressed pepper (*Capsicum annum* L.) plants resulted in higher levels of proline. The increase in proline improved water affinity under water-stressed conditions (Lobato *et al.*, 2009, Pereira *et al.*, 2013). Other underlying mechanisms involved in drought stress alleviation are increased root water uptake and aquaporin activity in Si-treated plants (Liu *et al.*, 2014, Chen *et al.*, 2018).

2.2.4. Salinity toxicity

The detrimental effects of salinity may be diminished by Si fertilization. Matoh *et al.* (1986) reported that Si application to rice grown in hydroponic culture with the addition of NaCl or polyethylene glycol at concentrations up to the equivalent of 20% sea water alleviated the effects of salt toxicity. Silicate concentration 86 mg L^{-1} reduced the translocation of sodium ions to the shoots and increased the dry matter production of the salt-stressed plants. Experiments conducted on wheat (Ahmad *et al.*, 1992) and barley (Liang *et al.*, 1996) have shown similar findings. Si application restricted salt stress by partially blocking the entry of Na^+ via apoplastic transport. Si counterbalances the reduction in gs (stomatal conductance) and A (photosynthetic rate) induced by salinity stress (Zuccarini, 2008, Zhu & Gong, 2014). In studies conducted on cucumber (Wang *et al.*, 2015), tomato (Li *et al.*, 2015) and sorghum (Yin *et al.*, 2013, Liu *et al.*, 2014) and maize (Parveen & Ashraf, 2010). Si applications alleviated salt-induced stress by improving leaf water content and transpiration rate, which translated into improved growth in cucumber and tomato (Wang *et al.*, 2015). In sorghum, the transpiration rate of Si treated plants was higher than the control plants (Liu *et al.*, 2014). Three mechanisms have been proposed for Si-mediated tolerance of salinity and drought: (1) An improvement in leaf water content, photosynthesis and root hydraulic conductivity (Rios *et al.*, 2017, Liu *et al.*, 2019), (2) Increased levels of antioxidant activities, and membrane integrity which affects osmotic pressure regulation, and (3) Si regulates stress markers by restricting Na^+ uptake and improving K^+ uptake (Zhu & Gong, 2014, Zhu *et al.*, 2019).

2.2.5. Low temperature stress

The application of Si to wheat under cold stress resulted in a significant increase in the leaf water content, which reduced water loss via transpiration (Gong *et al.*, 2003, Liang *et al.*, 2008). Si treatment of rice, maize, sunflower and wax gourd plants resulted in tolerance to cold induced stress (Liang *et al.*, 2006). The main mechanism for Si mediated tolerance of

cold stress has been associated with the enhanced retention of water in leaf tissues because the silica deposited in the outer walls of the epidermal cells on both surfaces of plant leaves formed a silica cuticle double layer, reducing water loss through stomatal transpiration (Matoh *et al.*, 1991). Another view is that the mechanisms include the protection of membranes by higher levels of antioxidants, and lower lipid peroxidation and membrane permeability, which enhance water retention in leaf tissues (Liang *et al.*, 2008). He *et al.* (2010) discovered a significant increase in sucrose and proline content in silicon treated plants. In contrast, Kidane (2008) reported decreased proline content in Si treated banana plants exposed to chilling injury. However, he also reported that Si treated banana plants enhanced levels of the sugars, sucrose and raffinose, which are known to provide for cryoprotection, which agreed with the findings of He *et al.* (2010).

Cold tolerance was examined in pistachio (*Pistacia vera sp*.) by Habibi (2015, 2016) using Si fertilizer applied as a foliar drench. Habibi (2015) found that foliar-applied Si significantly improved photosystem II (PSII) efficiency during cold stress while root-applied Si failed to enhance cold tolerance. In addition, foliar-applied Si in cold-stressed plants reversed membrane damage by increasing antioxidant activities in both pistachio and maize (Habibi, 2015, 2016). This agreed with an earlier study that demonstrated an increase in antioxidant activities in cold stressed cucumber plants subjected to Si treatment (Liu *et al.*, 2009). In plants treated with either foliar or root-applied Si, relative water content and plant growth improved relative to the control plants (Habibi, 2015, 2016). The mechanism proposed was an increase in amino acid content (osmolytes) in Si-treated cold-stressed plants, which protect membrane integrity (Krasensky & Jonak, 2012, Habibi, 2015, 2016).

In another study conducted on cold-stressed maize plants, applications of Si, Zn and Mn cold protectants resulted in the improvement in the regulation of plant signalling molecules

(abscisic, salicylic and jasmonic acids), phenolics, proline and antioxidant activities that were disrupted or inhibited in the control under cold stressed conditions. This resulted in an increase in yield. The ameliorative effects of Si was linked to the levels of the micronutrients Mn and Zn (Moradtalab *et al.*, 2018). Si induced improvement in several physiological processes in cold stressed rice has been associated with the over expression of Lsi1 (Azeem *et al.*, 2016).

2.3. Assessment of physiological responses in plants

2.3.1. Photosynthesis in plants

Photosynthesis has been defined as the primary fuel of life on earth. During the process of photosynthesis the sun's energy is used by plants to convert carbon dioxide and water into organic compounds (Pietrzykowska, 2015). Within photosynthesis, reactions are categorised as light and dark reactions: the light reaction takes place in the photosynthetic membrane of chloroplasts under long actinic irradiation, in the presence of H_2O and CO_2 molecules. The energy of light is captured and used to produce the energy molecules ATP and NADPH. During the dark reaction, these molecules reduce inorganic carbon dioxide to organic carbon (Hopkins & Hüner, 2008). Photosynthetic efficiency is measured as either quantum yield of CO_2 assimilation (ΦCO_2) or quantum yield of O_2 evolution (ΦO_2) (Huner *et al.*, 1998). Photosynthetic rates may be reduced by stress conditions, which disturb or block photosynthetic electron transport and affect the photosynthetic apparatus (Baker, 1996).

2.3.2. Chlorophyll fluorescence

Measurement of chlorophyll fluorescence is one of the rapid screening methods to assess the Photosystem II (PSII) efficiency in plants. Additionally, it enables the understanding of fundamental mechanisms of photosynthesis (Murchie & Lawson, 2013). The quantification of methods available to define and measure the effect of stress on plants is more difficult and more costly. Visible symptoms are usually a late manifestation of stress impact, whilst

biochemical analyses are slow and expensive (Smillie & Hetherington, 1983). Therefore, the focus has been on developing quantitative and qualitative rapid, sensitive, and non-destructive methods of stress assessment. The chlorophyll in the membranes of chloroplasts emits a red fluorescence, of which a part, the induced or variable chlorophyll fluorescence, is responsible for changes in activities of the PSII (Papageorgiou, 1975, Roháček & Barták, 1999).

The absorption of incident photons by molecules of antenna pigments (chlorophylls and carotenoids) is followed by the excitation of energy transfer via excitons to reaction centers of Photosystem I (PSI) and PSII (Roháček & Barták, 1999). The light energy absorbed by chlorophyll molecules in leaf undergoes one of three fates: (1) It can be used to drive photosynthesis (photochemistry); (2) Excess energy can be dissipated as heat (H); and (3) It can be re-emitted as light chlorophyll fluorescence (F). The relationship between these parameters can be expressed as: P+H+F =1. Therefore, the yield of chlorophyll fluorescence provides crucial information on the photochemistry efficiency and heat dissipated (Maxwell & Johnson, 2000, Baker, 2008).

2.3.3. Relationship between chlorophyll fluorescence and photosynthesis in plants

The relationship between steady-state fluorescence and photochemistry under low light conditions is well understood (Genty *et al.*, 1989). With increasing irradiance, the fraction of energy used for fluorescence increases and the fraction used for photochemistry decreases (Seaton & Walker, 1990, Maxwell & Johnson, 2000). Thus, at low light intensity, a negative correlation exists between the probability of fluorescence and photochemistry. The relationship is reversed under high light conditions. With increasing irradiance and moisture stress, chlorophyll fluorescence and photochemistry both drop due to deactivation of antennae, to prevent damage by harmful radicals formed in those conditions (Gilmore & Yamamoto, 1992, Maxwell & Johnson, 2000). Therefore, at high light intensity and stress, a

positive correlation exists between fluorescence and photochemistry. The fact that the relationship between fluorescence and photochemistry is different under stress and optimum conditions implies that the interpretation of the fluorescence signal is not straightforward. An observed increase in fluorescence may be interpreted either as an increase or a decrease of photochemistry, depending on the micro-environment of the observed vegetation. Both effects have been observed (Flexas *et al.*, 2002). This has given rise to the discussion of whether chlorophyll fluorescence is a measure of photosynthesis performance or of stress factors (Maxwell & Johnson, 2000). The knowledge of the micro-environment is needed prior to the translation from a chlorophyll fluorescence measurement to actual photosynthesis can be made, while the combination of photosynthesis and chlorophyll fluorescence measurements enables a better understanding of plant responses to stress (Maxwell & Johnson, 2000, Baker & Rosenqvist, 2004).

2.3.4. Photosystems as major components of the photosynthetic electron transport chain

Photosystems (PS) are arrangements of chlorophyll and other pigments packed into thylakoids. Eukaryotes have two complexes, PSI and PSII. PSI uses the light harvesting pigment chlorophyll a, referred to as P700. PSII uses a form of chlorophyll a known as P680 (Barber, 1992). Both are active forms of chlorophyll and function in photosynthesis due to their association with proteins in the thylakoid membrane.

Photophosphorylation is the process of converting energy from a light-excited electron into the ATP molecule. This occurs when the electrons from water are excited by the light in the presence of P680 (Frenkel, 1995). Light energy causes the removal of an electron from a molecule of P680 that is part of PSII. The P680 oxidizes a water molecule to diatomic O_2 that is released during the process of heat dissipation, which is measured by using the parameter named non-photochemical quenching (NPQ) (Demmig-Adams & Adams III, 2006). ATP is

produced by H^+ ions electron moving from high energy state to lower energy state and H^+ ions from water splitting at PSII forming hydrogen ion concentration gradient (Moser *et al.*, 1992, Evert & Eichhorn, 2013). As H^+ ions flow down their gradient into the stroma, then pass through the ATP synthase driving ATP production.

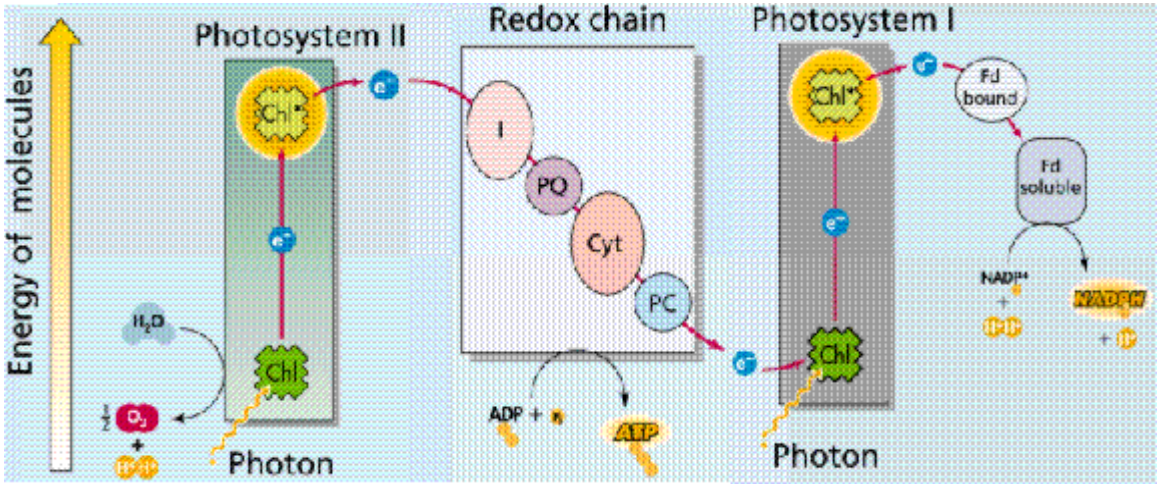

Figure 2. 3 A linear representation of the photosynthetic electron transport chain with the sequential arrangement of the three multi-molecular membrane complexes transfer electrons from water to the production of ATP and NADPH (Moser *et al.*, 1992).

2.3.5. Electron transport mechanism

Electron transport from water to NADP+ requires three membrane-bound protein complexes operating in series from in PSII, to the cytochrome bf complex and PSI (Figure 2.3). Electrons are transferred between these large protein complexes by small mobile molecules plastoquinone (PQ) and plastocyanin (PC) in plants. Because these small molecules carry electrons (or hydrogen atoms) over relatively long distances, they play a unique role in photosynthetic energy conversion. This is illustrated by plastoquinone (PQ), which has two main functions. PQ transfers electrons from the PSII reaction center to the cytochrome bf complex and carries protons across the photosynthetic membrane (Kallas, 1994). This is done by shuttling hydrogen atoms across the membrane from PSII to the cytochrome bf complex. Because PQ is hydrophobic, its movement is restricted to the hydrophobic core of the photosynthetic membrane. PQ operates by diffusing through the membrane until, due to

random collisions; it becomes bound to a specific site on the PSII complex. The PSII reaction center reduces PQ at the QB-site by adding two electrons and two protons, creating PQH2. The reduced plastoquinone molecule dissociated from PSII and diffuses randomly in the photosynthetic membrane until it locates a specific binding site on the cytochrome bf complex. The cytochrome bf complex is a membrane bound protein complex that contains four electron carriers, three cytochromes and an FeS center (Martinez *et al.*, 1994). The electrons are eventually transferred to the PSI reaction center. The protons released into the inner aqueous space contribute to chemical free energy transfer across the membrane (Huner *et al.*, 1998).

Electron transfer from the cytochrome bf complex to the PSI is mediated by a small Cu-protein, plastocyanin (PC), which is water soluble and operates in the inner water space of the photosynthetic membrane. Electron transfer from PSI to NADP+ requires ferredoxin, a small FeS protein, and ferredoxin-NADP oxidoreductase, a peripheral flavoprotein that operates on the outer surface of the photosynthetic membrane (Frenkel, 1995).

Photosynthetic membranes effectively limit electron transport to two dimensions. For mobile electron carriers, limiting diffusion to two dimensions increases the number of random encounters (Whitmarsh, 1986). Furthermore, because PC is mobile, any one cytochrome bf complex can interact with several PSI complexes. The same is true for PQ, which commonly operates at a stoichiometry of about six molecules per PSII complex (Baker, 1996).

CHAPTER 3

METHOD VALIDATION FOR SILICON ANALYSIS IN CITRUS AND HORTICULTURE LEAF TISSUE

Abstract

Silicon (Si) is classified as a beneficial element in higher plants. Although there is an abundance of literature on Si analysis and extraction procedures in graminaceous crops such as rice and sugarcane, there is limited information on the validation of these methods for most dicotyledonous horticultural crops. These methods include gravimetric extraction, which does not require sophisticated training but requires a large sample size, and sensitivity to Si content in the test plant. The autoclave inductive digestion method is considered effective by some and criticized by others due to the use of a strong oxidant and its sensitivity to the level of Si in the plant material. The microwave digestion method is a closed system that is quick and results in a high recovery rate due to its high extraction temperature and the avoidance of contamination. The aim of this experiment was to validate silicon measurement by means of inductively coupled plasma optical emission spectrometry (ICP-OES) against a conventional spectrophotometer method of analysis. The recovery efficiency using ICP-OES and spectrophotometer were 82%-96% and 85-94%, respectively. Microwave digestion, followed by spectrophotometry, had a recovery efficiency of 85-94%. The ICP-OES results correlated with the spectrophotometry results but were consistently superior. The limit of detection for Si determination by means of ICP-OES and that for the spectrophotometry method were 71 µg L^{-1} and 330 µg $L^{-}1$, respectively.

Keywords: microwave digestion, ICP-OES, Spectrophotometer, detection limit

3.1. Introduction

Silicon (Si) is the second most abundant element in the earth's crust after oxygen (Sposito, 2008), and is found predominantly as silicon dioxide (SiO_2) in unweathered rocks and soil minerals (Birchall, 1995, Epstein, 1999). During weathering processes Si is released from primary and secondary minerals. Although Si is abundant in soil, it is only found in small quantities because of the low solubility of the complex it forms with Al (aluminosilicates) (Epstein, 1999, Haynes, 2014). Repeated cropping can further reduce the level of available plant silicon (Epstein, 1999, Savant *et al.*, 1999, Datnoff *et al.*, 2001, Ma & Takahashi, 2002). Silicon is absorbed by plants as monosilicic acid ($Si(OH)_4$), which can be found in the interstitial water of soils at concentrations of up to 28 mg L^{-1} (Epstein, 1999). Plants treated with Si fertilizers typically results in Si uptake similar to other macronutrients such as Ca, Mg and P (Raven, 2003, Ma, 2004). Silicon is not listed as an essential element in higher plants although it is well known to be beneficial for growth and development in certain plants by increasing plant development rate, vigor, biotic and abiotic stress alleviation (Liang *et al.*, 2005, Tubana *et al.*, 2016, Rao & Susmitha, 2017). For example, in wheat (*Triticum aestivum L.*), photosynthesis and water use efficiency are significantly inhibited during frost conditions, but these effects can be reversed if Si fertilizer is applied (Zhu *et al.*, 2006). Similar findings were registered in maize (*Zea mays L.*) (Habibi, 2016) and in pistachio (*Pistacia vera sp.*) (Habibi, 2015). Adatia & Besford (1986) observed several positive effects of high-Si treatments of cucumber (*Cucumis sativus* cv. Corona), including greater leaf thickness, greater dry weight per unit area of leaf, and increased drought resistance. In another study conducted on tomato (*Solanum lycopersicum*), Si application alleviated the effects of salinity stress nutrient deficiencies at the reproductive stage (Miyake & Takahashi,

1978, Romero-Aranda *et al.*, 2006, Li *et al.*, 2007, Liu *et al.*, 2014, Li *et al.*, 2015, Wang *et al.*, 2015).

Many extraction methods have been developed for the quantification of Si from plant material including the gravimetric method, autoclave induced digestion (AID), HCl-HF digestion and microwave-assisted digestion using nitric acid, hydrogen peroxide or sodium hydroxide (Van der Vorm, 1987, Elliott & Snyder, 1991, Haysom & Ostatek-Boczynski, 2006). The first published method for Si extraction was for rice *(Oryza Sativa L.*) (Fox *et al.*, 1969). The method entails oxidizing the organic compounds of the plant material and solubilisation of silica using a long digestion time (Fox *et al.*, 1969). Although the gravimetric method is tedious and time consuming, it is adequately efficient for high-Si accumulator such as rice and sugarcane (*Saccharum officinarum L.*). However, it is not suitable for most horticultural crops with a lower Si content (King *et al.*, 1955).

The autoclave induced digestion (AID) method for Si extraction with sodium hydroxide and hydrogen peroxide was modified by (Elliott & Snyder, 1991). This method has the advantages of a low cost and rapid extraction. It works well with rice but generates highly variable results for sugarcane, possibly because of the lower Si content in sugarcane and the slow solubilisation of polysilic acid into monosilicic acid (Taber *et al.*, 2002, Ostatek-Boczynski & Haysom, 2003, Kraska, 2009). Taber *et al.* (2002) also reported that AID extraction gave consistently low recoveries of Si from maize stalks (12.1%), peach leaves (*Prunus persica*) 41.2%, and bluegrass (*Proa Pratensis*) 12.1%. The AID method has also given consistently lower Si values when compared to alternative extraction techniques such as hydrofluoric acid (HF) and microwave assisted digestion because it only extracts Si in its ionic form (Haysom & Ostatek-Boczynski, 2006, Kraska & Breitenbeck, 2010).

The solubilisation of Si for analysis using HF was initially demonstrated by Novozamsky *et al.* (1984). However, this extraction procedure can lead to the formation silicon tetrafluoride (Si F_4) complex which reduces the soluble Si. Hydrofluoric acid may also chemically attack and degrade the nebulizer and torch, which results in damage to the ICP-OES, resulting in errors in measurements. Moreover, the routine handling of HF requires usage of HF-resistant protective laboratory clothing and equipment (Taber *et al.*, 2002).

The microwave assisted digestion technique using nitric acid, hydrogen peroxide and sodium hydroxide was developed by Ostatek-Boczynski & Haysom (2003). This method has been demonstrated to be a safer and more reliable extraction method for Si from rice, sugarcane and pasture grasses due to its reduction in chemicals costs, avoidance of HF, rapidity of extraction, and its efficiency in extracting Si due to the use of a closed vessel, a reduction of the matrix effect and reduced contamination. There is a strong linear relationship between Si extractions using microwave assisted digestion, and Si extracted by means of dry ashing (Ostatek-Boczynski & Haysom, 2003, Haysom & Ostatek-Boczynski, 2006).

Various methods can be used for silicon quantification, for example, colorimetry, atomic absorption spectroscopy (AAS), electron beam analysis (EBA; scanning electron microscopy coupled with energy dispersive X-ray analysis), X-ray fluorescence (XRF) and inductively coupled plasma optical emission spectrometry (ICP-OES) (Elliott & Snyder, 1991, Haysom & Ostatek-Boczynski, 2006, Frantz *et al.*, 2008, Kraska & Breitenbeck, 2010). However, the accuracy of all these methods depend on the initial extraction procedure (Emich *et al.*, 1932).

The inductively coupled plasma optical emission spectrometry (ICP-OES) analytical method is stable and sensitive, leading to excellent accuracy and precision (Lichte *et al.*, 1980, Hou *et al.*, 2006). The ICP-OES measures the total silica by completely dissociating the molecules

confined in the sample during preparation (Kraska & Breitenbeck, 2010). The ICP-OES is a well-established analytic method against which to compare the older colorimetric methods (Elliott & Snyder, 1991, Taber *et al.*, 2002, Kraska, 2009). Taber *et al.* (2002) reported that when the Si content in plants such as apple (*Malus domestica*), peach, maize and bluegrass was determined with the colorimetric and ICP-OES analytical methods, there was a correlation of 0.99 between the two methods. The sensitivity of the colorimetric analysis was 6% lower than ICP-OES analysis (Taber *et al.*, 2002, Kraska & Breitenbeck, 2010).

Plant tissue analysis determines the mineral content in plant parts. However, foliar contamination may affect the reliability of the results. To determine accurate Si uptake over time, the initial step should be to measure surface bound Si on leaves (Markert, 1996, Robert *et al.*, 1996).

The sensitivity is important and determined by quantifying the lower limit of detection (LOD) which is the minimum concentration level that can be determined to be statistically different from a blank at the 95% confidence level and distinguishable from the noise level of the system (Hou *et al.*, 2006, Sivakumar *et al.*, 2006).

The aim of this experiment was to validate an accurate and rapid procedure for silicon extraction and quantification in horticulture crops such as tomato, lettuce, and citrus by using a microwave digestion followed by ICP-OES analysis or spectrophotometer analysis.

3.2. Materials and Methods

3.2.1. Pot trial

The pot trial was conducted in a climatically controlled glasshouse at the Hatfield Experimental Farm of the University of Pretoria (S25º 44' E28°15'). The pots were placed on a rotary table to minimize the effect of climatic differences on the plants. Daily climatic

variables (temperature and humidity) were monitored and recorded with data loggers (Figure 3.1).

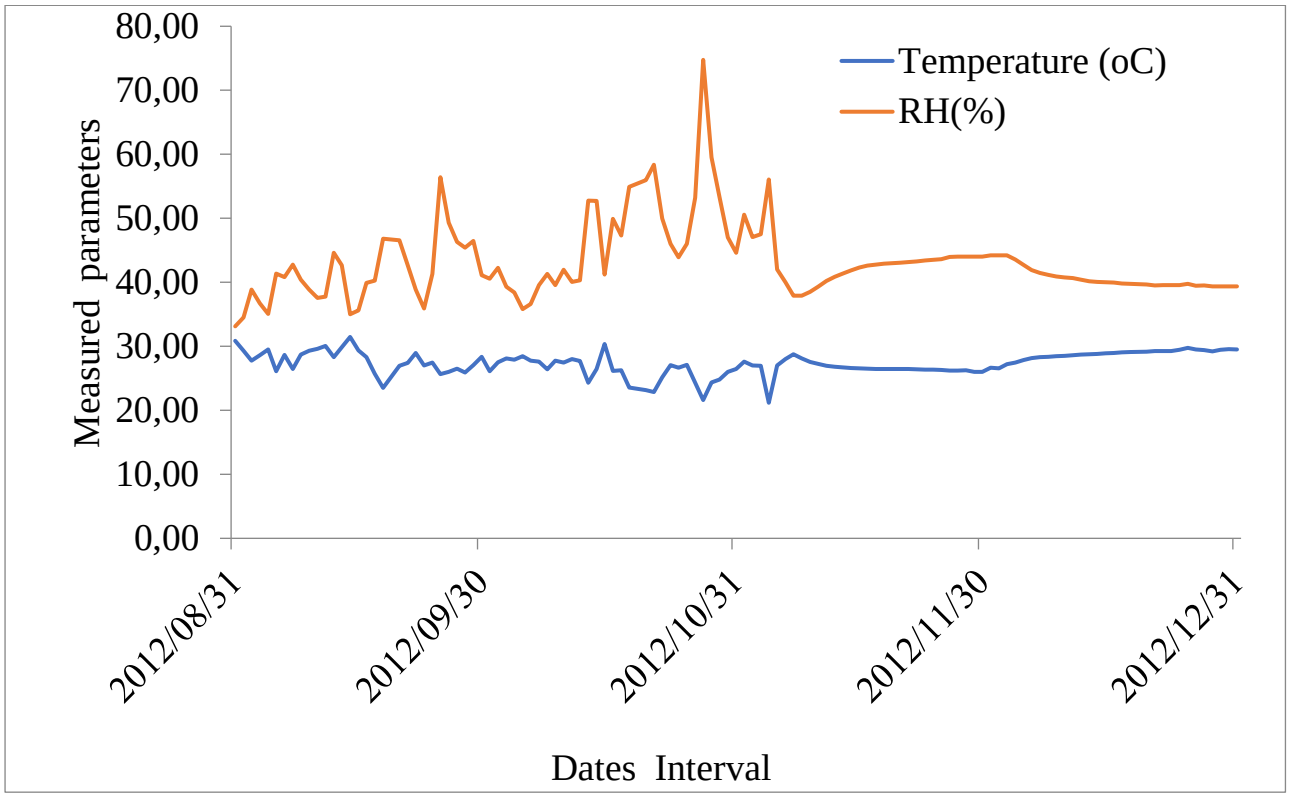

Figure 3. 1 Showing temperature (°C) and RH% recorded during the four months of pot trial.

Prior to Si experiment, the plants were left to establish for a month. Silicon was also applied as K_2SiO_3 at planting at a rate of 0 and 150 mg kg^{-1} of the growing medium to pot grown in (10 L) with sandy clay growing media. Subsequently, K_2SiO_3 was applied at a concentration of 0 and 500 mg L^{-1} as drenching fortnightly over a three-month period onto lettuce (*Lactuca sativa* L.), tomato (*Solanum lycopersicum* L.), and citrus trees: Valencia (*Citrus sinensis*) and Clementine (*Citrus reticulata*) until runoff. The pots were arranged in a randomized complete block design with four replications. The test plants constituting of three replicates subjected to Si rate of 150 mg kg^{-1} at planting and root-Si of 500 mg L^{-1} for three months were selected to determine whether there was a cuticle effect on Si uptake. Valencia and Clementine; leaves are polylamellate (100-25 nm), the outer region of lettuce is amorphous (22 nm), whereas the

outer region of tomato is reticulate (200 nm) (Holloway 1982). The trees were irrigated to field capacity every 3 days. The field capacity was determined by watering selected pots till drainage and weighing them after 8 h, to estimate subsequent irrigation needs. The trees were fertilized with NPK at 85-115-40 kg ha^{-1}, which represents the local fertiliser recommendation for two-year citrus trees (FSSA-MVSA., 2007). NPK was applied in the form of ammonium nitrate (NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4) and potassium nitrate (KNO_3).

The Si content in citrus trees was determined in young and old leaves to compare the pattern of uptake found in previous experiments (Matichenkov *et al.*, 2001, Ma & Takahashi, 2002).

3.2.2. Removal of surface bound Si

Three solvents were tested to remove surface bound Si from the leaves of the test plants: Valencia, Clementine, lettuce, and tomato leaves. The solvents used to remove the surface bound Si were deionised water, 10% acetone and 10% ethanol (Rossini Oliva & Raitio, 2003). The leaves were left for five minutes in each of the solvents to separate superficially adsorbed Si from Si incorporated into the biomass. The leaves were then oven dried at 60^{o}C for 48 h. The experiment was done in triplicate.

3.2.3. Microwave digestion

Polypropylene and Teflon containers were used for the preparation and digestion of the samples because glass contains Si. All containers were rinsed with 10% NaOH solution prior to use to minimize silicon contamination (Taber *et al.*, 2002). The microwave-assisted digestion method was adapted from Haysom & Ostatek-Boczynski (2006). Three mL of 65% HNO_3 (EMSURE) was added to 500 mg dry, ground leaf samples. Test tubes were capped and left to stand for five minutes to thoroughly wet the sample. Two mL of 30% H_2O_2 (AR grade) was then added and left to stand overnight. The capped Teflon tube (TFM) reaction

vessels were placed in the ceramic tube holders of a Mars 5 microwave system (CEM Corporation, North Carolina, United States of America). The rotor was placed in the cavity of the microwave unit which was set to a ramping temperature of 180^{o}C over 15 min, after which the temperature was maintained for 15 min and the acid digestion step conducted. On completion of the digestion cycle, the tubes were left to cool, and then 10% NaOH solution was added to the digestate tubes. The tubes were recapped and returned to the microwave system for the second heating step with a ramping temperature of 180^{o}C over 15min, after which then the temperature was maintained for 15min, which resulted in the solubilisation of amorphous Si. After the final cooling step, the contents of the tubes were neutralized with HNO_3 (2 M) in a plastic beaker, using phenolphthalein as an indicator, and then diluted to a volume of 250 mL with Type I deionised (DI) water (0.18 µS cm^{-1}) in a volumetric flask to reduce the matrix effect. The Si concentration of the final solution was determined using both colorimetric and ICP-OES analysis.

3.2.4. ICP-OES analysis procedure

An Inductively Coupled Plasma optical emission spectrometry (ICP-OES) analysis procedure was used to measure the Si concentration. The plasma power was 1000 W with a plasma flow rate of 15 L min^{-1} and an integration time of 1sec. Calibrating standards for the ICP-OES instrument (Agilent 720 series, Victoria, Australia) were prepared from a 1000 mg L^{-1} standard (Fluka). The ICP was fitted with an alkaline resistant torch, Sturrman Master spray-chamber and a V Groove nebulizer assembly in axial view with a highly sensitive ICP-OES setting.

Selectivity

The selection of wavelengths was based on the two Si sensitive lines of 251.611 nm and 288.158 nm. The wavelength 251.611 nm was selected as the most sensitive wavelength

based on the correlation accuracy with respect to relative standard deviation. This wavelength was used for both the analysis of samples and standards.

To reduce signal interference and obtain the net analyte signal, the background corrections were done to the right side of peak at 0.01 nm. Matrix interferences were accounted for by preparing the sample and standard in a similar matrix. The working range was determined by preparing a solution of the standard beyond the highest expected concentration in the samples. A linear curve (r = 0.999) was established with 0, 0.5, 1, 2, 5, 10 and 15 mg L^{-1} Si standards.

Sensitivity of the method

According to Froes *et al.* (2009) the detection limit of ICP-OES is estimated as the limit of quantification (LOD) using the equation:

$$LOD = (3 \times RSD_{Blank} \times BEC) /100 \qquad \text{Eq 1,}$$

where BEC is the background equivalent concentration and RSD_{Blank} is the relative standard deviation of the blank (n = 10).

The BEC equation is expressed as,

$$BEC=C_{Element}/SBR \qquad \text{Eq 2,}$$

Where $C_{Element}$ is the concentration and SBR is the signal to background ratio.

An interval correction was done after every ten consecutive samples run by running a known Si standard concentration to monitor the stability of the ICP-OES and to detect any drift in the readings. When this occurred, the ICP-OES was re-calibrated. After twenty consecutive samples were run, a known Si standard and a blank were introduced into the sequence to reduce contamination and to make automatic correction of any drift in the readings.

Accuracy

The recovery efficiency of Si was determined for the microwave assisted digestion. Known amounts of Si (0, 2000 and 4000 mg kg^{-1}), based on the sample Si content, were added to three replicates of leaf samples for the different crops. An interval correction was done after every ten consecutive samples run by using a known Si standard concentration to monitor the stability of the ICP-OES and to detect any drift in the readings. When this occurred the ICP-OES was re-calibrated. In addition, after twenty consecutive samples run, a known Si standard and a blank were introduced in the sequence to reduce contamination and make automatic correction of any drift in the readings.

Precision

The samples and standards were measured in triplicate and the relative standard deviation (RSD) was < 3%.

3.2.5. Colorimetric analysis

Fifty mL of the aliquot extracted using microwave digestion were placed in a polycarbonate test tube. Thirty-five mL of 20% acetic acid and 10 mL of ammonium molybdate (54 g L^{-1}, pH 7.0) was added, mixed and the resulting solution was left to stand for five minutes. Then 5 mL of tartaric acid (20%) were added to the solution, followed by the addition of 1 mL of a reducing solution, which consisted of two parts: A (2 g of Na_2SO_3 and 0.4 g of 1-amino-2-naphthol-4-sulfonic acid in 25 mL deionised water), and B (25 g of $NaHSO_3$ in 200 mL deionised water). Parts A and B were combined and brought to 250 mL in a volumetric flask with deionised water. After the addition of the reducing solution and mixing of the test tube contents, the mixture stood for only 30 min to avoid the polymerization of Si. The absorbance was taken using a UV/Vis spectrophotometer (Beckman Coulter DU 530) calibrated at 650 nm (Elliott & Snyder, 1991).

Selectivity

A linear standard curve (r = 0.999) was established by adding 0, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}.

Limit of detection

The limit of detection (LOD) was calculated based on the formula 3.3 δ/S, where δ was the standard deviation of a blank replicate determined under the same conditions, and S was the sensitivity, taken from the slope of the calibration graph (Al-Ghannam & Al-Olyan, 2008).

Accuracy

The recovery efficiency of Si was determined using microwave assisted digestion followed by Si quantification using the spectrophotometer. Known amounts of Si (0, 1000 and 2000 mg kg^{-1}), based on the sample Si content range determined in the Si treated leaves, were added to three replicates of leaf samples for the different crops.

3.2.6. Statistical analysis

The collected data was subjected to analysis of variance by using Statistical Analysis System software (SAS) version 9.4 (Cary, NC, USA) to determine treatment mean effects. Differences between treatments were determined using Fisher's Least Significant Difference (LSD) at 5% level of significance.

3.3. Results

3.3.1. Foliar bound Si removal

The most effective method of removing Si bound to the leaf surfaces was investigated using three solvents and a control. The Si content of the unwashed control and ethanol-washed lettuce and tomato leaves were significantly higher than leaves washed with deionised water and acetone for all leaf tissues (Table 3.1). Within solvent treatment, Si content of the Valencia and Clementine leaves were not significantly different from each other (Table 3.1).

Table 3. 1. The effect of foliar bound Si removal on Si leaf content (mg kg^{-1})

	Washing Solvents			
Leaf Samples	**Acetone**	**Deionized water**	**Ethanol**	**Unwashed Control**
Valencia	1389[b]	1404[b]	2136[a]	2177[a]
Clementine	1380[b]	1415[b]	2093[a]	2104[a]
Lettuce	883[b]	873[b]	1251[a]	1258[a]
Tomato	2029[b]	2045.70[b]	2368[a]	2369[a]

*The means in rows followed by similar letters do not differ at ($P \leq 0.05$) according to Fisher's LSD test. LSD: 50.01, CV%: 25.6 and Sed: 7.2. Values are means of silicon content **(**mg kg^{-1}) in three replicates of leaves of the four species.

3.3.2. Validation of inductively coupled plasma optical emission spectroscopy

After microwave extraction, Si levels were analyzed using ICP-OES. Its recovery efficiency ranged between 82- 96% and the recovery rate increased as the amount of Si added to the leaf samples increased (Table 3.2). The highest recovery rates were observed for the leaves of Clementine and Valencia. The coefficient of variation was generally low, in the range of 0.4-3.5%.

Table 3. 2. Silicon recovery (%) from mature plant tissue extracted with microwave technique using nitric acid/hydrogen peroxide and sodium hydroxide, followed by analysis in triplicate using ICP-OES

Plant tissue	Si added (mg kg^{-1})	Recovered Si (mg kg^{-1})	SD	CV (%)	Recovery (%)
Valencia	0	2358	41	1.7	-
	2000	3954	92	2.30	91
	4000	5956	43.7	0.73	94
Clementine	0	2269	42	1.30	-
	2000	3930	48	1.20	92
	4000	5998	30	0.50	96
Lettuce	0	967	20	3.50	-
	2000	2675	82	0.75	82
	4000	4702	51.51	1.09	95
Tomato	0	5201	14	1.40	-
	2000	6169	21	0.40	86
	4000	8422	48.18	0.57	91

*****Values in the four species are means of three replicates with CV% and SD. The recovery efficiency was calculated from Si recovered and applied Si.

The ICP-OES sensitivity was tested by measuring the lowest concentration of an analyte in a sample that is detectable and distinguishable from the noise level of the system. The limit of detection was 0.071 mg L^{-1} (Table 3.3)**.**

Table 3. 3. Silicon limit of detection using ICP-OES analysis

Parameters for detection limit quantification	**ICP-OES detection limit**
BEC	0.075 mg L^{-1}
RSD_{Blank}	28.7%
LOD	0.071 mg L^{-1}

*Values are Si measured by determine Si concentration from the blank. BEC= background equivalent concentration and LOD= Limit of detection

3.3.3. Validation of colorimetric analysis

After a standard extraction, the Si levels in leaf tissues were analyzed using a spectrophotometer, which was evaluated for accuracy and precision. The recovery efficiencies ranged between 85-94%. Recovery % were lower at the higher Si level added except for lettuce (Table 3.4). The standard deviation was generally low with exception of the control treatments. The CV% was in the range of 0.35-12.01%. In addition, lower CV% were observed in all leaf tissues at the higher Si level added.

Table 3. 4. Silicon recovery (%) from mature plant tissue extracted with nitric acid/hydrogen peroxide and sodium hydroxide analyzed in triplicate using a spectrophotometer

Plant tissue	Si added (mg kg^{-1})	Recovered Si (mg kg^{-1})	SD	CV (%)	Recovery (%)
Valencia	0	1957.75	19.80	1.01	-
	1000	2758.70	0.05	5.40	92
	2000	3455.90	0.003	0.35	85
Clementine	0	1433.39	41.10	2.87	-
	1000	2334.75	0.01	1.10	94
	2000	3221.40	0.01	1.08	92
Lettuce	0	811.17	5	0.62	-
	1000	1701.70	0.01	1.45	94
	2000	2658.7	0.013	1.34	94
Tomato	0	2429.23	291.87	12.01	-
	1000	3122.4	0.0544	5.82	93
	2000	3951.7	0.028	3.06	91

*Values in the four species are means of three replicates with CV% and SD. The recovery efficiency is calculated from Si recovered and applied Si.

The spectrophotometer detection limit (LOD) was computed from the sensitivity and standard deviation of the set measurements. The LOD was 0.3 mg L^{-1} (Table 3.5), which was five times higher than the detection limit of ICP-OES (Table 3.3).

Table 3. 5. Silicon limit of detection using Spectrophotometer analysis

Parameters for detection limit quantification	**Spectrophotometer detection limit**
Sensitivity	0.0067
Standard deviation	0.06
LOD	0.3 mg L^{-1}

The regression analysis of the citrus leaf data indicated a linear relationship of 99% between colorimetric analysis and ICP-OES (Figure 3.2). These two analytical methods have a strong relationship implying as the Si levels measured by ICP-OES increased, the same pattern was observed with colorimetric analysis.

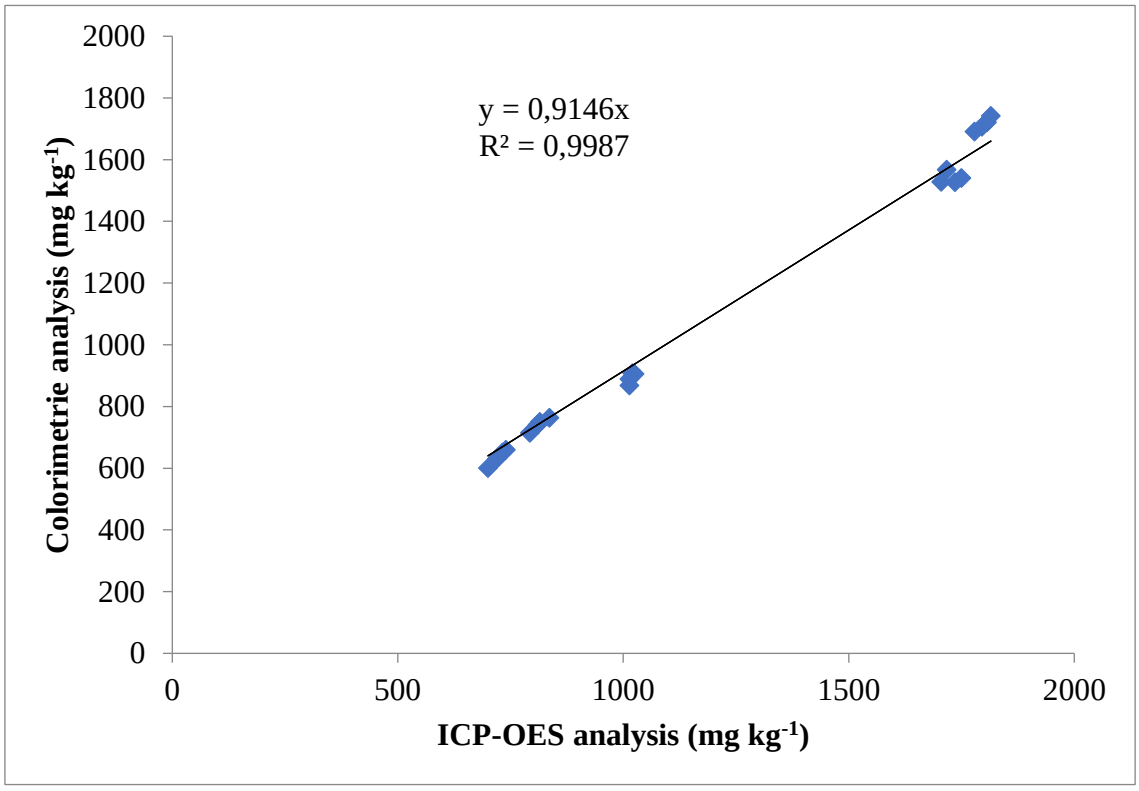

Figure 3. 2 The correlation between results from the ICP-OES analysis and results from the colorimetric analysis of Si in citrus leaf tissues.

Silicon uptake was investigated in young and mature leaves in both citrus species (Table 3.6). A significant increase in Si content was observed in old leaves compared to young leaves in both species.

Table 3. 6. Silicon content (mg kg^{-1}) in young and old Valencia and Clementine leaves

Varieties	Leaf maturity	Si (mg kg^{-1})
Valencia	Young	462^{b}
Valencia	Old	1311^{a}
Clementine	Young	439^{b}
Clementine	Old	1260^{a}

* LSD: 15.48 means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's LSD test. Values are means of three replicates.

3.4. Discussion

Leaf surface cleaning assists in the removal of dust particles, which is made up of elements such as Si, Al, Mg, K, Ca, S and Fe (Shi *et al.*, 2008). The removal of foliar bound Si was most effective using deionised water or acetone treatments for all leaf types. It proposed that the deionized water was able to dissolve surface-bound Si. In contrast, acetone is non-polar, and it is hypothesized that it dissolved the epidermal waxes binding superficial Si. Use of one of these treatments is needed before leaves are analysed for their Si content in uptake studies (Rossini Oliva & Raitio, 2003).

Method accuracy in analytical determination is conducted by evaluating the recovery efficiency (González & Herrador, 2007). Silicon concentration correlated with recovery efficiency and CV%, hence the samples with the highest Si contents were extracted and analysed with the most precision (Table 3.2). The high recovery efficiency by ICP-OES analysis is more likely due to the measurement of total Si (George *et al.*, 2000, Haysom & Ostatek-Boczynski, 2006). The low CV% values confirmed the consistency of the analysis it provided.

The sensitivity of a method is linked to the lowest level of detection by the instrument (Al-Ghannam & Al-Olyan, 2008). The ICP-OES limit of detection was 71 µg L^{-1} (Table 3.3). The detection limit was 12 times lower than the lowest Si concentration measured in the samples, which gave an indication of high signal to noise ratio of ICP-OES, which reflects the high sensitivity and accuracy of this analytical method (Hou *et al.*, 2006, González & Herrador, 2007). The recovery efficiency of Si using the spectrophotometer ranged between 85-94%, which was within the range of experimental error, and it also had a low variability (Table 3.4). In this study, the limit of detection for the spectrophotometer technique was 0.3 mg L^{-1} (Table 3.5), which suggested that ICP-OES is a more sensitive analytical method, possibly because it has fewer steps and less interferences (Lichte *et al.*, 1980).

The two analytic methods were compared by the correlation between results from the ICP-OES and colorimetric analysis. The correlation of Si in the leaf tissues was 99% and the regression equation shown in Figure 3.2 had a slope of 0.914. The strong linear relationship between ICP-OES analysis and colorimetric analysis technique corroborates with previous studies (Taber *et al.*, 2002). This suggested that ICP-OES can be used to determine Si content in the leaves of horticultural crops such as citrus. The Si content of leaves using the colorimetric technique was lower than those detected by ICP-OES, probably due to the instability of colour development. Another explanation could be that ICP-OES measures all forms of Si in solution, and the fact that Si standards were prepared in the same matrix as the samples, which resulted in the greater accuracy, as observed by Kraska & Breitenbeck (2010). In contrast, the development of the molybdenum blue colour measures Si ions only, therefore, it underestimates the total Si content (Taber *et al.*, 2002, Kraska & Breitenbeck, 2010).

In this experiment, the pattern of Si uptake in Valencia and Clementine was investigated to verify the reliability of the validated method. The Si content was significantly higher in the older leaves of both Valencia and Clementine than young leaves (Table 3.6). This agrees with previous results that reported Si content increases significantly with maturity in citrus, suggesting that Si is not easily redistributed within citrus plants (Wutscher, 1989, Matichenkov *et al.*, 2001, Ma & Takahashi, 2002). This implies that Si accumulated in plants cannot be used during stress period because it polymerizes into silica gel, which makes it immobile in the plants (Epstein, 1994, Ma & Yamaji, 2006). The distribution pattern of Si within the citrus leaves can be explained by the transportation of Si along the transpiration stream as identified in cucumber and barley (Ma & Takahashi, 2002).

Conclusions

Use of water or acetone removed much more of the foliar bound Si prior to extraction than ethanol. The inductively coupled plasma optical emission spectrometry (ICP-OES) analysis agreed with the conventional Spectrophotometer (colorimetric analysis) method. The ICP-OES analysis had a detection limit 10 times lower than the lowest Si concentration in the leaf tissues. It was more sensitive, precise, and faster than the colorimetric analysis. Microwave-assisted digestion extraction followed by ICP-OES analysis can be used as a sensitive, precise, and fast method for the analysis of horticultural crops for their Si content.

CHAPTER 4

FACTORS INFLUENCING SILICON UPTAKE, AND THE EFFECT OF SILICON LEVELS ON DRY MATTER PRODUCTION IN POTTED CITRUS TREES

Abstract

Although a considerable amount of evidence exists on the role that silicon (Si) plays in alleviating stress effects and increasing the growth and yield of many agricultural crops, especially in monocotyledonous species, little information on factors affecting Si uptake and Si impact on dry matter production in citrus can be found. Additionally, there is no information on the optimum concentration of Si application for citrus, which is important if Si is to be applied in commercial citrus production. Therefore, two citrus cultivars, 'Delta' Valencia (*Citrus sinensis*) and 'Nules' Clementine (*Citrus reticulata*) were treated, either with two Si sources (K_2SiO_3 and $Si(OH)_4$), as foliar applied Si rates and drench applied Si rates, during a summer and a winter season. The effects of the treatments were measured in terms of Si uptake and dry matter production. There were no significant differences in the uptake of potassium silicate (K_2SiO_3) and silicic acid ($Si(OH)_4$) formulations. Seasonal effects were observed, with significantly higher Si uptake in summer grown citrus, which indicated the active uptake of Si, requiring physiologically activity for the citrus plants. The Si uptake was significantly higher for the root drench than the foliar application, with the highest uptake of Si into leaves and roots resulted from a root drench at 1,000 mg Si L^{-1}. Dry matter production was positively influenced by the application of Si at 1,000-2,000 mg Si L^{-1}. This study provided evidence that Si uptake influences dry matter content in citrus cultivars.

Keywords: Clementine, Valencia, leaf age, season, Si uptake, Si concentration, root drench

4.1. Introduction

Silicon is the second most abundant element after oxygen (O) in the earth's crust (Ma & Takahashi, 2002, Sposito, 2008). Together they form the basic structure of aluminosilicates, the building blocks of clay minerals, where the strong Si-O bonds reduces the solubility and therefore, the availability of Si to plants (Richmond & Sussman, 2003). Plant available Si can be found in the soil solution in an undissociated form as monosilicic acid ($Si(OH)_4$) in a concentration range of 90-150 mg L^{-1} in soils with a pH lower than 8.0 (Jones & Handreck, 1967, Epstein, 1999, Ma & Yamaji, 2006). These levels can be reduced by repeated cropping and the removal of plant material containing Si (Birchall, 1995, Datnoff *et al.*, 2001). In highly weathered soils, Si availability in soil solution is reduced considerably because of leaching, soil acidification, organic complexes, the presence of aluminium, iron and phosphate ions, soil temperatures, exchangeable/dissolution reactions and soil moisture (Haynes, 2014).

Silicon provides a beneficial role as a growth promoter, mainly in monocotyledons, and also in some dicotyledons, due to protection it provides against abiotic and biotic stresses in higher plants (Adatia & Besford, 1986, Li *et al.*, 1989, Rafi *et al.*, 1997, Liang *et al.*, 2005, Liang *et al.*, 2006, Epstein, 2009, Zhu & Gong, 2014, Tubana *et al.*, 2016).

Several reports have described the beneficial role of Si application on yield and dry matter production, including trials on cotton (*Gossypium hirsutum L.*), banana (*Musa acuminata L.*) and bamboo (*Bambusa vulgaris L.*) (Li *et al.*, 1989, Motomura *et al.*, 2002, Henriet *et al.*, 2006), tomato (*Solanum lycopersicum L.*) and cucumber (*Cucumis Sativus* L.) (Miyake & Takahashi, 1978, 1983, Adatia & Besford, 1986, Marodin *et al.*, 2014), potted strawberry plants (*Fragaria ananassa L.*) (Silva *et al.*, 2013), rice (*Oryza Sativa L.*) (Tamai & Ma, 2008,

Detmann *et al.*, 2012, Dorairaj *et al.*, 2017) and sugarcane (*Saccharum Officinarum L.*) (Elawad *et al.*, 1982).

Silicon can be applied to the roots as a soil drench or using granular formulations or applied as a foliar spray (Epstein, 1999). In one study, on rice plants infected with brown spot (*Alternaria alternata*), the plants absorbed more Si from a root drench than from foliar-applied Si, which resulted in better control of the leaf disease (Rezende *et al.*, 2009). In another study conducted on cucumber, root applications of Si resulted in better control of powdery mildew infection than foliar-applied Si (Liang *et al.*, 2005). In contrast, Treder & Cieslinski (2005) found that foliar-applied Si increased yields more than root-applied Si in strawberry plants. In another study conducted in rice, foliar-applied-Si increased growth parameters such as the number of tillers, plant height, panicle length and leaf surface area (Prakash *et al.*, 2011). In a study conducted on pepper (*Capsicum annum*) plants, root-Si fertilization was more effective in the control of anthracnose lesions than foliar sprays (Jayawardana *et al.*, 2014).

The beneficial role of Si in citrus has been demonstrated in only a few studies. Taranovskaia (1939) reported on research on lemon (*Citrus limon*), conducted in Russia 77 years ago, that Si fertilisation increased the amount of fruit, accelerated growth by 30-80% and accelerated fruit ripening by two to four weeks. A similar study conducted in grapefruit (*Citrus Paradisi*) revealed that fertilisation with calcium silicate slag increased root and shoot mass by 19-40% (Matichenkov *et al.*, 1999, Matichenkov *et al.*, 2001). In addition, Matichenkov *et al.* (2001) reported a 14-41% increase in tree height and 31-48% increase in branch growth for Valencia (*Citrus sinensis*). Similarly, Wutscher (1989) found that potassium silicate (K_2SiO_3) applications increased fresh shoot mass by 30-40% in one-year-old and two-year-old sweet

orange (*Citrus sinensis* (L.) Osbeck) trees over a six-month period under greenhouse conditions.

The aim of this study was to determine Si uptake and distribution, and plant dry matter production in two citrus cultivars subjected to two Si concentrations, two Si sources and methods of application in two seasons.

4.2. Materials and Methods

4.2.1. Soil sampling and analysis

Soil (top ±20 cm) from the Hatfield Experimental Farm of the University of Pretoria was classified as Hutton soil and used as potting medium in the Si uptake experiment (Soil Classification Working Group, 1991). The samples from the collected soil were air dried, milled and sieved through a 2 mm sieve before conducting physical and chemical analyses to determine soil fertility and acidity (Soil & Council, 1992). Soil texture was determined using the hydrometer method. Soil EC and pH were measured in a 1:2.5 soil:water suspension. Ca, Mg, K and Na were extracted with 1 mol L^{-1} ammonium acetate solution and concentrations determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES). The phosphate content of the soils was extracted using the Bray I method and the concentration determined with ICP-OES. NO_3^- and NH_4^+ were extracted with 1 mol L^{-1} KCl and analysed with the Kjeldahl method. Extractable Si was extracted with 1 mol L^{-1} KCl (Crusciol *et al.*, 2018). Results from the soil analysis are presented in Table 4.1.

Table 4. 1. Physical and chemical properties of selected soil

Textural class	**Water content (%)**	**Sand (%)**	**Silt (%)**	**Clay (%)**	**pH**	**EC (mS m^{-1})**	
Sandy clay	11.7	58	6	36	5	19	
Element analyses (mg kg^{-1})							
K	Ca	Mg	Na	P	NO_3^-	NH_4^+	Si
25	104	18	9	78	28	9	8

4.2.2. Silicon uptake in plants

4.2.2.1. Investigation of two Si formulations on Si uptake

A preliminary study was conducted to assess the effect of two Si formulations on Si concentration in two-year-old citrus trees in a glasshouse on the Experimental Farm of the University of Pretoria (S25^{o} 44' E28°15'). The cultivars 'Delta' Valencia oranges (*Citrus sinensis*) and 'Nules' Clementine (*Citrus reticulata*), grafted onto Carrizo citrange rootstocks, were subjected to K_2SiO_3 and H_4SiO_4 applied as a nutrient solution fortnightly at three concentrations of Si (0, 100 and 500 mg L^{-1}) to (20 L) over a three months period. The pots were filled with sandy clay soil from the Hatfield Experimental farm (Table 4.1). The pots were arranged in a randomized complete block design with four replications. The trees were irrigated to field capacity every 3 days. The field capacity was determined by watering selected pots till drainage and weighing them after 8h, to estimate subsequent irrigation needs. The trees were fertilized with NPK at 85-115-40 kg ha^{-1}, which represents the local fertiliser recommendation for two-year citrus trees (FSSA-MVSA., 2007). NPK was applied in the form of ammonium nitrate (NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4) and potassium nitrate (KNO_3). The trial was conducted over summer and winter seasons, to determine the impact of seasonal variability in Si uptake.

4.2.2.2. Comparison of two Si application methods and seasons effects on Si uptake

These experiments were conducted in a glasshouse on the Experimental Farm of the University of Pretoria (S25^{o} 44' E28°15'). Foliar and root applications of potassium silicate were conducted on two-year-old citrus trees 'Delta' Valencia oranges (*Citrus sinensis*) and 'Nules' Clementine (*Citrus reticulata*) cultivars grafted onto Carrizo citrange rootstocks grown in 20 L plastic pots filled with a sandy clay soil (Table 4.1). Potassium silicate was sprayed on the leaves until runoff to ensure uniform coverage every 14 days over three months period. Three Si rates (0, 100 and 500 mg L^{-1}) were tested in a randomised complete

block design with six replications. To prevent runoff of the sprays onto the soil, plant stems and the surfaces of the pots were covered with a plastic sheet. Additional K that was introduced by K_2SiO_3 was corrected by adding K_2O to the control trees. During the experiment, dark green, fully-grown leaves (old) and lighter, new fully expanded (young) leaf samples were taken separately.

In another set of trees, K_2SiO_3 was mixed with the soil at planting at three Si rates (0, 75 and 150 mg kg^{-1} of soil). K_2SiO_3 was subsequently applied as a solution (1 L pot^{-1}) at three Si concentrations (0, 100 and 500 mg L^{-1}) were applied fortnightly over three months period. The pots were arranged in a randomized complete block design with six replications. The trial was conducted in summer and winter, to account for seasonal variability in Si uptake. Daily climatic variables (temperature and relative humidity) were monitored and recorded hourly with data loggers.

4.2.2.3. Determination of Si maximum absorption rate in citrus plants

To determine the optimum Si application rate, K_2SiO_3 was applied as a nutrient solution every 14 days for three months at the following Si rates (0, 100 and 500, 1000 and 2000 mg L^{-1}) to pot grown (10 L) 'Delta' orange and 'Nules' clementine plants. The pots were arranged in a randomized complete blocks design with four replications. These samples were washed in deionised water to remove all impurities and samples were oven dried (Labotec, South Africa) at 70^{o}C for 48h. Samples were milled and sieved for Si analysis using ICP-OES.

4.2.3. Sample preparation and Si determination

At the end of the above three experiments, all plants were sampled. The leaves and roots were oven dried at 70^{o}C for 48h. Si content and dry matter production were determined. Plant material samples were prepared according to the method described by (Haysom & Ostatek-Boczynski, 2006). Polypropylene and Teflon containers were used for the preparation and

digestion of plant samples to minimize contamination risks when using glassware. All containers were rinsed with NaOH (10%) prior to use. Leaf samples of 500 mg were placed in the Teflon microwave digestion tubes and three mL of 65% HNO_3 (AR grade) was added. The Teflon tubes were capped and left to stand for five minutes to thoroughly wet the samples. Then two mL of 30% H_2O_2 (AR grade) were added to the tubes and left overnight. The Teflon tubes were sealed and placed in ceramic tube holders of a CRM 500 microwave system (CEM Corporation, North Carolina, United States of America). The rotor was placed in the cavity of the microwave unit with a ramping temperature of 180^{o}C for 15 min, then the temperature was maintained for 15min and the acid digestion step conducted. On completion of the acid digestion cycle, the Teflon tubes were left to cool. NaOH (10%) solution was then added to the tubes and they were returned to the microwave system for the second heating step to solubilise amorphous Si. The contents of the Teflon tubes were transferred into plastic beakers to minimize Si contamination, then neutralized with HNO_3 (2 M), using phenolphthalein as an indicator, and then diluted to 250 mL in volumetric flasks. A 10 mL sample was taken from each tube for Si determination ICP-OES spectrometry (ICP-OES, Varian Liberty 200, Thermo Fisher Scientific, Massachusetts, United States of America), fitted with a hydrofluoric acid (HF) resistant torch, a Sturrman Master spray-chamber and a V Groove nebulizer assembly. The plasma power was 1000 W with a plasma flow rate of 15 L min^{-1} and an integration time of 1s. Two Si sensitive wavelengths, 251.611 nm and 288.158 nm were used to detect Si. The wavelength of 251.611 nm was selected as the most sensitive wavelength, based on its higher correlation with the calibration curve. Matrix interferences were accounted for by preparing the sample and standard in a similar matrix.

4.2.4. Statistical analysis

The collected data was subjected to analysis of variance by using Statistical Analysis System software (SAS) version 9.4 (Cary, NC, USA) to determine treatment mean effects.

Differences between treatments were determined using Fisher's Least Significant Difference (LSD) at a 5% level of significance.

4.3. Results

4.3.1. The effect of two Si formulations on Si uptake

The Valencia and Clementine cultivars took up Si at similar levels for both the Si sources (Figures 4.1). Therefore, K_2SiO_3 was used in subsequent research because it was cheaper and easier to access. In Valencia, the Si content increased significantly with the Si application rate, the highest uptake rate was observed with an application rate of 500 mg L^{-1} for both Si sources (Figure 4.1 A). In clementine Si uptake also increased significantly with Si applications at both rates and Si sources (Figure 4.1 B). However, there was no significant difference resulting from the two Si application rates for both Si sources.

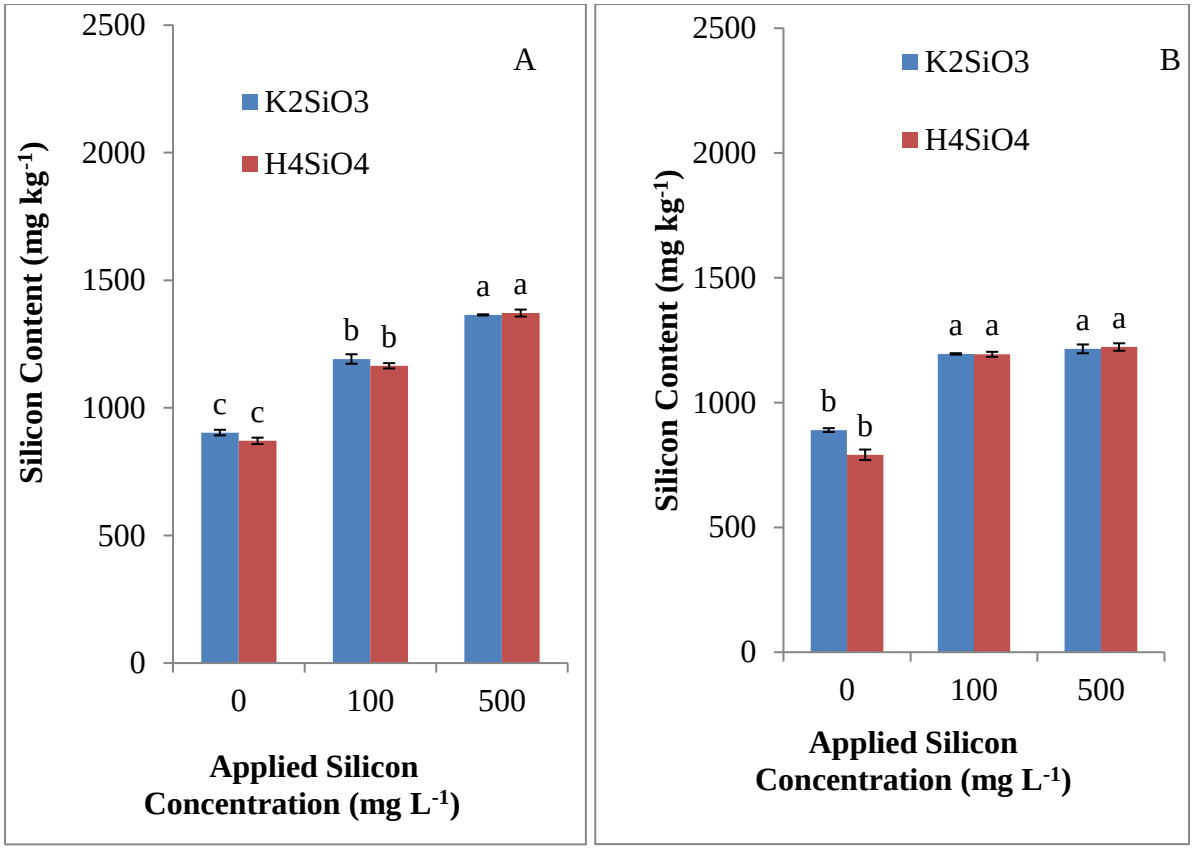

Figure 4. 1 Silicon content in (A) Valencia leaves and (B) Clementine leaves, after treatment with two Si sources at two concentrations, applied using drench applications

4.3.2. Season and leaf age impact on Si uptake in root-Si applied plants

The investigation on the effect of season, on Si uptake in both cultivars demonstrated that season significantly impacted on Si uptake (Figures 4.2). Si application in summer resulted in significantly higher uptake of Si than a winter application of Si in both young and old citrus leaves. Levels of Si were significantly higher in old leaves than young leaves in both cultivars (Figures 4.2). The Si application rate of 150 mg kg^{-1} resulted in greater Si uptake of Si than a rate of 75 mg kg^{-1}, especially with old leaves.

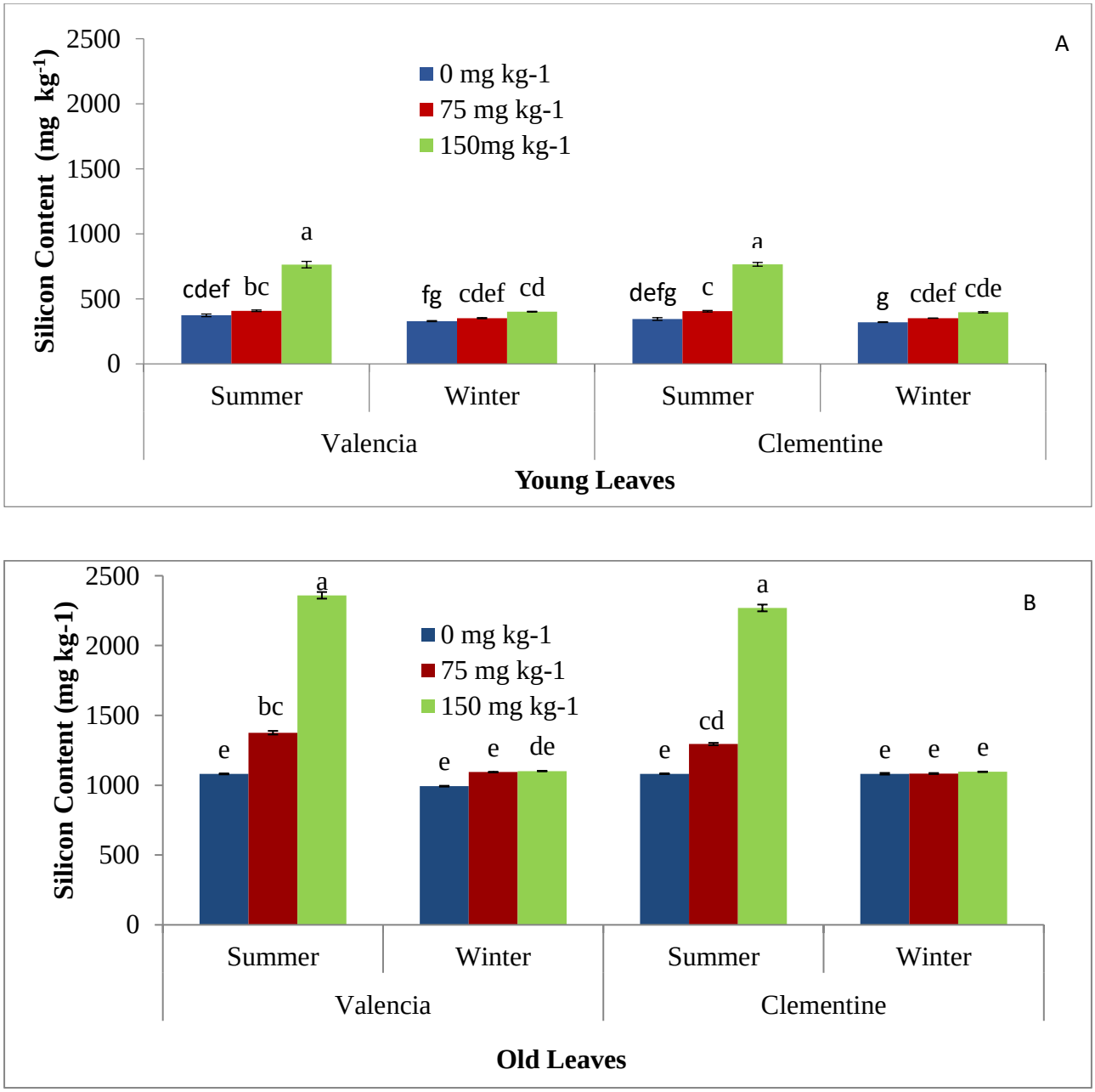

Figure 4. 2 Silicon content of (A) young citrus leaves and (B) old leaves with a drench application of K_2SiO_3. Bars sharing a letter are not significantly different. Data are means ± standard errors.

4.3.3. Impact of season and leaf age on Si uptake in foliar-Si applied plants

The investigation on the effect of season, leaf age and foliar-Si application on Si uptake in both cultivars demonstrated that Si application rate, season and leaf age significantly impacted on Si uptake (Figures 4.3). Silicon uptake responded positively to Si application drenches at concentrations of 100 and 500 mg L^{-1}, with more uptake after applications at 500 mg L^{-1}. Summer application of Si resulted in significantly more uptake of Si than winter

application of Si in both young and old citrus leaves (Figures 4.3). After foliar application of Si at 500 mg $L^{-1,}$ the levels of Si in old leaves was 2192 mg kg^{-1} compared to 560 mg kg^{-1} in young leaves. Drench applications of Si resulted in more Si uptake than foliar applications of Si regardless of citrus tree types (Figures 4.2 and 4.3).

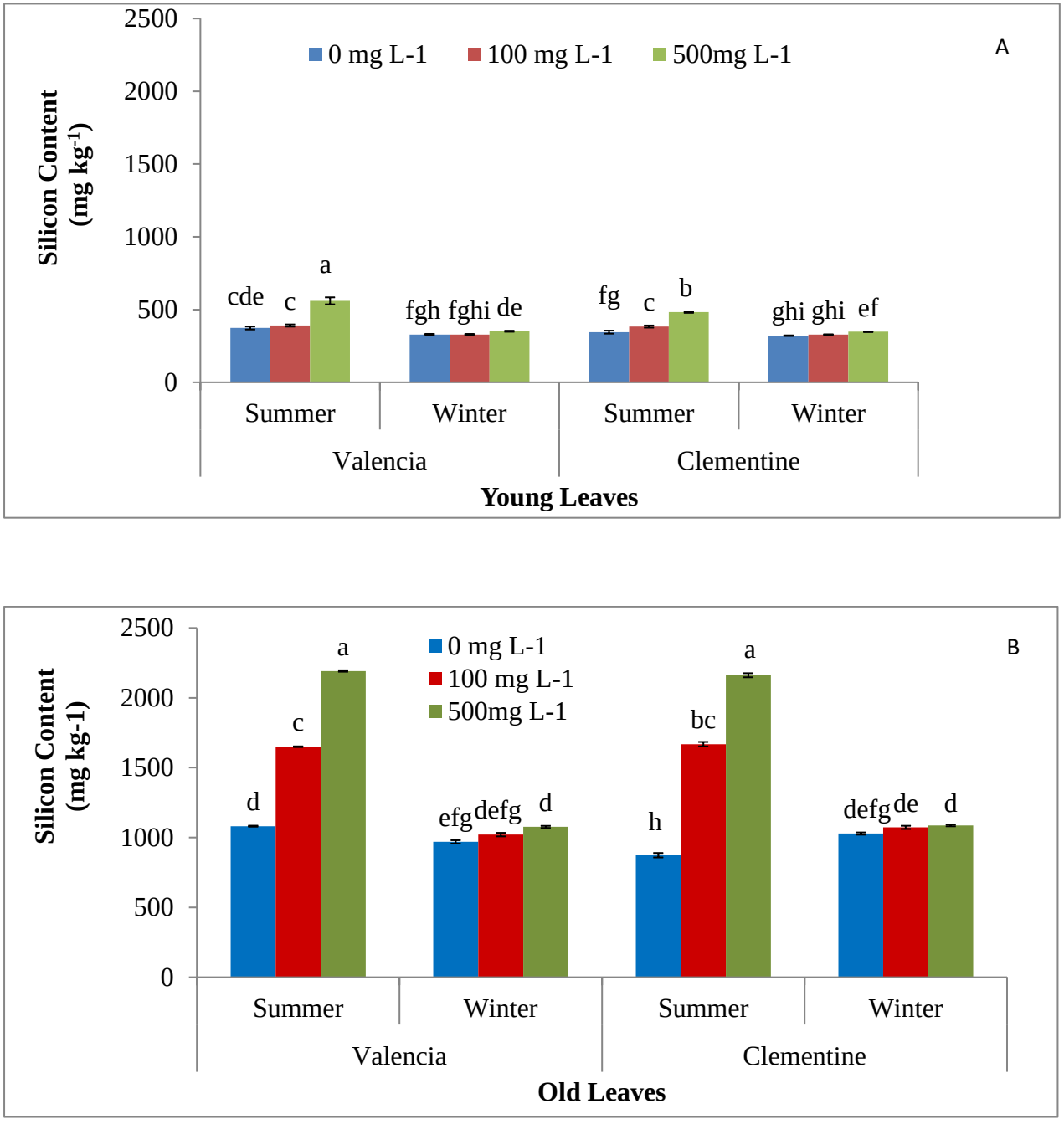

Figure 4. 3 Silicon content of (A) young citrus leaves and (B) old leaves after foliar applications of K_2SiO_3. Bars sharing a letter are not significantly different. Data are means ± standard errors.

4.3.4. Determination of an optimum application concentration of Si for citrus plants

Si absorption increased significantly with increases of soil drenches of Si, reaching the highest concentration in the roots and leaves of both citrus cultivars at 1000 mg Si L^{-1} (Figure 4.4). The Si concentration in both leaves and roots organs of the two cultivars decreased at a Si application rate of 2000 mg L^{-1} (Figure 4.4). Si levels were significantly higher in roots than leaves, irrespective of Si application rate.

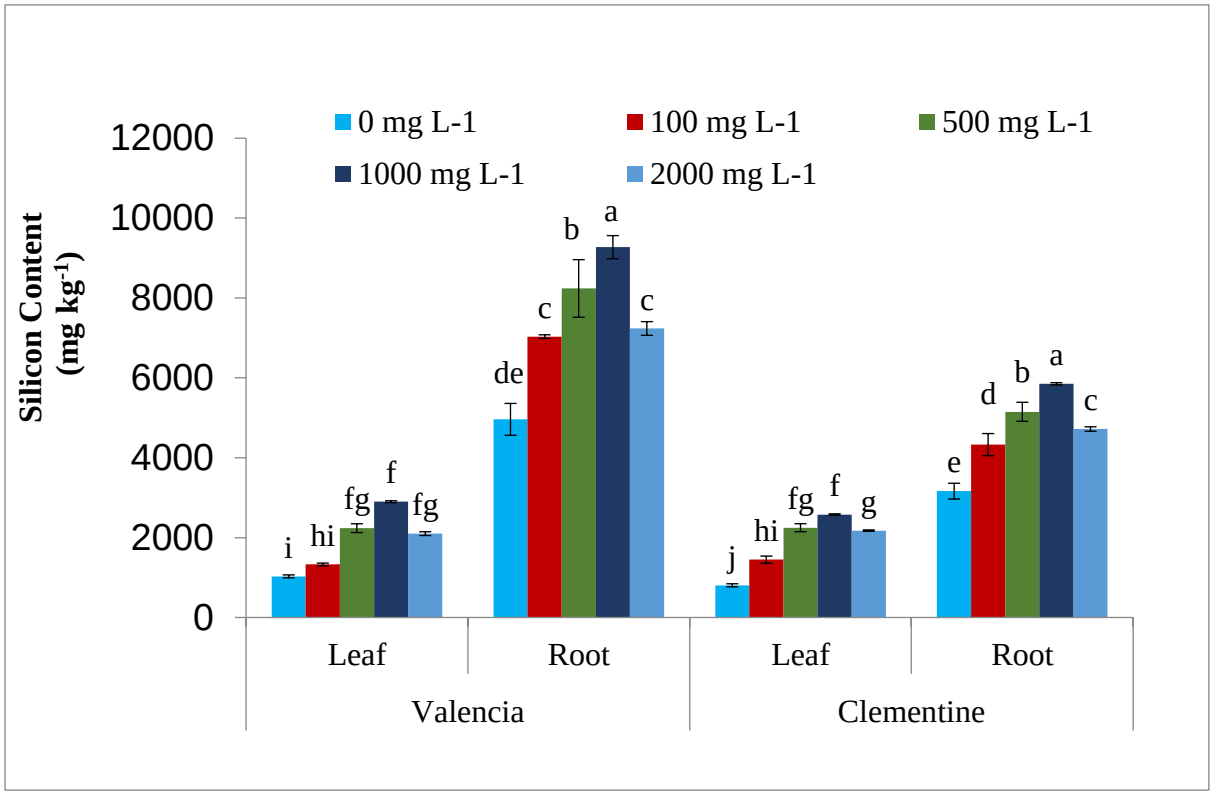

Figure 4. 4 Si uptake in roots and leaves of potted Valencia and Clementine trees subjected to Si drench treatments. Bars sharing a letter within a cultivar are not significantly different. Data are means ± standard errors.

4.3.5. Influence of Si applications on dry matter production in citrus trees

Dry matter production of leaves and roots in both cultivars did not increase when Si was applied at concentrations of 0, 100 and 500 mg L^{-1} (Table 4.2). However, there were significant increases in leaves and roots dry matter production in both cultivars when Si was applied at concentrations of 1000 mg L^{-1} and 2000 mg L^{-1} (Table 4.2). The root dry mass of

Valencia was consistently higher than leaf dry mass, whereas the inverse was observed in Clementine.

Table 4.2. Response of leaves and roots dry matter production to Si soil application in Valencia and Clementine cultivars

	Valencia		Clementine	
Si application rate (mg L^{-1})	**Leaf dry mass (g)**	**Roots dry mass (g)**	**Leaf dry mass (g)**	**Roots dry mass (g)**
0	65.90^{i}	95^{d}	60.50^{cde}	36.80^{h}
100	66.80^{hi}	97.80^{cd}	61.80^{cd}	38.60^{gh}
500	69.90^{gh}	98.70^{bc}	62.40^{bc}	40.20^{fg}
1000	72.80^{f}	118.70^{a}	66^{a}	46.90^{e}
2000	74.90^{ef}	115.50^{a}	68.40^{a}	47.40^{de}
Lsd	3.32		2.76	
CV%	2.6		3.6	
Sed	2.3		1.91	
P value	<0.001		<0.001	

Data are means. For each cultivar, values sharing the same letter vertically are not significantly different.

4.3.6. Influence of Si application on Si uptake in citrus cultivars

In a confirmatory trial, the uptake of Si was assessed in trees of two citrus cultivars treated with Si at four concentrations. Significant increases in uptake of Si in the roots and leaves of both cultivars were measured, for all concentrations of Si used. The highest level of Si uptake

was observed at Si application rates of 1000 mg L^{-1} and 2000 mg L^{-1} (Table 4.3). Additionally, Si uptake was significantly higher in roots than leaves.

Table 4. 3. Silicon uptake in root and leaves organs of two citrus cultivars

Organs	**Valencia**		**Clementine**	
Si application rate (mg L^{-1})	**Leaves**	**Roots**	**Leaves**	**Roots**
0	67.8^{ghi}	126.1^{fgh}	48.5^{e}	53.2^{e}
100	149.7^{fg}	282.1^{d}	138.4^{cd}	100^{d}
500	146.5^{f}	492.3^{c}	135.9^{cd}	126.6^{cd}
1000	216.6^{e}	974.2^{a}	168.3^{bc}	246.4^{a}
2000	156^{f}	836.6^{b}	149.6^{c}	220.1^{a}
Lsd	40		50.15	
CV%	25.89		25.6	
Sed	7.11		7.2	
P value	<0.001		<0.001	

Data are means. For a given cultivar, values sharing the same letter vertically are not significantly different.

4.4. Discussion

In a study on the influence of Si applications on the effects of drought stress in grasses, it was found that K_2SiO_3 was the best source of Si, and silica gel the least effective (Eneji *et al.*, 2008). In another study conducted on wheat, K_2SiO_3 was again a better source of Si than silica gel. This may be explained by the rapid formation of orthosilicic acid from K_2SiO_3 (Mecfel *et al.*, 2007). In the current study, two Si sources, K_2SiO_3 and H_4SiO_4, were equally

effective as Si sources, which suggests that both sources of Si readily dissociated to form orthosilicic acid, which can be taken up by plants.

Si uptake has been found to increase with increased Si supply in several plants (Wutscher, 1989, Epstein, 1999, Ma & Takahashi, 2002, Mitani & Ma, 2005, Ma & Yamaji, 2006, Wang *et al.*, 2015, Tubana *et al.*, 2016). Previous studies demonstrated that Si is not relocated within citrus plants (Wutscher, 1989, Ma & Takahashi, 2002). In our study, Si content significantly increased with leaves maturity in the two citrus cultivars, irrespective of the Si application level, confirming this hypothesis.

A similar trend in Si uptake has been found in other studies conducted on the leaves of bamboo and banana, where Si concentration increased with leaf age, even after maturation (Motomura *et al.*, 2002, Henriet *et al.*, 2006).

Soil temperature influences root growth and the ability of roots to absorb and translocate nutrients (Lawlor, 2004). In the current study, in both the young and mature leaves of the two citrus cultivars (Figures 3.2), a clear seasonal effect was observed, irrespective of the Si source or application method. Si uptake was significantly higher in summer than in winter. These findings are supported by previous reports, which demonstrated a rapid silica uptake in bamboo during the summer season (Motomura *et al.*, 2002). This implies that there is involvement of an active uptake of Si, which is dependent on soil temperature, requiring physiologically activity by the citrus plants.

Si content was higher after drench applied Si than after foliar applied Si, which corroborates previous research (Liang *et al.*, 2005, Rezende *et al.*, 2009). In the foliar applied Si, the absorption is affected by the cuticle barrier that severely restricts diffusion from the external leaf surface into the bulk of the leaf, the mobility of the element in the entire plant, the

polymerization of silica on the leaf surface and the possible involvement of transpiration in Si uptake in plants (Ma & Takahashi, 2002, Treder & Cieslinski, 2005, Buck *et al.*, 2008).

Plants absorb Si as monosilicic acid, $Si(OH)_4$, which has a upper limit saturation concentration of 500 mg L^{-1}. In hydroponic studies, at higher concentrations monosilicic acid polymerizes into silica (Jones & Handreck, 1967, Epstein, 1999, Xu *et al.*, 2001, Mitani *et al.*, 2005). In this study, the Si uptake saturation limit in citrus plants was demonstrated at a concentration of between 1000 mg L^{-1} and 2000 mg L^{-1}. A similar trend was found in another study, conducted with wheat, where it was reported that 1000 mg L^{-1} was the optimum Si concentration for drenching wheat (Casey *et al.*, 2004). In another study on banana, the optimum Si application rate was 2000 mg L^{-1} (Kidane, 2008). The optimum Si application rate for zucchini and zinnia plants grown hydroponically, with constant uptake being possible, was 50 mg L^{-1} (Tesfagiorgis & Laing, 2013). The significant difference observed between the two citrus cultivars is accounted for by the original differences in size between Valencia and Clementine cultivars because the later variety was half the size of the former.

Several studies have reported on the effect of Si in dry matter production in other crops (Adatia & Besford, 1986, Detmann *et al.*, 2012, Silva *et al.*, 2013, Marodin *et al.*, 2014). The positive response of dry matter content to Si uptake was only registered under the two highest Si application rates of 1000 mg L^{-1} and 2000 mg L^{-1} with no significant differences between these two Si application levels in either leaves or roots. A positive correlation between Si and dry mass weight has been demonstrated in previous studies (Adatia & Besford, 1986, Detmann *et al.*, 2012, Silva *et al.*, 2013, Marodin *et al.*, 2014, Dorairaj *et al.*, 2017). Growth of Si-treated banana plants plant was not influenced by the level of Si supplied (Henriet *et al.*, 2006).

Conclusions

The current study presented Si uptake of citrus plant and its influence on dry matter production. The study focused on the influence of leaf age, Si formulations, application method, season, and applied Si on Si uptake in two citrus species. The study provided evidence on the maximum Si uptake. The study also demonstrated the influence of plant organs and Si application rate on Si uptake. The distribution pattern of Si within the citrus plants was in the order of young leaves<old leaves<roots. This suggests that Si is not relocated within the citrus plants, more details on the uptake mechanism is found in Chapter V. Based on the findings, root-Si application was the preferred method of application in subsequent experiments. Additionally, citrus dry matter production was positively influenced by Si application rates of 1000 and 2000 mg L^{-1}.

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CHAPTER 5

THE INVESTIGATION OF SILICON LOCALIZATION AND UPTAKE IN CITRUS

Abstract

Several studies have demonstrated Si absorption in monocotyledonous and dicotyledonous species. Regarding Si uptake, studies in monocotyledons have identified Si deposition around the cell wall, cuticle layer bulliform cells, silica cells and endodermal cells. In previous studies with different citrus species there was evidence of Si uptake, however no information on Si deposition was found. Therefore, in this study, Si was applied (0 and 1000 mg L^{-1}) to the roots of two citrus species, 'Delta' Valencia (*Citrus sinensis*) and 'Nules' Clementine (*Citrus reticulata*). Si uptake was investigated in young and mature leaves, and roots. Si deposition was investigated through scanning electron microscopy using energy dispersive analysis X-ray, environmental scanning electron microscopy and light microscopy. Si uptake was significantly higher in Si treated leaves in comparison to both young and mature leaves of the untreated Si plants. Si uptake increased with leaf age, which indicated the involvement of active uptake of Si, and was significantly higher in roots than in leaves.

With respect to Si deposition, granules were identified in the epidermal cells through SEM and ESEM studies. The light microscopy identified the presence of Si granules on the surface and around the outer cell surface, forming the cuticle-silica double layer of the upper epidermis in Si treated plants.

Keywords: Clementine, energy dispersive X-ray analysis, Silicon uptake, Valencia

5.1. Introduction

Silicon (Si) is the second most abundant element in the earth's crust after oxygen. These two elements are combined in the aluminosilicates of rocks, clays and soil minerals (Birchall, 1995). Plant available Si can be found in the soil solution in an undissociated form as monosilicic acid ($Si(OH)_4$) in a concentration range of 90-150 mg L^{-1} in soils with a pH lower than 8.0 (Jones & Handreck, 1967, Epstein, 1999, Ma & Yamaji, 2006). In highly weathered soils, Si availability in soil solution is reduced considerably because of soil acidification, organic complexes, presence of aluminium, iron and phosphate ions, temperature, sorption/dissolution reaction and soil moisture (Epstein, 1999, Haynes, 2014). This leads to the reduction in plant available Si and the need to supplement with silicon fertilizer in order to improve the quality and yield of agricultural crops under abiotic and biotic stress conditions (Epstein, 1999, Ma & Takahashi, 2002, Mitani & Ma, 2005, Tubana *et al.*, 2016). Silicon has not been defined as an essential element for higher plants, although it significantly improves fitness in nature, increases agricultural productivity, and is present in plants in amounts equivalent to certain macronutrients such as Ca, Mg and P (Raven, 2003).

There are two general mechanisms for Si uptake and transport (active and passive uptake) co-existing in a plant, with their relative contribution being dependent much upon the plant species and external Si concentration (Liang *et al.*, 2005, Ma & Yamaji, 2006). *Oryza sativa* L. and *Zea mays* L., representatives of monocots, have Si in their tissues in the order of 5% or higher (dry weight basis) and are known as Si accumulators. On the other hand, *Helianthus annuus* L. and *Benincasa hispida* L., representatives of dicots, contains about 0.1% Si on a dry weight basis, and are described as intermediate types (Jones & Handreck, 1967, Ma & Takahashi, 2002). In another study conducted by (Mitani & Ma, 2005), Si concentration in the root cell symplast of rice was higher than the external Si in the soil solution. This suggests

that silicic acid is taken up against the concentration gradient from the external solution to the cortical cells, which requires an active uptake process. In dicots, the coexistence of both a passive and active transport dependent upon the external Si supply has been identified in cucumber (Liang *et al.*, 2005). In another study, uptake of Si by cucumber (*Cucumis Sativus*) and tomato (*Solanum lycopersicum*) was hypothesized to be by passive uptake, based on their inefficient radial transporters which do not enable them to take up as much Si as rice using transporter proteins for xylem loading in the latter (Mitani & Ma, 2005). This implies that for plant Si, the initial uptake , translocation from the xylem and distribution in the leaves may be influenced by at least three transporters in higher plants (Takahashi *et al.*, 1990, Ma & Yamaji, 2008, Yamaji *et al.*, 2008, Ma *et al.*, 2011, Mitani *et al.*, 2011, Yamaji *et al.*, 2012, Rao & Susmitha, 2017).

The Si influx transporter Lsi1 is mainly expressed in the main roots and lateral roots but not in root hairs and is responsible for the transport of Si from the external solution to the root cells (Ma & Yamaji, 2006, Ma *et al.*, 2011). Homologs of Lsi1 have also been reported in barley (*Hordeum vulgare*), maize, pumpkin (*Cucurbita Pepo*) and wheat (*Triticum aestivum*) (Chiba *et al.*, 2009, Mitani *et al.*, 2011, Montpetit *et al.*, 2012). While, the Si efflux transporter Lsi2 is found in the endodermis of rice roots (Ma *et al.*, 2011). Homologs of Lsi2 transporter have also been reported in barley, maize and pumpkin (Mitani *et al.*, 2009, Mitani *et al.*, 2011). The transporter Lsi6 is a homolog of Si influx transporter Lsi1 in rice, barley and maize (Yamaji *et al.*, 2008, Yamaji *et al.*, 2012). It is a plasma membrane protein localized in xylem cells of leaf sheaths and blades and plays a role in the unloading of Si from xylem vessels into leaf tissue (Yamaji *et al.*, 2008, Mitani *et al.*, 2009).

Silica deposition in plants may be found in the outer cell surface of leaves, which creates a cuticle-silica double layer that has been hypothesized to be dependent on the transpiration rate (Raven, 2003, Ma & Yamaji, 2008). The role of transpiration in Si uptake implies that Si

should be densely deposited in the mesophyll tissue where most of the transpiration takes place (Motomura *et al.*, 2000). However, Si is deposited in both the mesophyll regions and epidermal cells in Poaceae, implying that in addition to the influence of transpiration on Si uptake, plants also positively controlled the Si deposition process (Motomura *et al.*, 2004).

Silicon deposits have been found in leaf blades and inflorescence bracts tissues (Motomura *et al.*, 2004, 2006, de Melo *et al.*, 2010). Other areas of Si deposition have been identified around the cell surface: cell lumens, cell wall, guard cells, intercellular spaces, root endodermal cells regions and bulliform cells (Ma & Takahashi, 2002, Raven, 2003, Motomura *et al.*, 2004, Heckman, 2013). Silicon is also found in the upper and lower epidermis of leaves as silica bodies that eventually constitute a cuticle-silica double layer (Kaufman *et al.*, 1985, Motomura *et al.*, 2000, Ma & Takahashi, 2002, Richmond & Sussman, 2003, Motomura *et al.*, 2006).

The beneficial role of Si in citrus has been demonstrated in a few studies. Si fertilisation has been reported to increase fruit yield, and to accelerate growth and fruit ripening by two to four weeks (Taranovskaia, 1939). In a study of grapefruit (*Citrus paradisi*), fertilisation with calcium silicate slag increased root and shoot mass (Matichenkov *et al.*, 1999). (Matichenkov *et al.*, 2001) reported an increase in tree height and shoot mass for Valencia trees. In greenhouse studies, potassium silicate (K_2SiO_3) applications improved fresh shoot mass in young sweet orange (*Citrus sinensis* (L.) Osbeck) trees over a six-month period (Wutscher, 1989). However, none of these citrus studies provided information on the Si deposition. Therefore, the aim of this study was to examine the site of Si deposition in two Si accumulating citrus species.

5.2. Materials and methods

5.2.1. Plant material

Two citrus species, Valencia 'Delta' and Clementine 'Nules', were used for the study. These two widely cultivated commercial citrus cultivars in South Africa, were selected in order to compare two different citrus species, namely orange and soft citrus (mandarins) (Saunt, 2000). Both species were grafted onto Carrizo citrange rootstocks.

5.2.2. Silicon uptake experiment

Seventy-two 2-year citrus seedlings were planted in 10 L pots containing an artificial growing medium of Coir-Perlite (60% - 40%). These were fertilized with NPK at 85-115-40 kg ha^{-1}, which represents the local fertiliser recommendation for two-year citrus trees (FSSA-MVSA., 2007). NPK was applied in the form of ammonium nitrate (NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4) and potassium nitrate (KNO_3). During this period, the water holding capacity of the medium was determined by watering selected pots till drainage and weighing them, to estimate subsequent irrigation needs. Each pot was irrigated to field capacity with 800 mL of distilled water every three days. The trees were left to acclimatize for three weeks prior to the Si uptake study.

The Si uptake experiment was performed over a period of three months in two summer seasons (January- April 2014 and 2018) in a glasshouse at the Experimental Farm of the University of Pretoria (S25^o 44' E28^o 15'). The citrus plants were drenched once a month for three months with potassium silicate (1000 mg L^{-1}). In the control (0 mg L^{-1}), the level of K introduced by K_2SiO_3 to the other treatment was corrected by adding K_2O to the trees. Each treatment consisted of six replications (in groups of 3 plants per replicate). The 72 trees were organised in a randomised complete block design. These pots were kept in a climatic-controlled greenhouse, on a rotary table to minimize greenhouse effects on the plants. A month after the third drenching application, young and mature plant leaves were collected.

Roots were collected 10 mm from the root base (Lux, 2003). These samples were washed in deionised water to remove all impurities and samples were oven dried (Labotec, South Africa) at 70^{o}C for 4h. Samples were milled and sieved for Si analysis using ICP-OES.

5.2.3. Si analysis

Polypropylene and Teflon containers were used for the preparation and digestion of plant samples to minimize contamination risks when using glassware. All containers were rinsed with NaOH (10%) prior to use. Leaf and root samples (500 mg) were placed in Teflon microwave digestion tubes and 3 mL of 65% HNO_3 (AR grade) was added. The Teflon tubes were capped and left to stand for five minutes to thoroughly wet the sample. Then 2 mL of 30% H_2O_2 (AR grade) were added to the tubes and left overnight. The samples were placed in the microwave unit to the ramping temperature of 180^{o}C for 30min and the acid digestion step conducted. NaOH (10%) solution was then added to the tubes and they were returned to the microwave system for the second heating step to solubilise amorphous Si. The contents of the Teflon tubes were transferred into a plastic beaker to minimize Si contamination, then neutralized with HNO_3 (2 M), using phenolphthalein as an indicator, and then diluted to 250 mL in a volumetric flask. A 10 mL sample was taken for Si determination with ICP-OES (Varian Liberty 200); fitted with a hydrofluoric acid (HF) resistant torch, Sturrman Master spray-chamber and V Groove nebulizer assembly. The plasma power was 1000 W with a plasma flow rate of 15 L min^{-1} and an integration time of 1s. Two Si sensitive wavelengths, 251.611 nm and 288.158 nm were used to detect Si. The wavelength of 251.611 nm was selected as the most sensitive wavelength, based on its higher correlation with the calibration curve. Matrix interferences were accounted for by preparing the sample and standard in a similar matrix.

5.2.4. Data collection

Scanning electron microscopy (SEM) and EDAX analysis

Silicon deposition was investigated inside leaves using scanning electron microscopy (SEM) was used to determine and identify Si precipitates within the leaf. Mature citrus leaves were collected after three months of Si application; washed with distilled water, wiped, and then fixed with glutaraldehyde (primary fixation) overnight and osmium tetroxide (post fixation) for 1h. This was followed by a wash with a 0.05 M sodium cacodylate buffer, and then subjected to dehydration in an ethanol series. The specimens were placed in hexamethyldisilazane, left to air dry overnight, then mounted onto a stub using a carbon double-sided tape, and sputter coated with gold using an EMTECH K550X Coater (Quorum Limited, Kent, United Kingdom) and viewed at 3kV with a Zeiss Crossbeam 540 FEG scanning electron microscope (ESEM) (Carl Zeiss, South Africa). Energy dispersive analysis was conducted with an SEM-EDAX (XL30, Phillips, Eindhoven, Holland).

Environmental scanning electron microscopy (ESEM)

The energy dispersive analysis unit attached to the ESEM was used to determine the Si content in the adaxial and abaxial epidermal tissues of citrus leaves (Valencia and Clementine). Mature citrus leaves were collected a month after the last Si drench, washed with distilled water and wiped dry, before one square centimetre of leaf was excised with a razor blade and coated with gold in a vacuum coating unit (Polaron E5200C, Watford, UK), mounted on a carbon planchette and viewed on an ESEM instrument (Phillips XL30, Eindhoven, Holland). The operating settings were: voltage energy of 20 keV, with pump detection of 500 µm, the diffusion pump was set to a pressure between 1 and 2 Torr, wet mode, purge custom, spot size 6, room temperature (approximately 20^{o}C), a working distance of 50 µm and the mechanical pump had a pressure of 10 mm (Hg). A gaseous secondary detector was used to determine Si.

Light microscopy

Plant material (approximately 2 mm^2) of each sample was cut from the region between the leaf margin and midrib of the middle sections. The tissues were fixed in 3% glutaraldehyde for 24 h at 1^oC. The tissues were then washed in a 0.05 M sodium cacodylate buffer for 30 min. The samples were then post-fixed overnight in 2% osmium tetroxide with a pH of 7.2. After this, they were washed twice in 0.05 M Na-cacodylate buffer for 30 min. The specimens were dehydrated through a series of ethanol with 10, 30 and 50% (v/v) ethanol, for 15 min at each dehydration step. The samples were left overnight in the 50% (v/v) ethanol. The next day, the samples were dehydrated through 70, 90, 100% (v/v) ethanol with 15 min intervals between each step. The dehydration series were completed with two rinses of 15 min each in absolute alcohol. The Agar low viscosity (LV) resin (Advanced Laboratory Solutions, Johannesburg, South Africa) is not miscible with alcohol and, therefore, the samples were rinsed twice and left overnight in propylene oxide. To ensure adequate infiltration of the material with the embedding mixture, the tissues were left overnight in 25:375 LV resin/propylene oxide. The next day, the tissues were placed in 50:50 LV resin /propylene oxide overnight, 75:25 LV resin/ propylene oxide for 2 h and twice into 100% LV Resin for 1h.

The tissues were finally placed in labelled moulds and polymerized for 16h at 60^oC. After removal of the tissues from the oven the tissues were cooled at room temperature before sectioning. Selected tissue regions were sectioned in the range of 0.5 -1.0 μm. Thick sections were cut using an LKB Ultramicrotome III (Stockholm, Sweden) with a knife clearance angle set at 5^o to produce purple sections.

The sections were mounted on glass slides and stained with Ladd's stain for 20s and rinsed in distilled water. The tissues were viewed using an Olympus BH2 light microscope (Wirsam Scientific, Johannesburg) at 40X and 100X magnification.

5.2.5. Statistical analysis

The collected data was subjected to analysis of variance by using Statistical Analysis System software (SAS) version 9.4 (Cary, NC, USA) to determine treatment mean effects. Differences between treatments were determined using Fisher's Least Significant Difference (LSD) at 5% level of significance.

5.3. Results

5.3.1. Si uptake in citrus species

Si uptake increased significantly with age in both Valencia and Clementine leaves, regardless of Si treatment (Figure 5.1). For both species, drenching with 1000 mg L^{-1} Si resulted in significantly higher Si contents in both roots and leaves (Figure 5.2). Additionally, levels of Si in roots were significantly higher than in leaves, regardless of Si treatment, and in both citrus species.

Figure 5. 1 Si levels in young and mature leaves of two citrus species. Bars sharing a letter are not significantly different. Data are means ± standard errors.

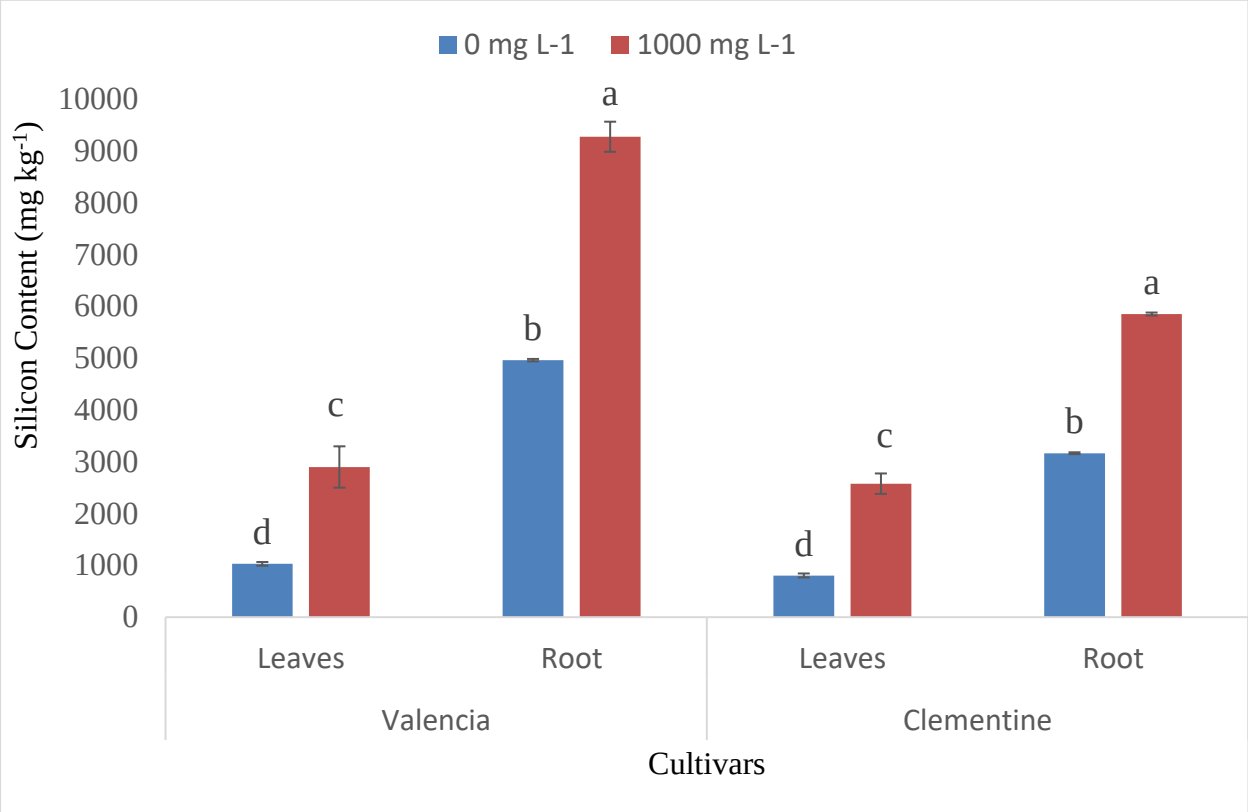


Figure 5. 2 Si levels in mature leaves and roots of two citrus species. Bars sharing a letter are not significantly different. Data are means ± standard errors.

5.3.2. Scanning electron microscopy with energy dispersive X-rays analysis

Scanning electron microscopy investigation in citrus leaves demonstrated the absence of silica bodies in the control leaves (Figure 5.3A). In contrast, silica bodies (granules) were present in epidermal cells of Si-treated leaves (Figure 5.3B). Elemental analysis using EDAX demonstrated the presence of Si in Si treated leaves while negligible amounts of Si were found in Si untreated leaves (Figures 5.3C and 5.3D).

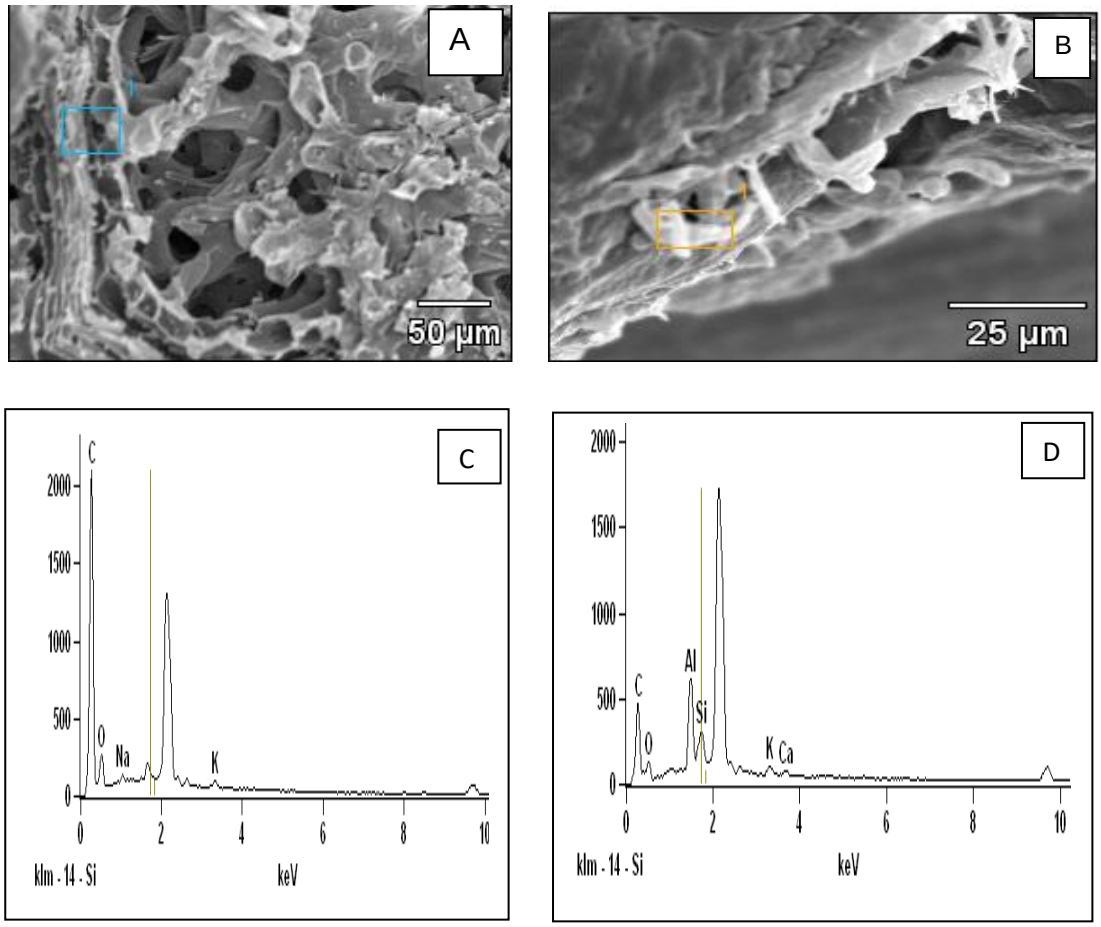


Figure 5.3 Scanning electron microscopy images of A) –Si and B) +Si mature leaves, white granules are areas of silicon detection. The selected areas (blue and orange blocks) were examined for elemental composition by EDAX C) -Si leaves D) + Si leaves.

5.3.3. Environmental scanning electron microscopy (ESEM)

ESEM was used to investigate the presence of Si in adaxial and abaxial leaf surfaces. Si was located on the adaxial surface as white granules, and more granules were found in Si treated leaves than in the control (Figure 5.4). Despite evidence of Si in epidermal cells, ESEM of the adaxial leaf surface did not provide the exact area of Si deposit within the cell structure.

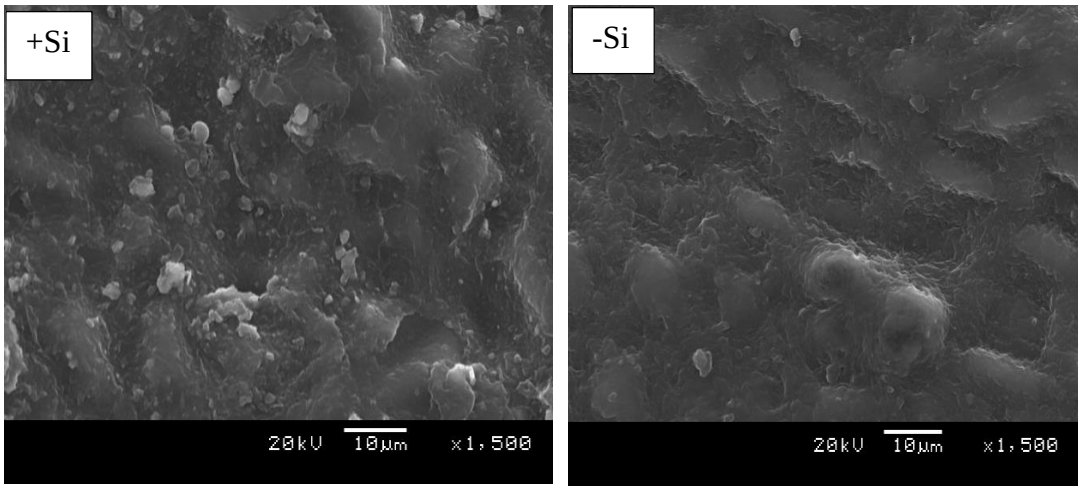


Figure 5.4 ESEM micrographs of +Si and -Si mature citrus leaves, the white granules are areas of silicon detection on the adaxial leaf surfaces.

In the investigation of the abaxial region where stomata are found on citrus leaves, there were no Si deposits identified, irrespective of the Si treatment (Figure 5.5).

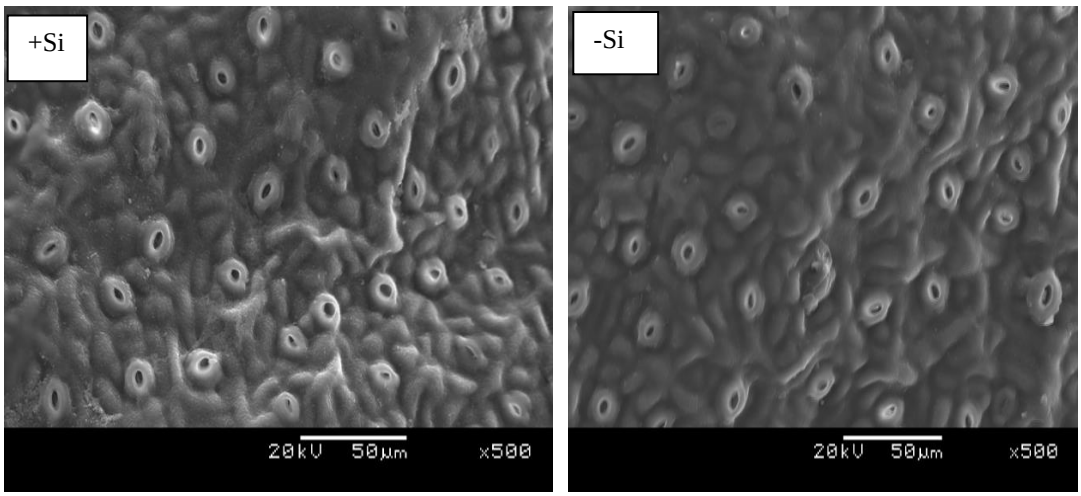


Figure 5. 5 ESEM micrographs of mature citrus leaves subjected to +Si and -Si treatments; stomata of the abaxial leaf surfaces.

5.3.4. Light microscopy

Light microscopy provided further detail with regards to Si deposits in the cell structure. In the current study, cell structures were examined in both 40 and 100X magnifications, the later provided a better view. Silica granules were located on the adaxial epidermal cell of Si treated leaves. Additionally, silica deposits were found on the outer cell surface that constitute a Si double layer in treated Si leaves (Figures 5.6). With respect to the control leaves, no Si deposits were located on the leaf surface (Figure 5.7).

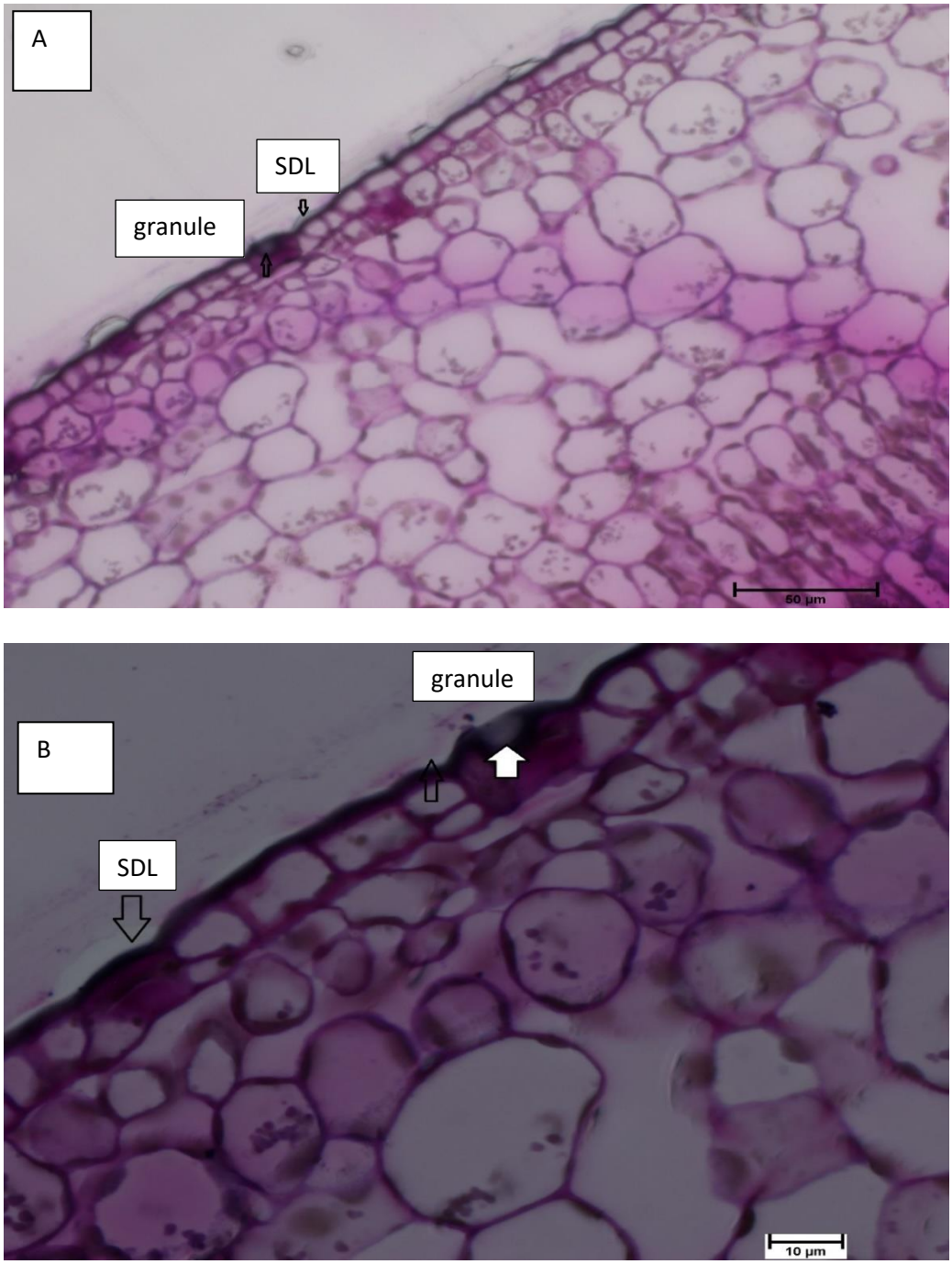


Figure 5. 6 Si treated matured citrus leaves subjected to light microscopy, viewing the upper epidermal surface at (A) 40X, and (B) 100X magnification: arrows in diagram mark silica granules in the epidermal surface; Si deposits in the outer cell regions constitute the cuticle- silica double layer (SDL).

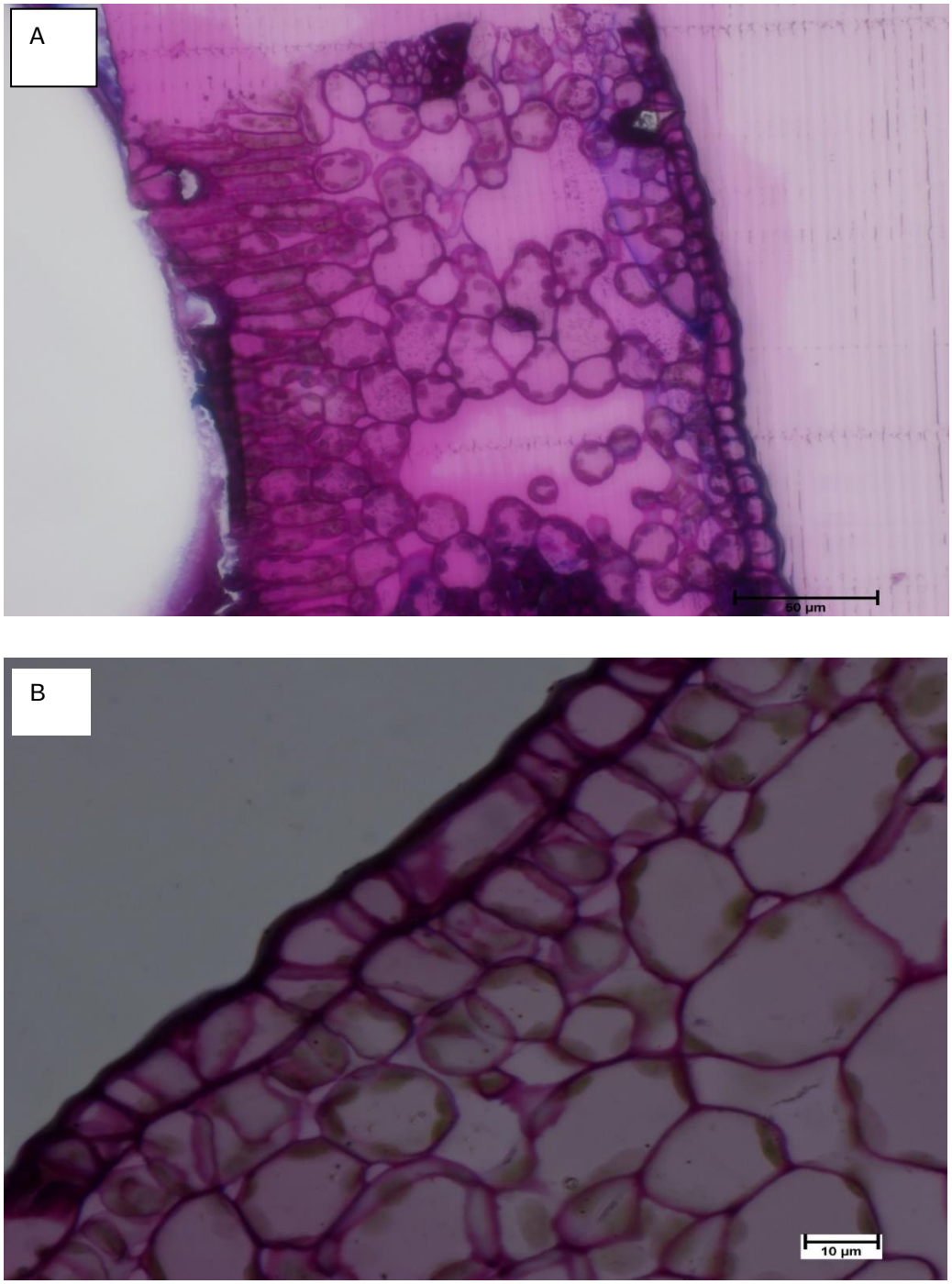


Figure 5.7 Untreated mature citrus leaves subjected to light microscopy. A) 40x and B) 100X magnification: no silica deposits were located.

5.4. Discussion

The uptake and deposition of Si in plant tissues is related to several factors such as species, leaf age, external Si supply, root uptake ability, transporters and transpiration (Takahashi *et*

al., 1990, Epstein, 1999, Heine, 2005, Mitani & Ma, 2005, Yamaji *et al.*, 2008, Ma & Yamaji, 2015, Rao & Susmitha, 2017). Si uptake and deposition in leaves significantly increased in both cultivars when treated with Si. These results agree with previous studies conducted in tomato (Marodin *et al.*, 2014); rice (Ma & Takahashi, 2002, Mitani & Ma, 2005); citrus (Wutscher, 1989, Matichenkov *et al.*, 1999, Matichenkov *et al.*, 2001); forage grass (de Melo *et al.*, 2010) and cucumber (Adatia & Besford, 1986), who demonstrated Si uptake is linked to external Si concentrations. The Si content of citrus leaves measured here was less than 5 g kg^{-1}, which is typical of a Si passive accumulator (Liang *et al.*, 2006, Tubana *et al.*, 2016).

In this study, Si content significantly increased with age in the two citrus species irrespective of Si application level. This finding supports the statement that Si is not relocated within citrus plants, as demonstrated by Wutscher (1989) and Ma & Takahashi (2002). A similar trend in Si deposition was found in other studies conducted on the leaves of bamboo (*Sasa veitchii*) and banana (*Musa acuminata*), where the Si concentration increased with leaf age, even after maturation (Motomura *et al.*, 2002, Henriet *et al.*, 2006), indicating that silica deposits are immobile within plants and cannot be translocated to young leaves (Ma & Takahashi, 2002, Tubana *et al.*, 2016).

In the current study, the Si levels were significantly higher in the roots than the leaves. This result agrees with Matichenkov *et al.* (1999) who demonstrated a similar pattern of Si levels, and speculated on the poor translocation of Si in citrus plants. In studies conducted on Si distribution, tomato demonstrated a similar uptake pattern, whilst in bitter gourd shoot Si levels were higher than in the roots (Heine, 2005). Ma *et al.* (2004) observed that Si levels were higher in the shoots than the roots in species with an active Si uptake, such as in rice, in which 90% of the Si taken up by the roots was translocated to the shoots (Ma *et al.*, 2011). A

similar trend was observed banana (Henriet *et al.*, 2006) and rice (Ma & Takahashi, 2002) Si fertilization studies.

Si deposition in plant cells depends on a number of factors, such as pH, Si concentration applied and transporter genes (Zhang *et al.*, 2013). When the pH is lower than 7.0, which is the usual range inside cells, silica particles form chained oligomers due to weak electrostatic repulsion. But at higher pH values, they form condensed, disordered polymers (Coradin & Lopez, 2003). In the current study, Si deposition was only found on adaxial surfaces of leaves. Similar results were observed in shoot tissue of tropical forage grass (*Brachiaria brizantha*), which was classified as a passive uptake species (de Melo *et al.*, 2010). In other previous studies conducted on bamboo and sugarcane, which have been classified as active uptake Si accumulators, Si deposits were found on the abaxial surface (Sakai & Sanford, 1984, Motomura *et al.*, 2006). Moreover, in rice, an active Si accumulator, Si deposits were found in both leaf surfaces (Zhang *et al.*, 2013). Therefore, the site of Si deposition in specific cell structure depends on the plant species (de Melo *et al.*, 2010).

In this study, Si deposits were only observed on the upper (adaxial) epidermis. Rice and a tropical forage grass, have more silica deposited in the upper epidermis than the lower epidermis (Takahashi *et al.*, 2006, de Melo *et al.*, 2010), whereas in sugarcane more silica is deposited in the lower epidermis (Naidoo *et al.*, 2009). This suggests that the quantity of silica deposited in the epidermis (lower or upper) depends on the number of silica cells present at the deposition site, which is specific to each plant species.

In the current study, silica granules were identified on the epidermal cells of Si-treated citrus leaves. This corroborates with previous studies that have identified silica bodies in the epidermis surface of rice, grass and bamboo (Kaufman *et al.*, 1985, Motomura *et al.*, 2000, Ma & Takahashi, 2002, Richmond & Sussman, 2003, Motomura *et al.*, 2006, Zhang *et al.*,

2013). Some studies reported that silica is deposited in the epidermal cell regions, which are the termini of transpiration stream, which provides substantiate evidence of transpiration involvement in Si deposition (Kaufman *et al.*, 1985, Ma & Takahashi, 2002, Ma & Yamaji, 2006). Silica deposits in outer cell surfaces that constitute cuticle Si double layer in Si-treated plants in this study has also been previously identified in Si fertilization studies conducted in *Brachiaria brizantha* and rice (Ma & Takahashi, 2002, Raven, 2003, de Melo *et al.*, 2010, Zhang *et al.*, 2013, Schurt *et al.*, 2014). It is likely that Si incorporates cell surfaces because the presence of an organo-silicon compound made up with lignin and carbohydrate to provide a physical barrier against abiotic and biotic stresses has been proposed to develop in rice (Inanaga *et al.*, 1995, Kim *et al.*, 2002). Moreover, Si distribution along the epidermal cells in this study resembled the pathway of transpiration flow in the cell walls and intercellular space (apoplasm) transported into the plasma membrane and its trajectory to the xylem (Hodson & Sangster, 1989, Ma & Takahashi, 2002, Marschner, 2012). The presence of barriers in apoplasmic movement such as endodermis cells and Casparian bands implies the possible involvement of transporters, such as Lsi1, known to facilitate Si uptake across the plasma membrane and plant cells (Marschner, 2012, Ma & Yamaji, 2015).

Conclusions

Si was deposited in upper epidermis cell wall regions as granules and silica deposits on the outer cell surface that constituted the cuticle-silica double layer in both species. Therefore, water loss through the epidermis could be reduced, especially if the treated plants were exposed to unfavourable abiotic conditions.

CHAPTER 6

EFFECTS OF SILICON APPLICATION ON PHOTOSYNTHESIS AND CHLOROPHYLL FLUORESCENCE PARAMETERS OF CITRUS UNDER COLD STRESS CONDITIONS

Abstract

Cold stress is a limiting factor in the photosynthetic performance and yield of a crop. The objective of this study was to determine the effect of silicon (Si) applications on leaf gas exchange and chlorophyll fluorescence parameters of citrus subjected to cold stress. Two experiments were conducted involving foliar and root Si applications followed by cold stress treatments of 0 and 4^{o}C on Valencia and Clementine citrus trees. Leaf gas exchange and chlorophyll fluorescence parameters were measured: A, gs, Tr, Ci, iWUE, F_o, F_m, F_v/F_m, F'_v/F'_m, ETR, NPQ and ETR/A. Foliar-Si application of 100 and 500 mg L^{-1} at 4^{o}C improved F'_v/F'_m and F_v/F_m in Valencia. Contrastingly, foliar Si treatment of 100 and 500 mg L^{-1} improved F_o in Clementine at 4^{o}C. NPQ was significantly improved in Clementine by foliar Si application of 100 mg L^{-1} after 2h cold stress, while ETR foliar Si application of 500 mg L^{-1} was improved after 16h cold stress of 4^{o}C. Root-Si application of 1000 mg L^{-1} at 0^{o}C reduced gs, Tr and C_i, but improved iWUE and F_o in both cultivars. The NPQ and ETR were improved in Valencia, while A, F_m and ETR/A were improved in Clementine following 1000 mg L^{-1} root-Si application and prolonged stress of 0^{o}C. Root-Si applications in cold stressed conditions improved the photosynthetic rate of Clementine and water use efficiency in both cultivars.

Keywords: Clementine; low temperature; stress tolerance; Valencia.

6.1. Introduction

Silicon (Si) is a non-essential element in higher plants, but it is well known to be involved in growth and developmental processes of plants subjected to biotic (e.g. diseases) and abiotic (e.g. heat, drought and low temperature) stresses (Liang *et al.*, 2005, Liang *et al.*, 2015). For example, in wheat (*Triticum aestivum* L.), photosynthetic rate and water-use efficiency were significantly inhibited under cold stress but improved after Si application (Zhu *et al.*, 2006). Similarly, Si application improved stomatal conductance and the photosynthetic rate of dry bean (*Phaseolus vulgaris* L.), under saline stress condition (Zuccarini, 2008). In another study conducted on young cucumber (*Cucumis sativus* L.) seedlings under osmotic stress, Si application significantly improved photosynthetic rate (Hattori *et al.*, 2008). On the contrary, Ma *et al.* (2004) demonstrated that Si application under drought stress in cucumber plants enhanced photosynthetic rate, but significantly reduced transpiration rate and stomatal conductance. Other studies reported that Si application significantly increased water content and dry mass in tomato (*Solanum esculentum* L.), grown under saline conditions (Romero-Aranda *et al.*, 2006). Similarly, exogenous Si application improved leaf water potential in rice (*Oryza sativa* L.) and wheat under water-stressed conditions (Matoh *et al.*, 1991, Gong *et al.*, 2003). It has been hypothesized that Si improves leaf water potential by the reduction in water loss due to transpiration because most of the silicon is deposited in the outer walls of the epidermal cells on both sides of the plant leaf (Matoh *et al.*, 1991). Improvement of the iWUE in relation to Si treatments has been observed in few studies, strawberry (*Fragaria ananassa L.*) (Dehghanipoodeh *et al.*, 2018); Kentucky bluegrass (*Poa pratensis*) (Saud *et al.*, 2014); maize (*Zea mays L.*) (Gao *et al.*, 2005) and tomato (Romero-Aranda *et al.*, 2006).With regards to chlorophyll fluorescence, Si treatment improved the efficiency in

abiotic stress experiments with saline (Al-aghabary *et al.*, 2005); and water stress (Maghsoudi *et al.*, 2015).

Chlorophyll fluorescence is a widely used tool for determining the activity of photosynthetic apparatus and allowing assessment of photo-protection mechanisms involved in plant responses under abiotic and biotic stresses (Sayed, 2003, Ribeiro *et al.*, 2009, Reeksting *et al.*, 2014, Hazrati *et al.*, 2016, Kalaji *et al.*, 2018). Parameters such as maximum quantum efficiency of PSII primary photochemistry (F_v/F_m), qP (photochemical quenching), non-photochemical quenching (NPQ), stomatal conductance (gs), transpiration rate (T), photosynthetic rate and intercellular CO_2 concentration (C_i) are important indicators of photosynthetic performance in crops subjected to stress factors (Maxwell & Johnson, 2000, Shen *et al.*, 2010, Hazrati *et al.*, 2016, Mashilo *et al.*, 2017). Analysis of leaf gas exchange and chlorophyll fluorescence can aid in identification and selection of cultivars with tolerance to biotic and abiotic stresses (Debona *et al.*, 2014, Reeksting *et al.*, 2014, Anjum *et al.*, 2017, Mashilo *et al.*, 2017, Kalaji *et al.*, 2018).

Citrus is an important fruit crop grown commercially in more than 100 countries worldwide (FAO, 2015). The optimum temperature for citrus leaf photosynthesis ranges between 25 to 30^{o}C (Ribeiro *et al.*, 2004). Under cold stress, a reduction in stomatal conductance has been reported in citrus (Ribeiro & Machado, 2007), which may impair photosynthetic activity by decreasing CO_2 availability to Rubisco (Machado *et al.*, 2002, Medina *et al.*, 2002). This consequently affects flower induction, resulting in reduced fruit yield and quality (Ribeiro & Machado, 2007). This affects the revenue of the citrus industry since high quality fresh fruits are essential in a global market (Ladaniya, 2008). Silicon fertilization of citrus plants has been responsible for a significant increase in root and green mass of germinated grapefruit (*Citrus paradisi*) because of the interaction between low temperature stress and Si fertilization (Matichenkov *et al.*, 2001). Previous studies showed that Si application in young

citrus improved plant height, branching and fruit yield (Wutscher, 1989, Matichenkov *et al.*, 1999). The effect of Si application on physiological processes linked to cold stress tolerance are not well understood and sparsely investigated in citrus. The application of Si may be a viable and cost-effective approach for boosting citrus production in frost-prone regions (Liang *et al.*, 2008). Therefore, the objective of this study was to examine the effect of Si application on leaf gas exchange and chlorophyll fluorescence parameters of citrus subjected to low temperature stress.

6.2. Materials and methods

6.2.1. Plant material

Two citrus cultivars, Valencia 'Delta' and Clementine 'Nules', were used for the study. These are two widely cultivated, commercial citrus cultivars in South Africa that were selected to represent two different citrus classes namely, oranges and soft citrus (mandarins) (Saunt, 2000).

6.2.2. Determining optimum foliar and root Si application rates

The experiments were conducted under glasshouse conditions at the Experimental Farm of the University of Pretoria (S25º 44' E28º 15'). The aim of these initial Si trials was to determine the optimum Si application rates for further use in the chilling studies. The initial Si uptake experiments were performed on two-year-old citrus trees of 'Delta' Valencia oranges (*Citrus sinensis*) and 'Nules' Clementine (*Citrus reticulata*), grafted onto Carrizo citrange rootstock. They were grown in 10 L polyethylene pots and were foliar-sprayed fortnightly using potassium silicate (0, 100, 500, 1000 and 2000 mg L^{-1}) in four replicates (in groups of 3 plants per replicate). Silicon content of the leaves was determined following six weeks of foliar Si application. Based on the initial results (Figure 6.1.1), Si application rates of 0 (control), 100 and 500 mg L^{-1} were selected for the main study because Si precipitate

build-ups were found on the leaves treated with 1000 and 2000 mg L^{-1} , and there were non-significant differences after treatments with 500, 1000, and 2000 mg L^{-1}. With regards to Si application to tree roots, Si was applied as a solution through drenching once a month over three months at 0, 100, 500, 1000 and 2000 mg L^{-1} in four replications (in groups of 3 plants per replicate). Based on the initial results (Figure 6.1.2), Si application rates of zero (control treatment) and the optimum dose (1000 mg L^{-1}) were selected for the root drenching study.

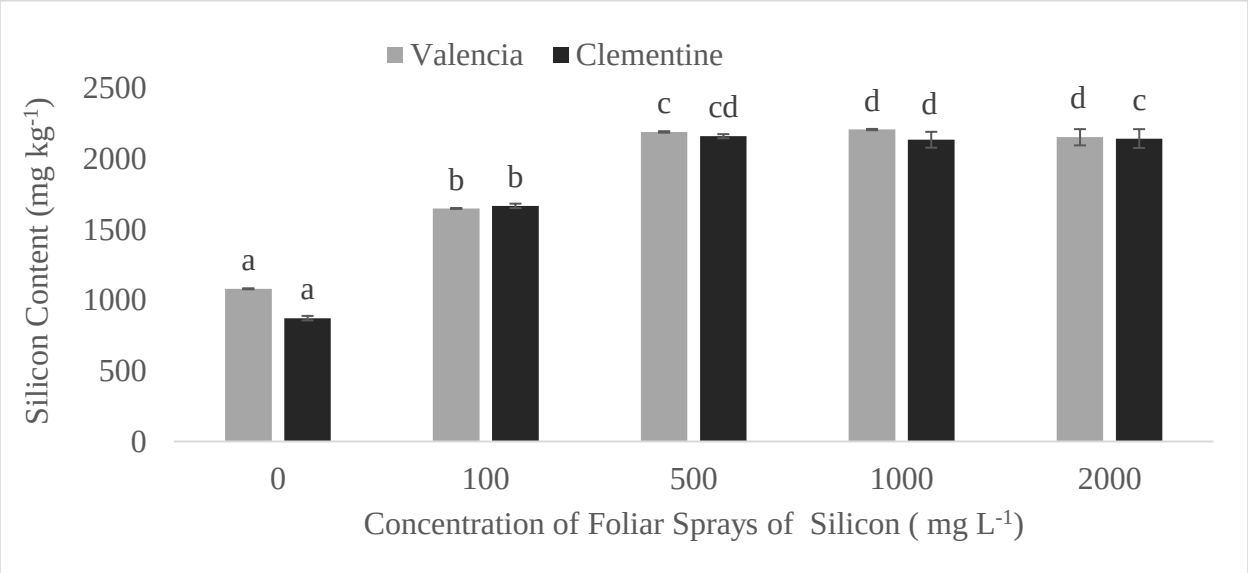


Figure 6.1.1 Silicon concentrations for foliar sprays applied to Valencia and Clementine leaves. Values are means ± S.E of four replicates. SE = standard error.

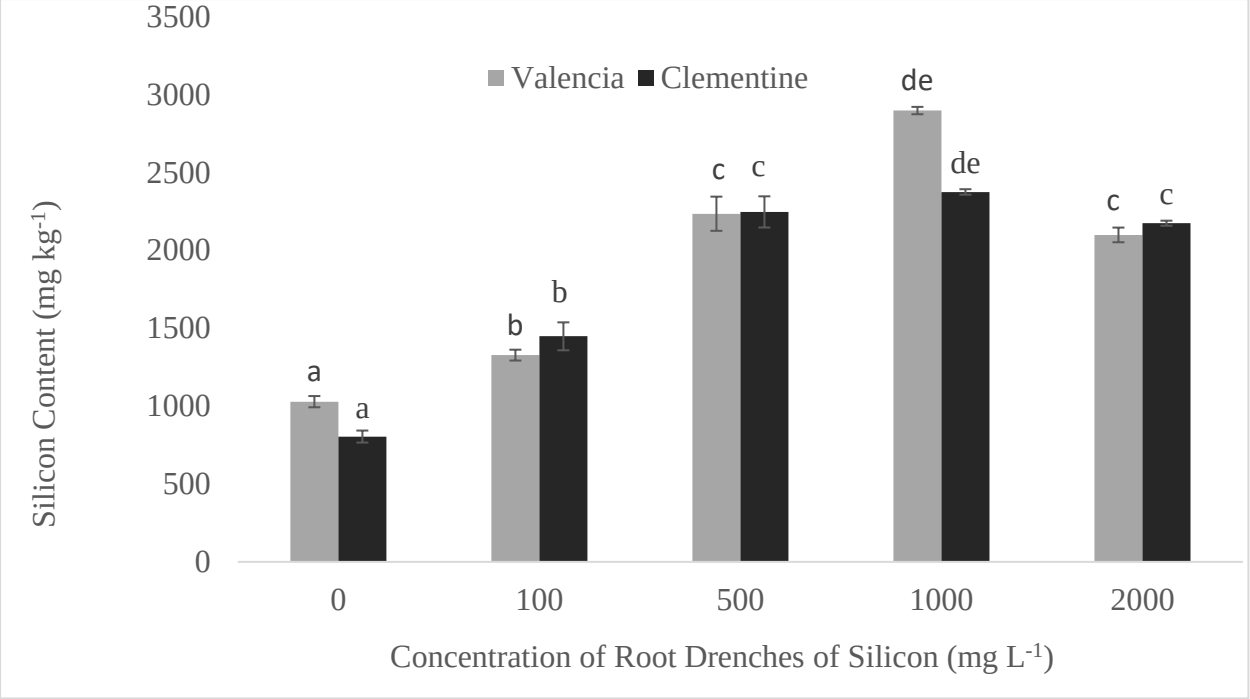


Figure 6.1.2 Silicon concentrations in Valencia and Clementine leaves after Si root drenches applied three times at four concentrations to Valencia and Clementine trees. Values are means ± S.E of four replicates. SE = standard error.

6.2.3. Chilling injury experiment

The chilling injury experiment was conducted using cold chambers at the University of Pretoria's Experimental Farm and at the Rosaly Commercial Farm near Krugersdorp. At the end of the sixth week of foliar Si application, the young citrus trees were transferred to a cold chamber set at a temperature of 4ºC and plants were exposed for 0, 2, 4 and 16h of cold stress. After three months of Si root drenches, the young trees were subjected to cold stress at 0ºC for 0, 2, 4, 48 and 72h.

6.2.4. Data collection

Si analysis

Si levels were determined using the microwave-assisted digestion method adapted from (Haysom & Ostatek-Boczynski, 2006). Three mL of 65% HNO_3 (EMSURE) was added to dry, grounded 500 mg leaf samples with three replicates per treatment (3 samples/replicate). The tubes were capped and left to stand for five minutes to thoroughly wet the sample. Two

mL of 30% H_2O_2 (AR grade) were then added and left to stand overnight. The capped Teflon tube (TFM) reaction vessels were placed in the ceramic tube holders of the CEM Mars 5 microwave system. The rotor was placed in the cavity of the microwave unit to the ramping temperature of 180^oC for 15min and the temperature maintained for 15min then the acid digestion step was conducted. On completion of the cycle, the tubes were left to cool, after which NaOH (10%) solution was added to the digestate. The tubes were recapped and returned to the microwave system for the second heating step to the ramping temperature of 180^oC for 15 min and the temperature was maintained for 15min and a second acid digestion step was conducted, which resulted in the solubilisation of amorphous Si. After final cooling, the contents of the tubes were neutralized with HNO_3 (2 M) in a plastic beaker, using phenolphthalein as an indicator, and diluted to a volume of 250 mL with type I deionised (DI) water 18MΩ cm^{-1} type I H_2O in a volumetric flask to reduce the matrix effect. The Si concentration was determined with ICP-OES (Varian Liberty 200).

Leaf gas exchange measurements

Leaf gas exchange measurements were performed on three fully expanded leaves selected per tree (6 trees/treatment) using a field portable LICOR 6400-40 leaf chamber pulse amplitude modulated fluorimeter attached to the gas analyser sensor head of the infrared LICOR-6400 XT (LI-COR Inc., Lincoln, NE, USA). The portable photosynthesis system was fitted with an LED (light emitting diode) light source that uses mixed LEDs to deliver both red and blue light to the leaves in the chamber. The CO_2 concentration of the reference air entering the leaf chamber was adjusted with a CO_2 mixer control unit such that the ‘‘sample’’ air entering the chamber contained 400 µmol CO_2 mol^{-1}. This resulted in CO_2 concentration of the reference air being close to 400 µmol CO_2 mol^{-1}. The flow rate of H_2O was set at 500 µmol$m^{-2}s^{-1}$. The quantum flux density was adjusted according to daily ambient conditions and was fixed at 1000 µmol$m^{-2}s^{-1}$. Leaf temperature and relative humidity were set at 25^oC and 50% within

the chamber, respectively. The following leaf gas exchange parameters were measured: stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration (E) and net photosynthesis assimilation rate (A) (Maxwell & Johnson, 2000, Shen *et al.*, 2010). Intrinsic water-use efficiency (IWUE) was measured as the ratio of A and gs (Martin & Ruiz-Torres, 1992).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were also determined using a LICOR-6400 XT Portable Photosynthesis System fitted with a chamber pulse amplitude modulated fluorimeter (6400-40). Four leaves from intact plants of each tree were dark-adapted for 30min by covering the leaf with aluminium foil. After the aluminium foil had been removed, the leaves were exposed to a 0.8s light flash. Following this, the dark-adapted leaves were light-adapted for one hour and were then exposed to actinic illumination for 6s to excite PSI and force the electrons to drain from PSII. Photosystem II activity was measured by the following parameters: F_o the minimal level of fluorescence was obtained with low intensity modulated light. The F_m was obtained by 6s pulses of saturating light, maximum quantum efficiency of photosystem II primary photochemistry of dark-adapted (F_v/F_m) and light-adapted (F'_v/F'_m) leaves, electron transport rate (ETR) and non-photochemical quenching (NPQ) (Genty *et al.*, 1989, Maxwell & Johnson, 2000). The relative measure of electron transport to oxygen molecule was measured as ETR/A (Fryer *et al.*, 1998).

6.2.4. Statistical analysis

The collected data was subjected to analysis of variance by using Statistical Analysis System software (SAS) version 9.4 (Cary, NC, USA) to determine treatment mean effects. Differences between treatments were determined using Fisher's Least Significant Difference (LSD) at 5% level of significance.

6.2. Results

6.2.1. Effect of foliar-Si application on chlorophyll fluorescence parameters under chilling temperature stress of 4°C

The effective quantum yield of light adapted leaves (F'_v/F'_m) in Valencia was significantly higher after the 100 (0.778) and 500 (0.779) mg L^{-1} treatments compared to the control (0 mg L^{-1}) (0.731) after 2h of exposure to 4°C (Figure 6.2.1). A Si application rate of 500 mg L^{-1} improved F'_v/F'_m significantly (0.792) compared to the rate of 100 mg L^{-1} (0.725) after exposure to 4h of cold stress of 4°C. After exposures of 2, 4 and 16h to cold stress of 4°C, the two Si treatments (100 and 500 mg L^{-1}) did not improve F'_v/F'_m in Clementine.

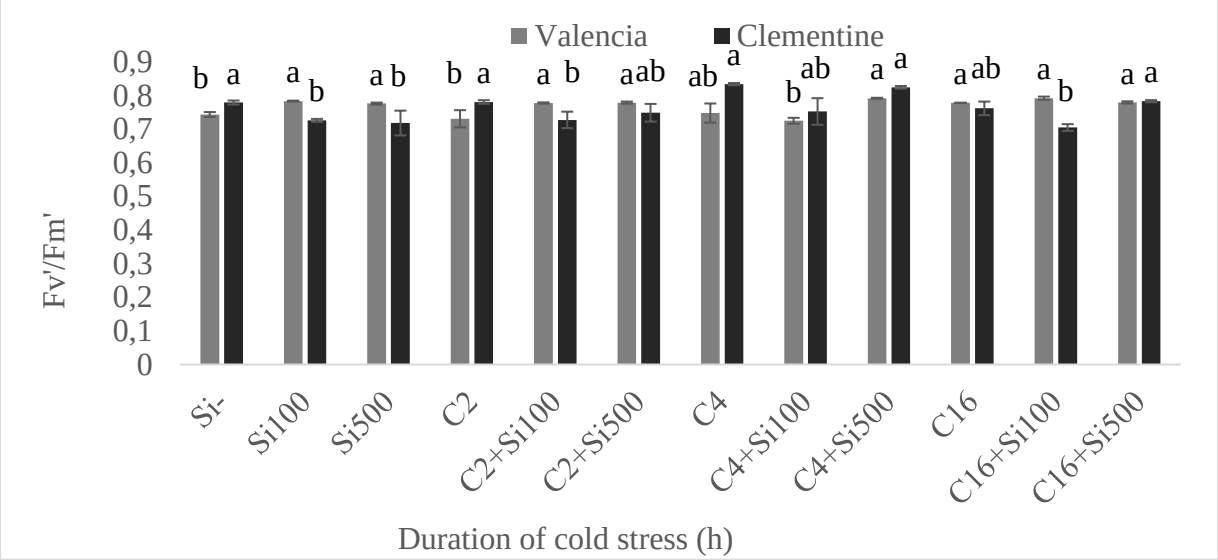


Figure 6.2.1 The effect of foliar-Si application Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1}) on the effective quantum yield of Photosystem II in 'Valencia' and 'Clementine' leaves during chilling injury stress of 4°C for 0, 2, 4 and 16h. Data are means ± standard errors, bars sharing a letter are not significantly different.

The maximum quantum yield of dark-adapted leaves (F_v/F_m) in Valencia were significantly higher after Si applications of 100 (0.8223) and 500 (0.8207) mg L^{-1}, compared to the control trees (0.7533) after 2h of exposure to cold stress of 4°C (Figure 6.2.2). After 4h of exposure to cold stress of 4°C, Si application of 500 mg L^{-1} significantly improved F_v/F_m (0.816) compared to treatments with 0 and 100 mg L^{-1}, respectively, measuring 0.748 and 0.7487. In

Clementine, the two Si treatments (100 and 500 mg L^{-1}) failed to improve F_v/F_m after exposure of 2, 4 and 16h to cold stress of 4ºC.

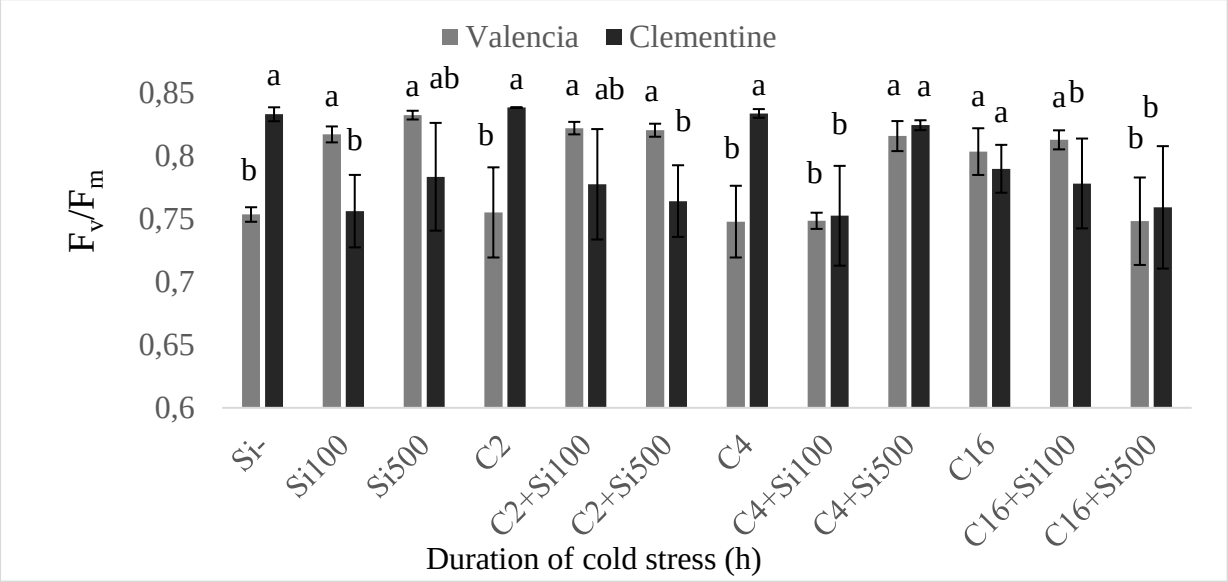


Figure 6.2.2 The effect of foliar-Si application on Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1}) the maximum quantum yield of Photosystem II efficiency of 'Valencia' and 'Clementine' leaves during chilling injury stress of 4ºC for 0, 2, 4 and 16h. Data are means ± standard errors, bars sharing a letter are not significantly different.

The minimum fluorescence of dark-adapted leaves (F_o) in Valencia was significantly lower after 2h of exposure to cold stress of 4ºC following foliar treatments with 100 mg L^{-1} (168.7) and 500 mg L^{-1} (179.1), relative to the control trees (233.4) (Figure 6.2.3). After 4h of exposure to cold stress of 4ºC, a significant reduction in F_o was observed after treatment with 500 mg L^{-1} (164.5) compared to the control (235.5) and 100 mg L^{-1} (265.5) Si. After 16h of exposure to chilling temperatures of 4ºC, a significant increase in F_o was observed in the control trees (253.5), compared to trees treated with 100 (158.4) and 500 (183.6) mg L^{-1} foliar Si applications. In Clementine, F_o increased significantly after 2h of cold stress with Si application rates of 100 (210) and 500 mg L^{-1} (235.2) compared to the control trees (162.9).

Additionally, after 4h of exposure to cold stress, a significant increase in F_o was observed after treatments with 100 mg L^{-1} (239.7) compared to the control trees (155.1) and after foliar

treatment with 500 mg L^{-1} (166.7). Similarly, after16h of cold stress, a significant increase in F_o was observed after treatment with Si at 100 mg L^{-1} (245.9) compared to the control trees (166.3) and after treatment with 500 mg L^{-1} (203.1).

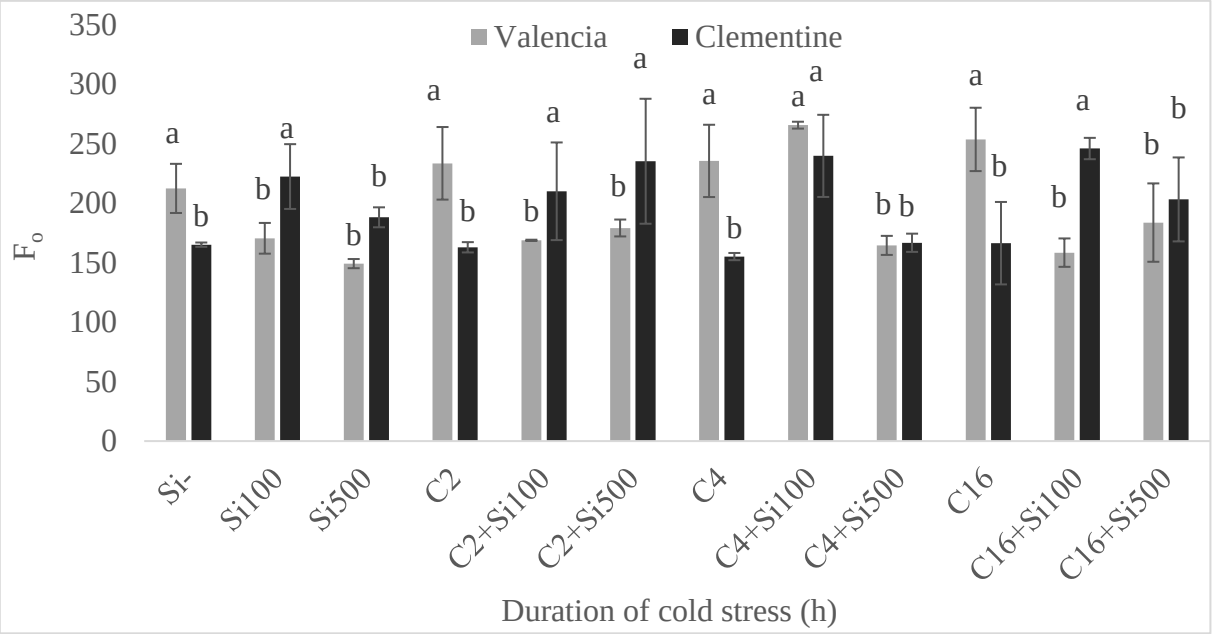


Figure 6.2.3 The effect of foliar-Si application Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1}) on the minimum fluorescence of dark-adapted of 'Valencia' and 'Clementine' leaves during chilling injury stress of 4^{o}C for the duration of (0, 2, 4 and 16h). Data are means ± standard errors, bars sharing a letter are not significantly different.

The maximum fluorescence of dark-adapted leaves (F_m) of Valencia trees was significantly increased after exposure to 4h cold stress after treatment of Si at 100 mg L^{-1} (1058), compared to control trees (930) and treatment at 500 mg L^{-1} (907). In Clementine, F_m was not affected by the Si treatments or exposure to cold stress (Figure 6.2.4).

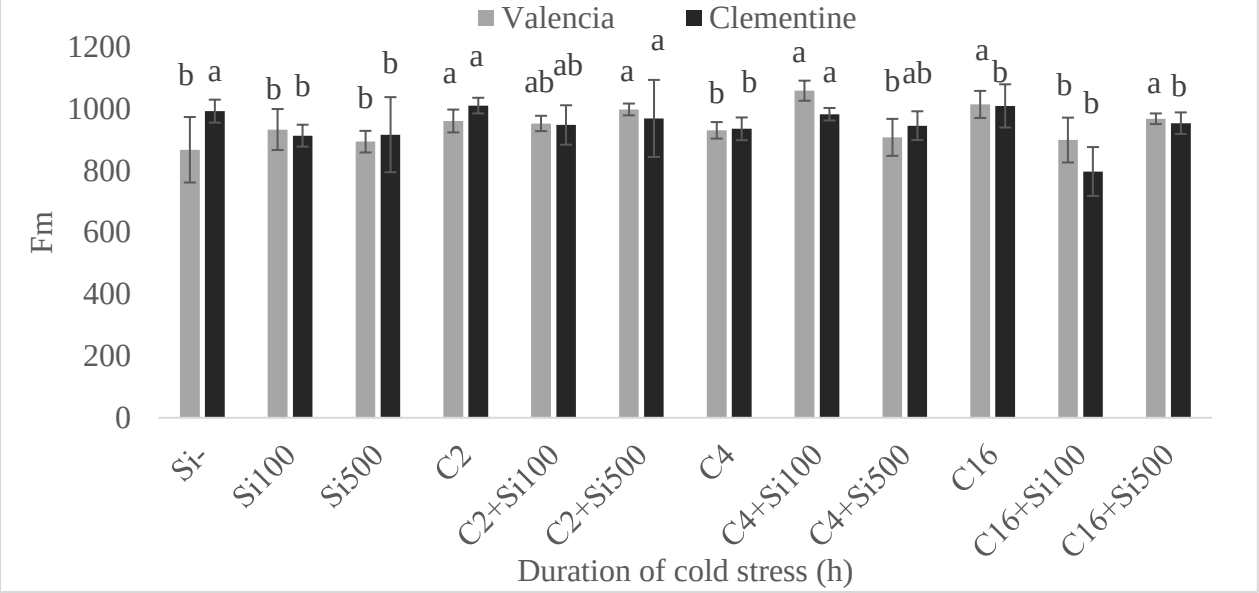


Figure 6.2.4 The effect of foliar-Si application Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1}) on the maximum fluorescence of dark-adapted 'Valencia' and 'Clementine' leaves during chilling injury stress of 4^{o}C for the duration of (0, 2, 4 and 16h). Data are means ± standard errors, bars sharing a letter are not significantly different.

Foliar-Si treatment of 100 mg L^{-1} reduced NPQ significantly after cold stress exposure of 2, 4 and 16h compared to non-stressed trees (0h of exposure to stress) (Figure 6.2.5). After 4h of exposure to cold stress of 4^{o}C, the NPQ of Valencia trees was significantly higher for trees treated with Si at 100 (1.252) and 500 mg L^{-1} (1.581) compared to the control trees (0.8252). In Clementine, a significant reduction in NPQ was observed after treatment with Si at 100 mg L^{-1} compared to the control trees after 2h of exposure to cold stress of 4^{o}C.

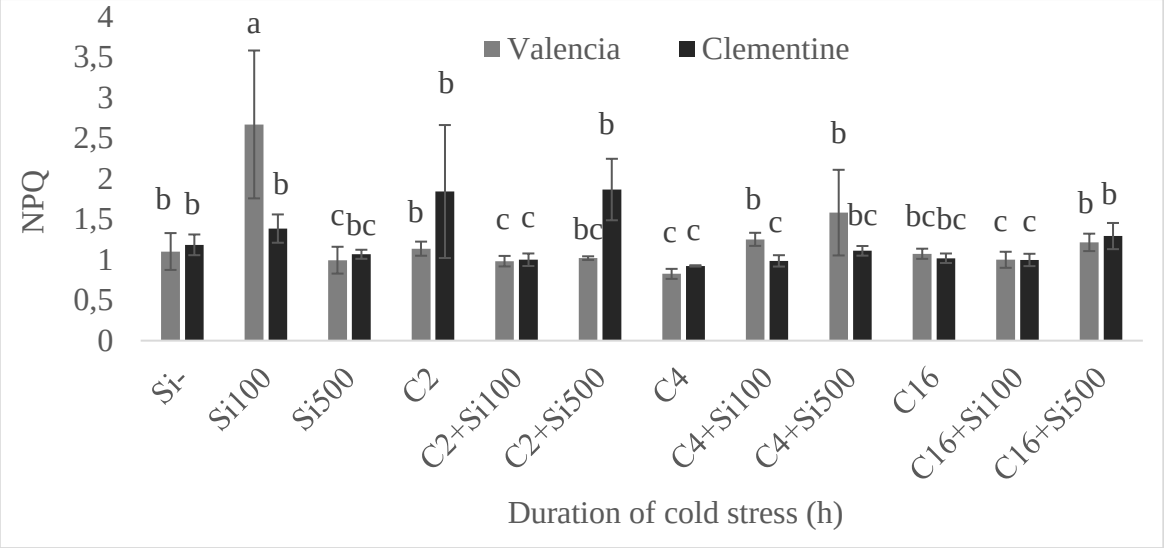


Figure 6.2.5 The effect of foliar-silicon application Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1})on the non-photochemical quenching of 'Valencia' and 'Clementine' leaves subjected to chilling temperature stress of 4^{o}C for the duration of 0, 2, 4 and 16h. Data are means ± standard errors, bars sharing a letter are not significantly different.

The electron transport rate (ETR) in Valencia was higher after treatment with 100 mg L^{-1} (5.326) compared to the control trees (5.005) and after treatment with 500 mg L^{-1} (4.535), following 2h of exposure to cold stress of 4^{o}C. In Clementine the ETR was significantly lower after treatment with 100 mg L^{-1} (4.714) than in control trees (5.19) and after treatment with 500 mgL^{-1} (5.19), following 2h of exposure to cold stress of 4^{o}C. The ETR was also significantly lower after treatment with 100 mg L^{-1} (4.625) than the control trees (5.206) and after treatment with 500 mg L^{-1} (5.423), following 4h of exposure to cold stress of 4^{o}C (Figure 6.2.6).

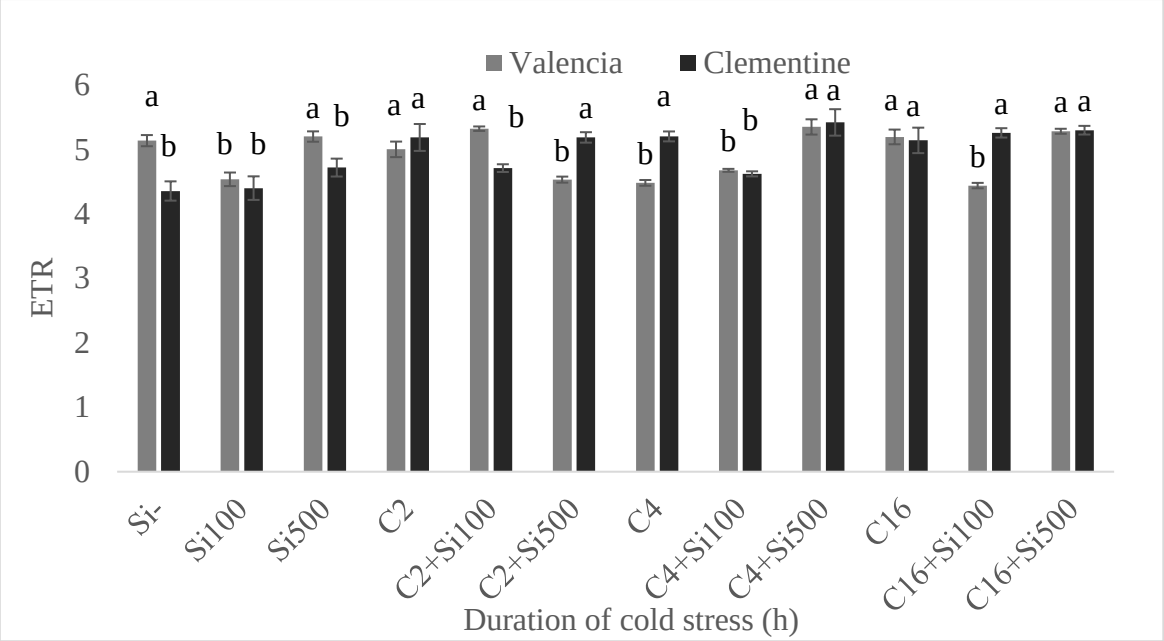


Figure 6.2.6 The effect of foliar-silicon application Si+ (100 and 500 mg L^{-1}) and Si- (0 mg L^{-1})on the electron transport rate of 'Valencia' and 'Clementine' leaves subjected to chilling temperature stress of 4^{o}C for the duration of 0, 2, 4 and 16h. Data are means ± standard errors, bars sharing a letter are not significantly different.

6.2.2. Changes in leaf gas exchange in response to cold stress of 0^{o}C and root-Si application

Leaf gas exchange parameters of citrus cultivars to root Si-application under chilling temperature stress of 0^{o}C are shown in Figures 6.3. The photosynthetic rate (A) was significantly higher in non-stressed trees than cold stressed trees exposed at 2, 4, 48 and 72h in both citrus cultivars (Figure 6.3.1). In Clementine, a reduction of 59% in the photosynthetic rate (A) was observed in Si drenched trees compared to the control trees after 2h exposure to cold stress. Contrastingly, a significant increase in A was observed in Si drenched trees (9.252 µmol CO_2 $m^{-2}s^{-1}$) relative to the control trees (6.123 µmol CO_2 $m^{-2}s^{-1}$) after 72h of exposure to cold stress.

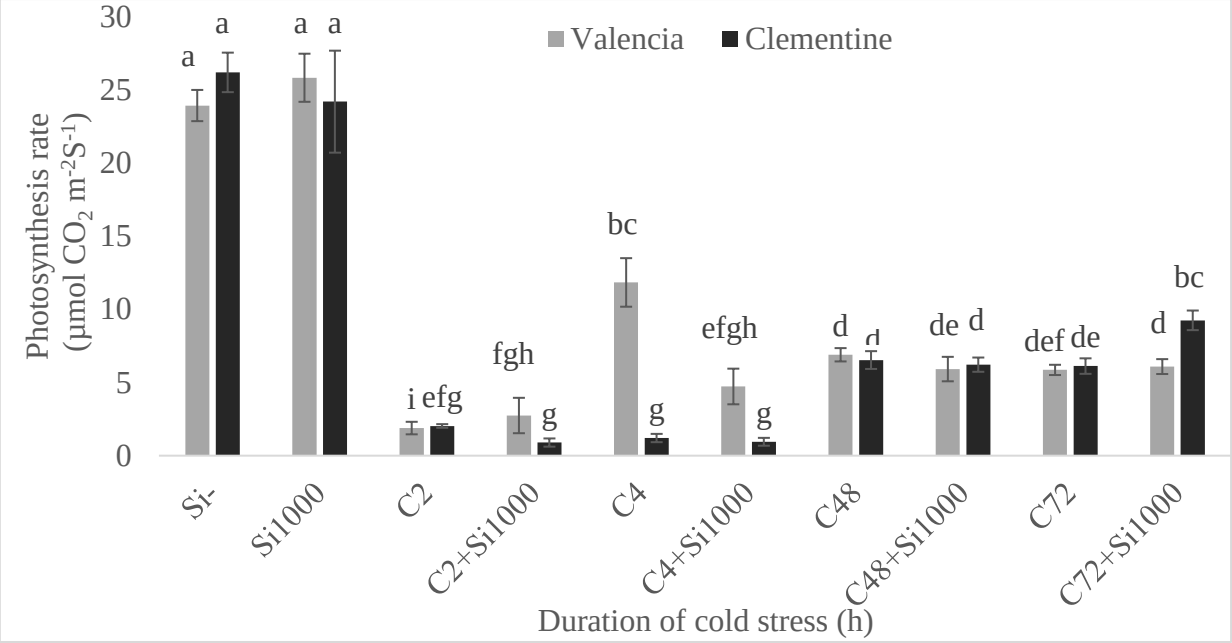


Figure 6.3. 1 Response of photosynthetic rate to root drench application of Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) in 'Valencia' and 'Clementine' leaves after chilling temperature stress of 0°C for 0, 2, 4, 48 and 72h. Data are means ± standard errors, bars sharing a letter are not significantly different.

Stomatal conductance (gs) in both cultivars were reduced significantly in trees drenched with Si compared to the control trees before exposure to cold stress of 0°C (Figure 6.3.2). Similarly, after 4h of exposure to cold stress, gs was significantly reduced in trees drenched with Si (0.0385 mol H_2O $m^{-2}s^{-1}$) compared to the control trees (0.1788 mol H_2O $m^{-2}s^{-1}$). In addition, a reduction in gs of 30 and 12% were observed at 48 and 72h, respectively, in trees drenched with Si compared to the control trees with a temperature stress of 0°C. In Clementine, a significant reduction in gs was observed in in trees drenched with Si (0.0099 mol H_2O $m^{-2}s^{-1}$) compared to the control trees (0.0195 mol H_2O $m^{-2}s^{-1}$) after 4h of exposure to cold stress of 0°C.

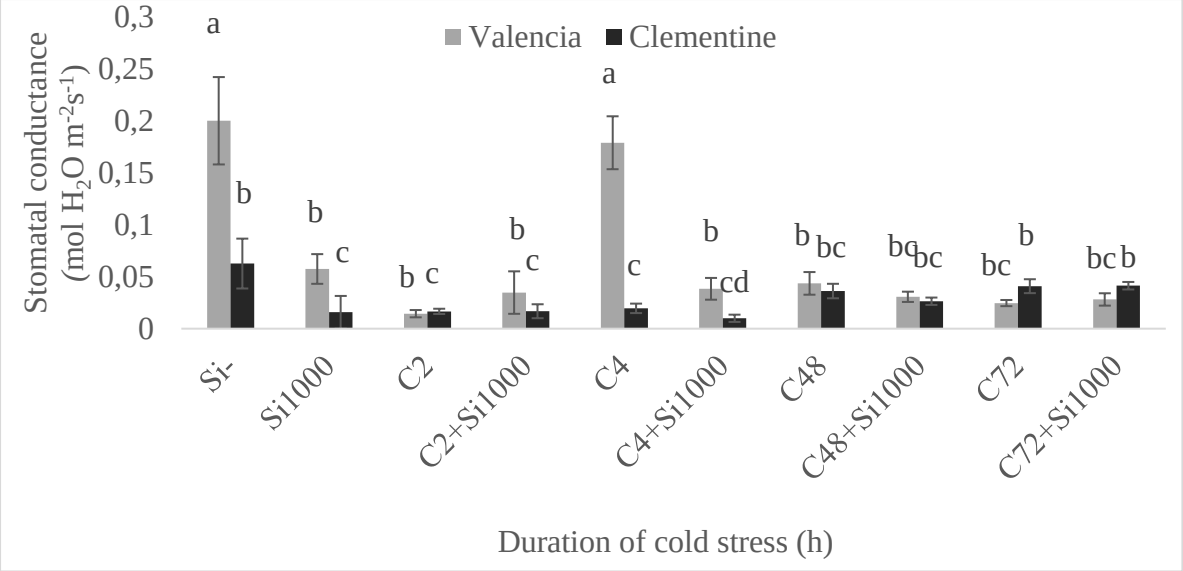


Figure 6.3.2 Response of stomatal conductance to root drench application of Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) in 'Valencia' and 'Clementine' leaves at chilling temperature stress of 0°C for 0, 2, 4, 48 and 72h). Data are means ± standard errors, bars sharing a letter are not significantly different.

The transpiration rate (Tr) was reduced in Valencia in both in trees drenched with Si (1000 mg L^{-1}) and the control trees subjected to cold stress of 0°C, compared to non-stressed trees, except in the control trees subjected to 4h of exposure to a cold stress of 0°C (Figure 6.3.3). In Clementine, Tr was reduced significantly before stress in trees treated with Si drenches compared to control trees. Additionally, Tr was reduced by 61% and 21%, respectively, at 4 and 48 h cold stress in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees (Figure 6.3.3). Meanwhile, a considerable increase in Tr by 59% was observed in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees after 2h of cold stress of 0°C.

Overall, a similar trend in reduction of A was observed in gs and Tr comparing cold stress trees to non-stressed trees in both cultivars. Another trend observed was an overall reduction of gs, Tr and A in trees treated with Si drenches compared to the control trees, regardless of cold stress exposure.

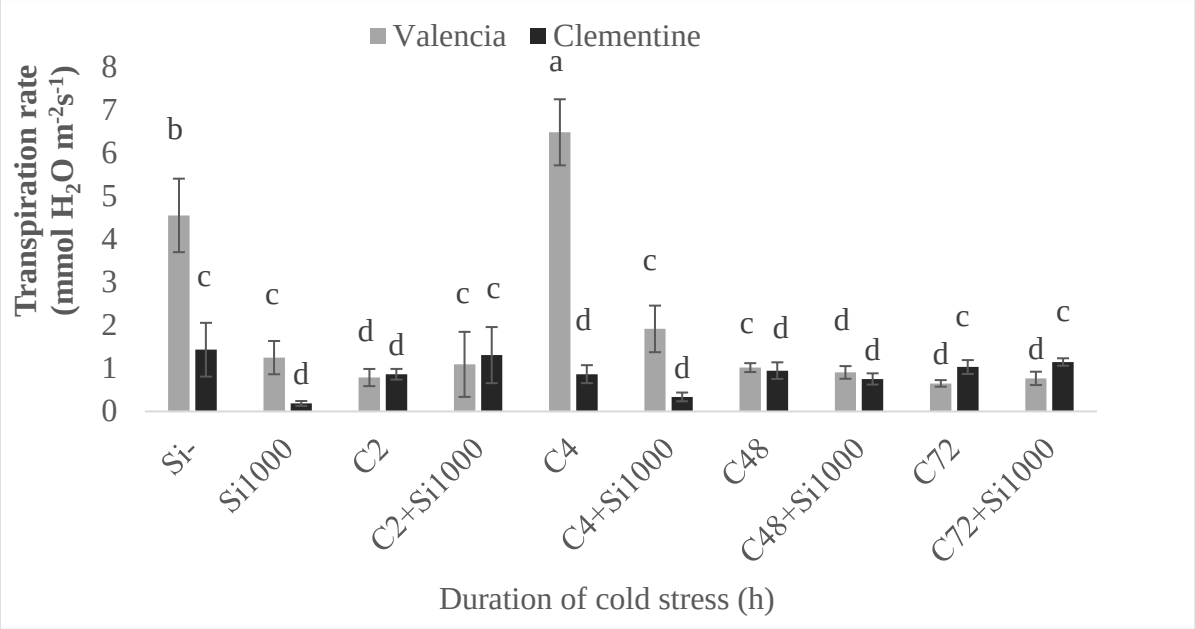


Figure 6.3.3 Response of transpiration to root-drench application of Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) in 'Valencia' and 'Clementine' leaves at chilling temperature stress of 0^{o}C for the duration of 0, 2, 4, 48 and 72h. Data are means ± standard errors. Bars sharing a letter are not significantly different.

Intercellular CO_2 concentration (C_i) of Valencia was significantly higher (559 µmol mol^{-1}) in trees treated with Si drenches (1000 mg L^{-1}) subjected to non-stressed conditions compared to cold stress conditions (Figure 6.3.4). Intercellular CO_2 concentration were increased after 48 and 72h of exposure to cold stress by 42 and 105% in trees treated with Si drenches (1000 mg L^{-1}) compared to the control Valencia trees. Intercellular CO_2 concentration (C_i) were reduced after 2 and 4h of exposure to cold stress of 0^{o}C by 26 and 44%, respectively in trees treated with Si drenches (1000 mg L^{-1}) compared to the control Valencia trees. Meanwhile, reductions of C_i by 48, 31 and 80% were observed in Clementine trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees, after 4, 48 and 72h of cold stress of 0^{o}C, respectively. In both cultivars, the C_i responses followed a similar trend to gs whereby reductions were observed in trees treated with Si drenches and the control trees subjected to cold stress of 0^{o}C compared to non-stressed conditions (Figures 6.3.2 and 6.3.4).

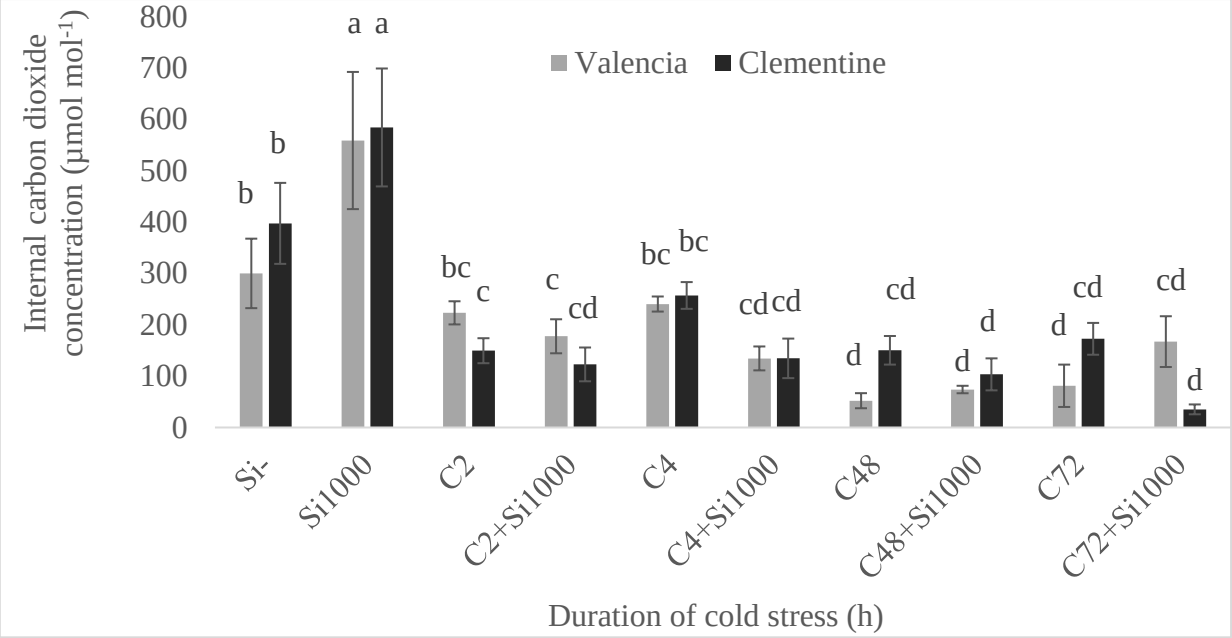


Figure 6.3.4 Response of internal carbon dioxide concentration to root-silicon application of Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) in 'Valencia' and 'Clementine' leaves at chilling temperature stress of 0^{o}C for the duration of 0, 2, 4, 48 and 72h. Data are means ± standard errors. Bars sharing a letter are not significantly different.

In both cultivars, a reduction in intrinsic water-use efficiency (iWUE) was observed after cold stress compared to non-stressed conditions, regardless of Si status. Additionally, there was an increase in iWUE of both cultivars in trees treated with Si drenches compared to the control trees, irrespective of chilling status. Meanwhile, non-significant differences were observed in trees treated with Si drenches compared to the control after 2h of cold stress in both cultivars and in Valencia trees after prolonged stress (Figure 6.3.5). This indicated that the improvement in iWUE in trees treated with Si drenches subjected to cold stress was linked to the increase in A of 51% in Si-treated Clementine trees subjected to prolonged cold stress (Figures 6.3.1 and 6.3.5).

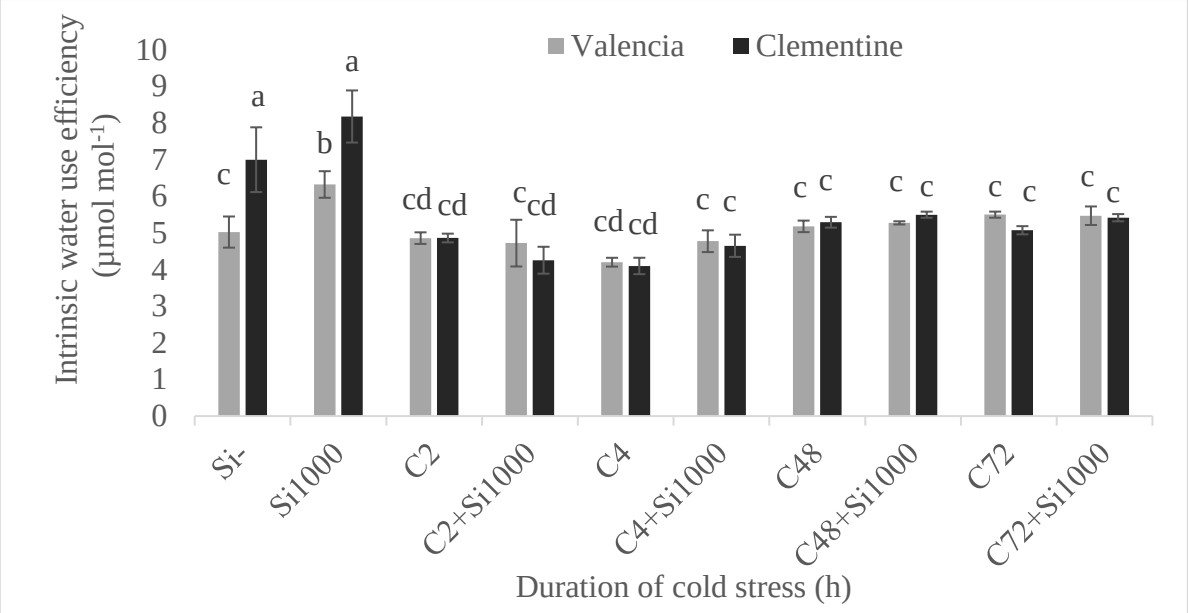


Figure 6.3. 5 Response of intrinsic water use efficiency to root-drench application of Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) in 'Valencia' and 'Clementine' leaves at chilling temperature stress of 0°C for the duration of 0, 2, 4, 48 and 72h. Data are means ± standard errors. Bars sharing a letter are not significantly different.

6.2.3. Changes in chlorophyll fluorescence parameters in response to cold stress of 0°C and root-Si application

The effective quantum yield of light adapted Valencia leaves (F'_v/F'_m) did not improve in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees, regardless of the growing conditions (0 and 96h of exposure to cold stress of 0°C). Overall, the interaction between cold stress and high light conditions resulted in a considerable reduction in F'_v/F'_m. With cold stress of 0°C, the F'_v/F'_m of trees treated with Si drenches (1000 mg L^{-1}) were not different to the control trees (Figure 6.4.1).

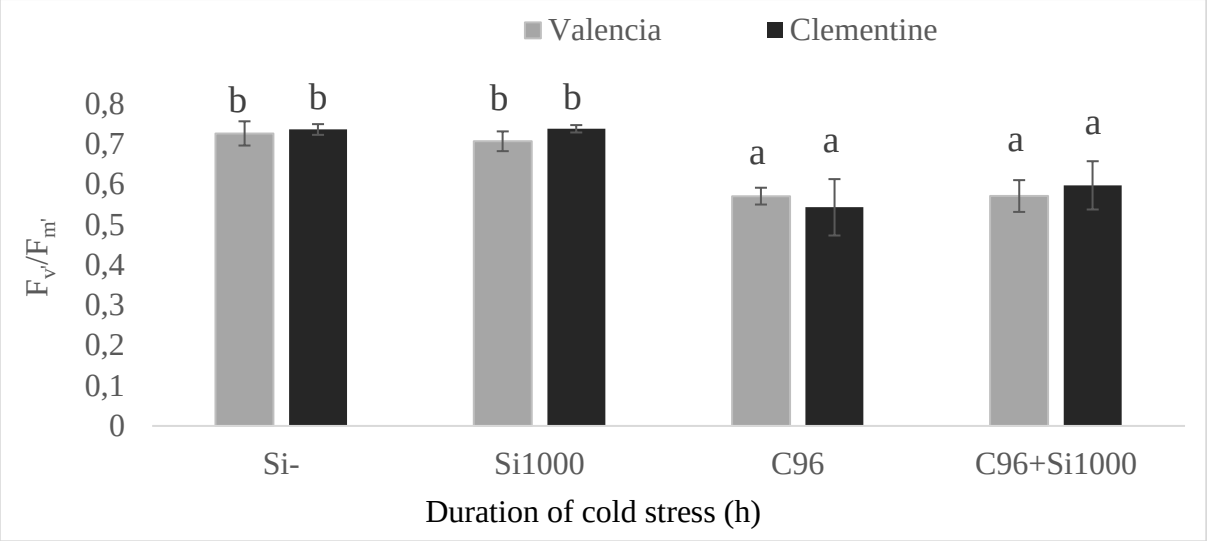


Figure 6.4. 1 The effect of root-drench application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the effective quantum yield of Photosystem II of 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0^{o}C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

The maximum quantum yield of dark-adapted Valencia leaves (F_v/F_m) was significantly reduced in trees treated with Si drenches (0.719) compared to the control trees (0.862) with a cold stress of 0^{o}C (Figure 6.4.2). The F_v/F_m of Clementine trees treated with Si drenches (1000 mg L^{-1}) was not different to the untreated control trees after cold stress of 0^{o}C (Figure 6.4.2).

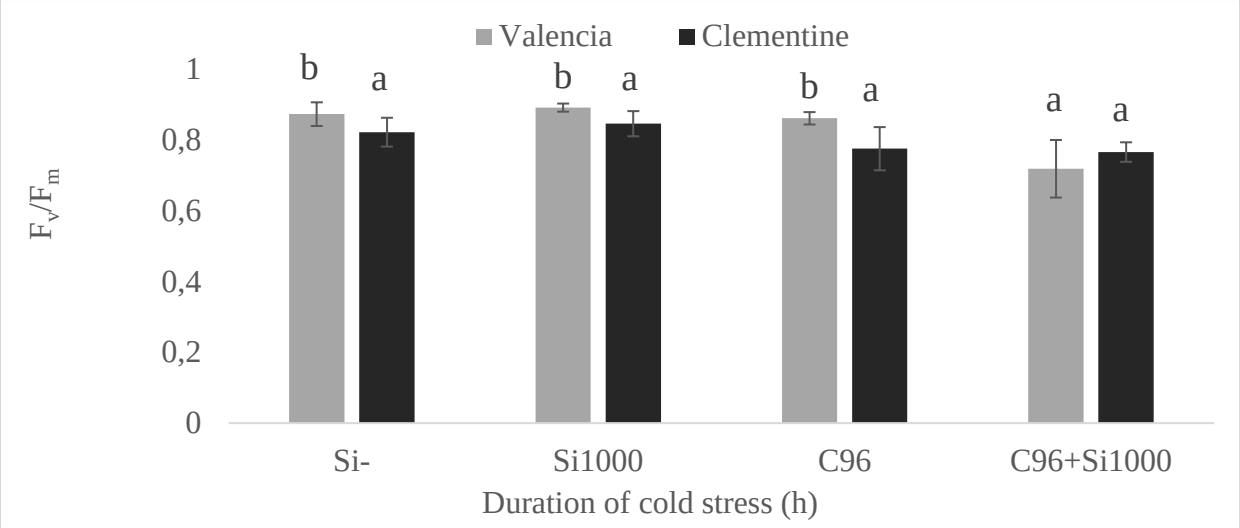


Figure 6.4. 2 The effect of root-drench application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the maximum quantum yield of dark-adapted 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0^{o}C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

With respect to chlorophyll fluorescence parameters after cold stress of 0^{o}C, the F_o in Valencia trees treated with Si drenches (1000 mg L^{-1}) was increased by 27% compared to the control trees (Figure 6.4.3). After cold stress of 0^{o}C, the F_o of Clementine was significantly increased in trees treated with Si drenches (1000 mg L^{-1}) (1175.86) compared to the control trees (409) (Figure 6.4.3).

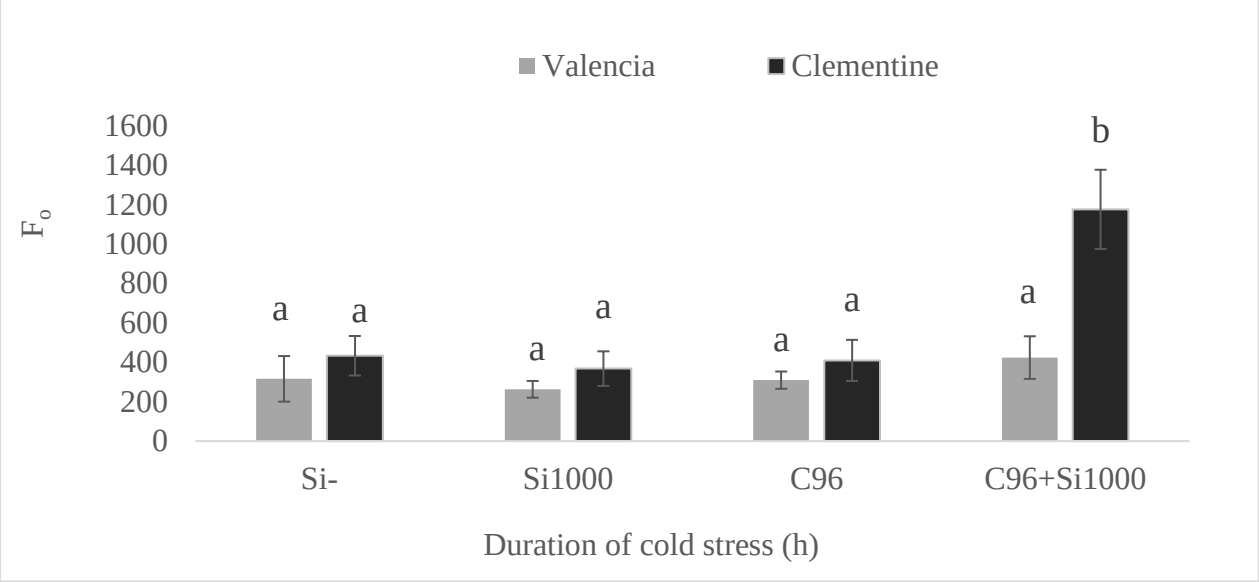


Figure 6.4. 3 The effect of root-drench application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the minimum fluorescence of dark-adapted 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0°C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

The maximal fluorescence (F_m) of Valencia leaves was reduced by 44% in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees after cold stress of 0°C (Figure 6.4.4). In Clementine, the F_m of the control trees was reduced by 30% under stressed conditions compared to non-stressed conditions (Figure 6.4.4). The Clementine F_m was significantly increased in trees treated with Si drenches (1000 mg L^{-1}) (4291) compared to the control trees (1875) after cold stress of 0°C (Figure 6.4.4).

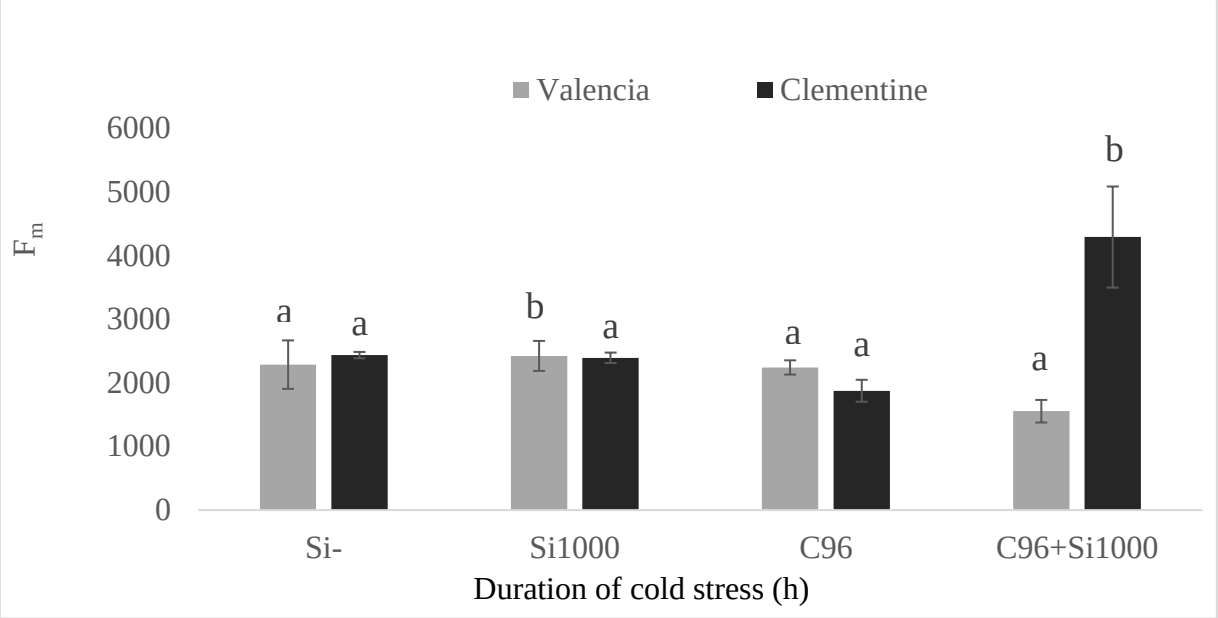


Figure 6.4. 4 The effect of root-silicon application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the maximum fluorescence of dark-adapted 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0^{o}C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

The non-photochemical quenching (NPQ) of Valencia was reduced by 24% in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees with a cold stress of 0^{o}C (Figure 6.4.5). The NPQ of Clementine leaves in non-stressed conditions was reduced by 99% in trees treated with Si drenches (1000 mg L^{-1}) compared to the control trees. In Clementine NPQ did not provide evidence of a positive effect of root treatments with Si under cold stress conditions.

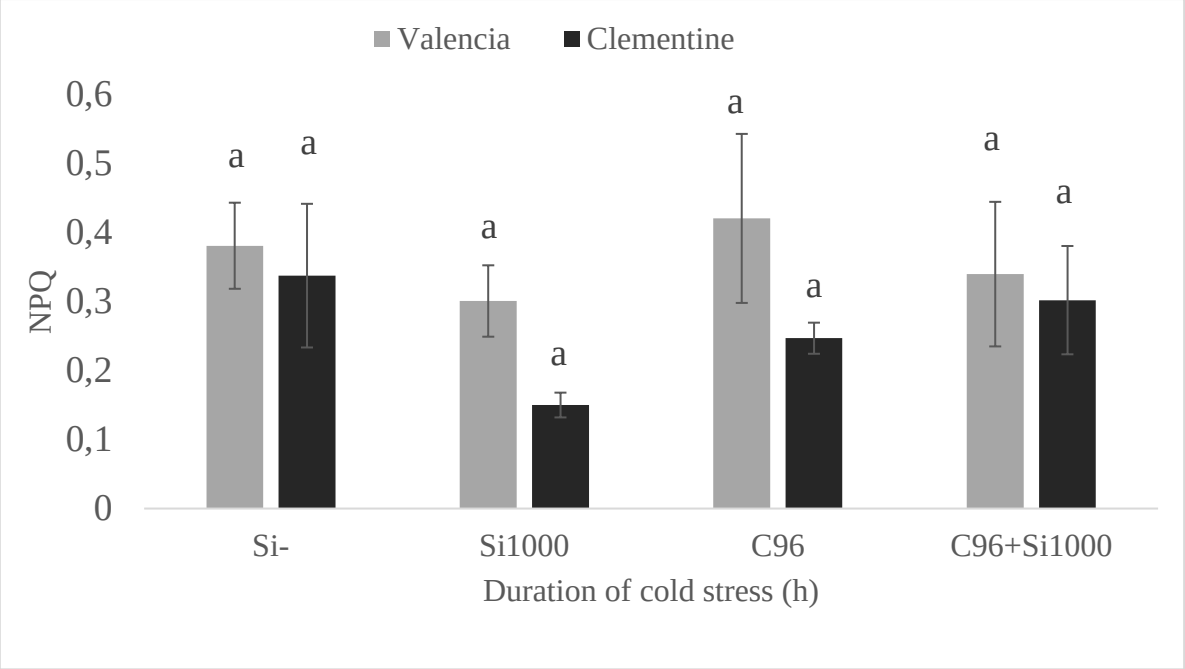


Figure 6.4.5 The effect of root-drench application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the non-photochemical quenching of 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0^{o}C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

The electron transport rate (ETR) of Valencia leaves in the control trees was reduced by 23.4% under cold stressed conditions compared to non-stressed conditions (Figure 6.4.6). Valencia ETR was increased by 21% in trees treated with Si drenches (1000 mg L^{-1}) compared to the control after cold stress of 0^{o}C (Figure 6.4.6). This was also linked to the reduction in heat dissipation (NPQ) of 24% in Valencia trees treated with Si drenches subjected to cold stress of 0^{o}C (Figure 6.4.5). In Valencia trees treated with Si drenches (1000 mg L^{-1}), ETR was increased by 26% after cold stress compared to non-stressed conditions. The ETR of Clementine leaves from trees subjected to Si drenches (1000 mg L^{-1}) was reduced by 35% after cold stress of 0^{o}C compared to non-stressed conditions.

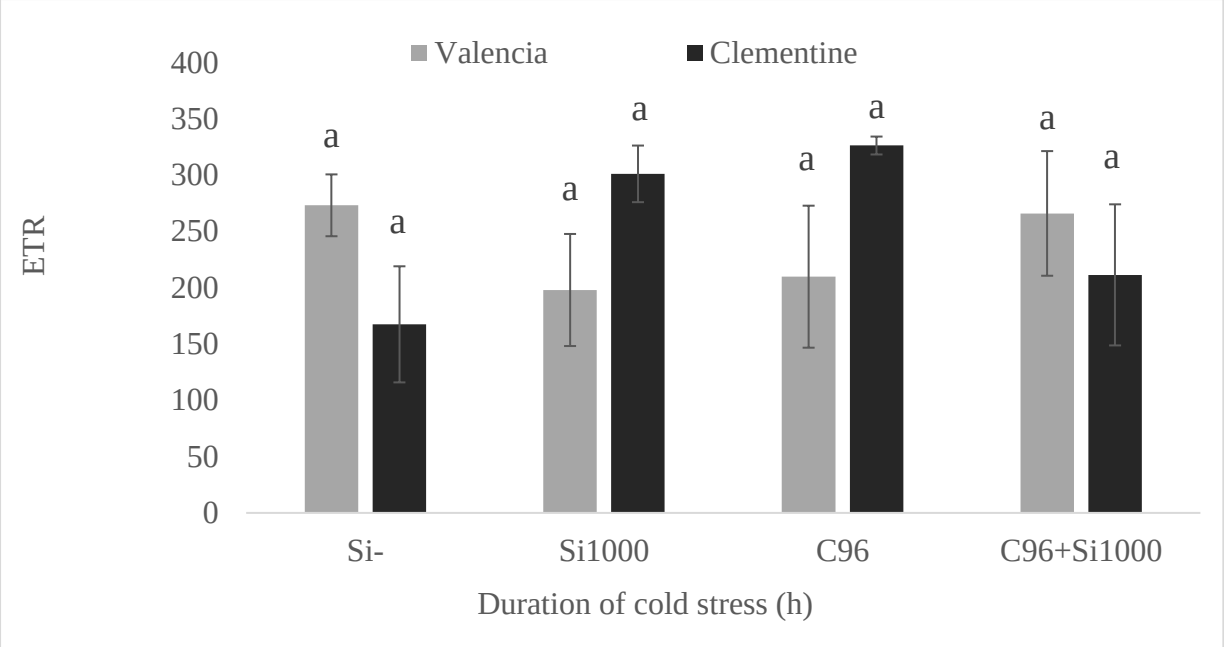


Figure 6.4. 6 The effect of root-drench application Si+ (1000 mg L^{-1}) and no Si- (0 mg L^{-1}) on the electron transport rate (ETR) of 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0°C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

In both cultivars trees exposed to cold stress of 0°C, the increase in photorespiration (ETR/A) observed was linked to a reduction in carbon assimilation rate (A) (Figure 6.3.1 & Figure 6.4.7). Valencia ETR/A was increased by 10% in trees treated with Si (1000 mg L^{-1}) compared to the control trees, after cold stress of 0°C (Figure 6.4.7). In Clementine, the ETR/A was significantly reduced for trees treated with Si (1000 mg L^{-1}) (21.61) compared to the control (56.84) trees after cold stress of 0°C (Figure 6.4.7). This reduction is also linked to the increase in A by 51% in Clementine trees treated with Si and subjected to prolonged cold stress of 0°C (Figure 6.3.1).

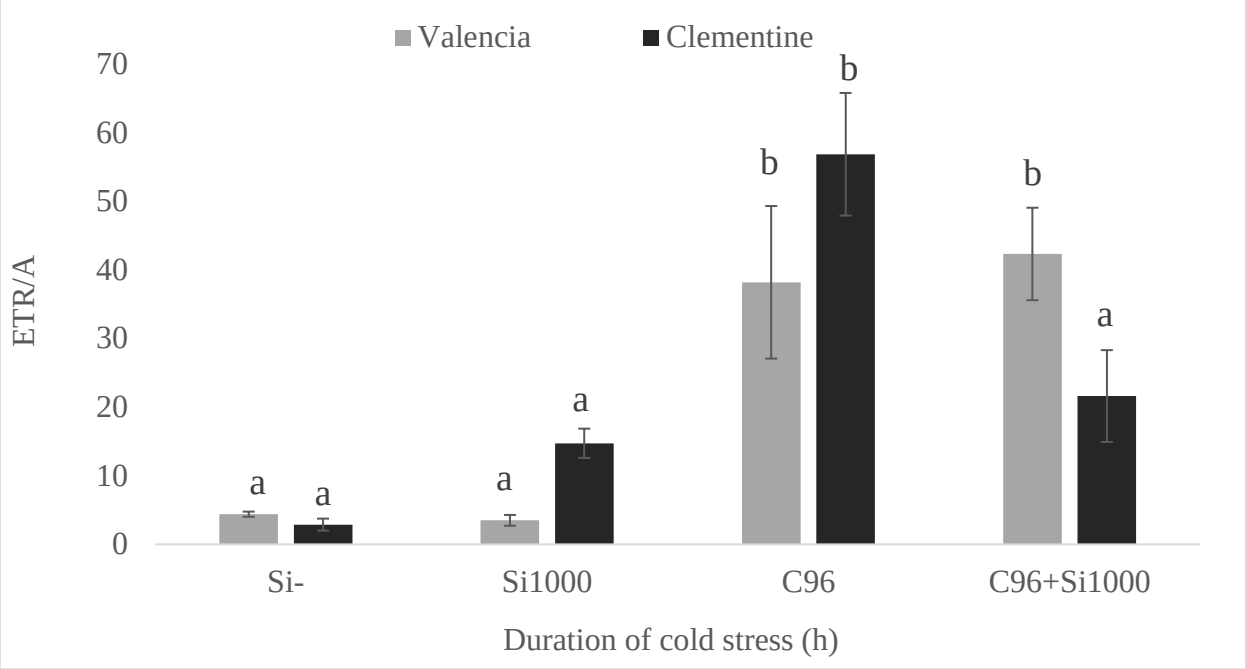


Figure 6.4.7 The effect of root-drench application Si+ (1000 mg L^{-1}) and Si- (0 mg L^{-1}) on the photorespiration of 'Valencia' and 'Clementine' leaves subjected to chilling injury stress of 0^{o}C for 96h. Data are means ± standard errors. Bars sharing a letter are not significant.

6.3. Discussion

Si applications are known to improve the photosynthetic and gas exchange of plants subjected to abiotic stresses such as cold, drought and heat (Gao *et al.*, 2005, Saud *et al.*, 2014, Maghsoudi *et al.*, 2015, Hazrati *et al.*, 2016). The current study examined the effect of Si application on leaf gas exchange and chlorophyll fluorescence parameters of citrus subjected to cold stress. In the present study, Valencia F'_v/F'_m improved after foliar treatment with Si at 100 and 500 mg L^{-1} after 2h of cold stress of 4^{o}C. A foliar treatment with 500 mg L^{-1} Si improved F'_v/F'_m after 4h of cold stress of 4^{o}C. On the contrary, foliar-Si applications had no significant effect on F'_v/F'_m in Clementine, which could be due to acclimation of the young citrus trees to cold stress (Figure 6.2.1). The maximum quantum yield of photosystem II (F'_v/F'_m and F_v/F_m) was indicative of improved photosynthetic capacity of plants (Maxwell & Johnson, 2000), and suggested that Si treatments improved the photosynthetic capacity of Valencia.

F_v/F_m of dark-adapted leaves in Valencia showed significant improvement after foliar treatments with 100 and 500 mg L^{-1} Si when the trees were subjected to cold stress of 4^{o}C. This implies that foliar-Si improved PSII efficiency of Valencia under cold stress. Negligible differences were observed in Clementine with regards to F_v/F_m. This implies that the exposure to cold stress of 4^{o}C for up to 16h did not reduce F_v/F_m of Clementine. The considerable reduction in PSII efficiency under cold stress observed is due to the reduction in electron transport efficiency which leads to photoinhibition and this is more pronounced in the presence of high light intensity (F'_v/F'_m) (Adams & Demmig-Adams, 1994). A root drench of Si failed to improve the PSII efficiency under cold stress in both cultivars (Figures 6.4.1 and 6.4.2). This implied that there may have been a reduction in reactive oxygen species after cold stress, as a result of root drenches with Si, this was masked due to the plant's capacity to increase antioxidant activities under cold strss condition (0^{o}C) (Fu & Huang, 2001, Kim *et al.*, 2017).

Photoinhibition is linked to the increase of F_o in stressed plants (Krause, 1988, Hazrati *et al.*, 2016). In this study, there was no increase observed in the F_o in Valencia subjected to cold stress of 4^{o}C at different foliar-Si application rates. This implied that Si is not involved in the photoinhibition process. Which led to the speculation that the plants were cold acclimatized, which resulted in the constant F_o (Krause, 1988). However, in Clementine F_o increased in Si treated plants at the onset of cold stress of 4^{o}C, suggesting that photoinhibition occurred. Similarly, in trees drenched with Si, an increase in F_o was observed in both cultivars after exposure to 0^{o}C (Figure 6.2.3). This implies that photoinhibition was a protective mechanism triggered by cold stress in trees treated with Si drenches (Porcar-Castell *et al.*, 2008, Maghsoudi *et al.*, 2015).

The maximal fluorescence (F_m) level is defined as the level reached after a high intensity flash application and its increase is linked to reduced heat dissipation (Maxwell & Johnson, 2000, Hazrati *et al.,* 2016). Valencia showed an increase in F_m following foliar-Si application at 100 mg L^{-1} rate after prolonged stress of exposure to 4^oC. This suggests that prolonged exposure to cold stress triggered cold sensitivity in the control trees, which was countered through Si application (Hazrati *et al.*, 2016).

In the experiment on trees treated with Si drenches, the reduction by 30% in F_m Clementine observed in the control trees subjected to cold stress may have been due to the protein deactivation in chloroplast structure (Hazrati *et al.*, 2016). However, in trees treated with Si drenches there was an increase of F_m due to a reduction in heat dissipation, which should lead to an improvement in PSII efficiency in cold stressed plants (He & Edwards, 1996, Mashilo *et al.*, 2017).

The current study demonstrated that the NPQ values in Valencia trees subjected to foliar-Si application of 100 mg L^{-1} were moderately higher when exposed to 2, 4 and 16h of cold conditions, relative to the NPQ values of trees under non-stressed conditions (Figure 6.2.5). Similarly, in Valencia trees treated with a root drench of Si (1000 mg L^{-1}), the NPQ values were moderately higher when exposed to 0^oC (Figure 6.4.5). This suggests that the interaction between foliar and root Si treatments of 100 and 1000 mg L^{-1}, respectively, and cold stress triggered a reduction in NPQ, translated by a reduction in heat dissipation. This corroborated with findings in wheat plants that demonstrated Si-supplied plants reduced their energy dissipation (Aucique-Pérez *et al.*, 2017). However, this improvement was not observed after the foliar-Si treatment of Valencia trees at 500 mg L^{-1}, possibly due to stomata being clogged. Contrastingly, in Clementine, the root drench of Si failed to reduce NPQ under cold stress conditions.

Electron transport rate (ETR) is an indication of the capacity of the plant to protect the PSII from oxidative damage (Lovelock & Winter, 1996). In the present study, the ETR in Valencia was higher after foliar treatment with 100 mg L^{-1} Si, compared to treatment with 500 mg L^{-1} at the onset of exposure to 4^{o}C (Figure 6.2.6). It is proposed that the higher foliar-Si application rate inhibited ETR due to stomatal clogging caused by a Si precipitate. Additionally, increases in ETR could be linked to the reduction in heat dissipation (NPQ) after foliar treatment of Si at 100 mg L^{-1}. In Clementine trees, ETR was improved after foliar treatment with Si 500 mg L^{-1} before the onset of cold stresses of 2 and 4h.

Application of Si as a root drench to Valencia trees increased ETR during cold stress of 0^{o}C, which showed that both the foliar and root Si applications improved ETR by reducing free radical development due to stress conditions (Flexas *et al.*, 2002). The reduced ETR observed in Valencia trees exposed to cold stress of 0^{o}C was linked to stomata closure that triggered an increase in NPQ as a photoprotective mechanism for the avoidance of over-excitation of PSII against photoinhibition (Demmig-Adams & Adams III, 1996, Ribeiro *et al.*, 2009). In Clementine trees drenched with Si, the ETR was reduced in stressed plants but increased by 80% in non-stressed conditions (Figure 6.4.6). This implied that Si failed to improve the ETR in the Clementine trees subjected to cold stress. These differences in ETR and NPQ between the two cultivars may be explained by the fact that plant tolerance to cold injuries varies greatly between species (Nagao *et al.*, 2005). Another explanation is the existence of specific physiological and biochemical response to photoinhibition under stress conditions in each citrus species (Santini *et al.*, 2012). Foliar application of Si could be used in citrus plants. However, there is potential for clogging of stomatal structures if high concentrations are used, which would diminish the value of Si for citrus trees under stress conditions. This finding was substantiated by an earlier study that demonstrated negative effects of several foliar chemicals on growth, associated with increases in the applied concentrations of the

compounds (Slatyer & Bierhuizen, 1964). Therefore, root drenches of Si are a safer option to achieve significant improvement of the metabolic activities of citrus trees subjected to abiotic stress.

The rate of photosynthetic was significantly reduced in cold stressed trees at 0^{o}C in both cultivars compared to non-stressed conditions. Cold stress results in a reduction in CO_2 fixation (Allen & Ort, 2001). This was substantiated by another study conducted on bottle gourd that demonstrated a reduction in the CO_2 assimilation rate for plants grown under water stress conditions (Mashilo, 2016). The observed improved photosynthetic activity in Clementine trees treated with Si drenches after prolonged stress of 72h may be linked to the improvement in iWUE efficiency in Si treated plants subjected to cold stress of 0^{o}C (Ma *et al.*, 2004).

The reduction in gs due to cold injury observed in the present study concurs with Ribeiro *et al.* (2009) who reported a significant reduction in stomatal conductance under cold stress due to stomatal closure. This can be explained by the reduction in CO_2 availability for Rubisco synthesis because of reduced stomatal conductance, which subsequently leads to a reduction in the photosynthetic capacity of citrus trees under cold stress (Medina *et al.*, 2002, Ribeiro & Machado, 2007). The reduction in stomatal conductance observed in the current study after 0, 4, 48 and 72h of exposure to cold stress following Si treatment agrees with Lobato *et al.* (2009) who reported a reduction in stomatal conductance in *Capsicum annum* under water stress condition in Si treated plants.

A reduction in transpiration rate for trees of both cultivars exposed to cold stress implied the existence of a protective mechanism to avoid photodamage after cold stress in citrus trees (Hussain *et al.*, 2018). It is well established that transpiration in leaves of some plants is reduced by Si application (Epstein, 1994, Ma *et al.*, 2001, Ma, 2004). The reduction in

transpiration rate in Valencia after Si application has been attributed to the deposition of Si around the cell walls, silica bodies formation and thickening of the cuticle layer which were findings of our previous study on Si deposition in citrus (Mvondo-She & Marais, 2019). In an earlier study, the reduced transpiration rate after Si treatment was explained by a well thickened layer of silica gel associated with the cellulose in the epidermal cell walls (Savant *et al.*, 1999).

This implies that the increase in transpiration rate observed in Clementine at the onset of cold stress was because the plant underwent shock and acclimatized, but during the prolonged stress of 72h, the plants lost their protective ability. The decrease in transpiration rate is linked to a decrease in stomatal conductance in trees treated with Si drenches is linked due to stomata closure (Gao *et al.*, 2006).

The sensible increase in C_i observed in Valencia after prolonged exposure to the cold stress can be explained by acclimation. The reduction in C_i at the onset of exposure to cold stress may have been because Valencia trees were temperature sensitive and unable to maintain an optimal internal carbon dioxide concentration. The effect of Si maximum absorption was demonstrated only when Valencia trees were subjected to prolonged stress. This agrees with a previous study that demonstrated Si effects occurred only in plants grown under severe abiotic and biotic stress conditions (Li *et al.*, 2007). The decrease in C_i cold stress in Clementine despite Si application may be attributable to a reduction in stomatal conductance (Farquhar & Sharkey, 1982).

In both cultivars, intrinsic water use efficiency (iWUE) was significantly reduced at cold stress of 0^oC regardless of duration of exposure (Figure 6.3.5). A decrease in iWUE has been reported in Kentucky bluegrass (Saud *et al.*, 2014) and bottle gourd (*Lagenaria siceraria*) (Mashilo *et al.*, 2017) when subjected to drought stress. A reduction in iWUE due to cold stress is caused by the reduction in the stomatal conductance and transpiration rate, which is

attributed to the decrease in soil water potential (Saud *et al.*, 2014). The reduction in iWUE observed in Clementine might be explained by cold stress shock, which eventually improved in the prolonged stress treatment, because of CO_2 assimilation rate improvement in Si-treated Clementine. Improvement of the iWUE in relation to Si treatments has been also observed in strawberry (Dehghanipoodeh *et al.*, 2018); Kentucky bluegrass (Saud *et al.*, 2014); maize (Gao *et al.*, 2005, Parveen & Ashraf, 2010) and tomato (Romero-Aranda *et al.*, 2006).

Photorespiration is defined as the process through which Rubisco binds to oxygen molecules and the reaction deviates from the regular metabolic pathway; therefore, no sugar and ATP molecules are synthesized (Peterhansel *et al.*, 2010). It is quantified by the ratio of electron transport rate (ETR) to photosynthetic assimilation (A) (Fryer *et al.*, 1998). In both cultivars there was a significant increase in ETR/A under stressed conditions, implying there was an increased in photorespiration rate (Figure 6.4.7). The increase in photorespiration rate is triggered by the decreased stomatal conductance as the stromal CO_2 concentration declines, hence increasing O_2 in stressed plants (Dehghanipoodeh *et al.*, 2018). Photorespiration is part of the dissipating mechanisms adopted by plants as a photo-protection against oxidative damage due to inefficiencies in electron transport system under cold stress conditions (Fryer *et al.*, 1998). The improved efficiency of the photosynthetic activity in Clementine trees treated with Si drenches and then subjected to prolonged cold stress is linked to the significant reduction in photorespiration; this results in a strong correlation between chlorophyll fluorescence and photosynthesis (Genty *et al.*, 1989).

Conclusions

The present study evaluated the photosynthesis and chlorophyll fluorescence parameters of two citrus cultivars subjected to foliar and root Si treatments, and then exposed to cold stress. A root drench with 1000 mg L^{-1} potassium silicate improved the photosynthetic rate in

Clementine and water use efficiency in both cultivars when subjected to cold stress. This implies that physiological and biochemical response to photoinhibition under cold stress conditions in citrus is species dependent. In addition, these findings suggest that root drenching with Si fertilizers is the most suitable application method to achieve significant improvement of growth of citrus species exposed to abiotic stress. This study also provided insight into physiological responses that occur in cold stressed citrus cultivars.

Chapter 7

SILICON UPTAKE AND ITS ROLE IN FRUIT QUALITY AND YIELD IN COLD STRESSED CITRUS TREES

Abstract

Citrus is an important fruit crop globally. However, cold stress events reduce citrus production and quality. Considering the threat of cold to crop quality and production, silicon role in fruit quality and production in other crops was evaluated. However, limited information is available on the impact of Si fertilization on fruit quality and yield in citrus. Therefore, experiments were conducted to determine the absorption of Si in citrus plants and to assess the role of Si uptake in citrus fruit quality and yield following cold stress conditions during flower induction.

The experiments on citrus fruit quality were conducted over two seasons: May 2011 to September 2012, and September 2012 to May 2013. In the first season potassium silicate solution was sprayed at three concentrations (0, 100 and 500 mg L^{-1}) onto the leaves of 'Cara Cara' navel orange trees (grown on Carrizo citrange rootstock) every three weeks until runoff.

In the second season potassium silicate solution was applied to the roots of 'Cara Cara' navel oranges on Carrizo citrange rootstock at three application rates (0, 500 and 1000 mg L^{-1}), fortnightly, with four rows per treatment. Fruit yield and fruit quality (total soluble sugar, juice content and acidity) were determined at harvest maturity.

Silicon absorption significantly increased $P \leq (0.05)$ with application rate in leaf tissues. However, there was no evidence of Si uptake in fruit, which explains why Si supply is not a suitable tool to enhance citrus fruit quality under either low temperature or normal growth conditions.

Key words: Cara-Cara, frost, low temperature, plant nutrition

7.1. Introduction

Citrus is one of the main fruit crops in the world, with an estimated production of more than 170 million tonnes (FAOSTAT, 2015). The attractiveness and high consumption of citrus fruits are due to health and taste attributes (Liu *et al.*, 2012, Lado *et al.*, 2018). Fruit quality is a very important parameter in the production of export citrus fruit, which relates to fruit size, morphology, maturity (including taste), pigmentation and shelf-life. Temperature is the most significant factor in citrus fruit quality (Ribeiro *et al.*, 2009, Lado *et al.*, 2018). Cold stress in citrus plants affects flower induction, which lowers the fruit quality and yield (Ribeiro & Machado, 2007). This affects the revenue from citrus because the high quality of fresh fruit results in the higher rate of acceptance in the market (Ladaniya, 2008).

Many field experiments under different soil, climate conditions and with diverse plant varieties have shown that applying Si fertilizers improves crop quality and yield (Matichenkov *et al.*, 2001, Motomura *et al.*, 2002, Henriet *et al.*, 2006, Barker & Pilbeam, 2015, Tubana *et al.*, 2016, Dorairaj *et al.*, 2017). Si application is known to enhance crop quality by improving the erectness of leaves, stems, and culms, thereby increasing the distribution of light within the canopy (Epstein, 1994, 1999). Silicon uptake often results in an increase in dry matter uptake and fruit quality in cucumber (*Cucumis sativus L.*) (Adatia & Besford, 1986, Abd-Alkarim *et al.*, 2017). In sugarcane (*Saccharum officinarum L.*), Si application improved growth from 17 to 30%, and the sucrose yield increased by 27% (Matichenkov & Calvert, 2002). In an experiment conducted in strawberry (*Fragaria ananassa L.*), Si application increases fruit production, values of titratable acidity and anthocyanins content (Silva *et al.*, 2013). In another experiment in tomato, Si applications improved fruit production (Marodin *et al.*, 2014). Two studies conducted on rice (*Oryza*

sativa L.), demonstrated there was an increase in yield of Si-treated plants compared to the control (Detmann *et al.*, 2012, Dorairaj *et al.*, 2017). In addition, Si application improved the harvest index in rice, which was linked to the improvement in nitrogen use efficiency (Detmann *et al.*, 2012). In a recent study conducted in melon, silicon fertilization improved fruit quality and yield (do Nascimento *et al.*, 2019). The yield increase was linked to the increased level of Si, and nutrient uptake, as a result of Si fertilization (do Nascimento *et al.*, 2019).

In a field investigation on the effect of Si fertilisers in citrus (*Citrus limon*) conducted in Russia 70 years ago, Si fertilisation accelerated the growth of citrus by 30 to 80%, fruit maturation by two to four weeks and increased the amount of fruit (Taranovskaia, 1939). In citrus plants, Si fertilization has been responsible for a significant increase in mass roots and green mass of germinated grapefruit, as a result of the interaction between cold stress and Si fertilization (Matichenkov *et al.*, 2001). Si application in young orange (*Citrus sinensis*) trees has also resulted in an increase in both the total tree height and the length of tree branches (Wutscher, 1989, Matichenkov *et al.*, 1999).

The aim of this experiment was to investigate the effect of Si uptake in citrus on fruit quality and yield post-frost conditions.

7.2. Materials and Methods

7.2.1. Site description

Saam Farm is in the Vaalharts region of the Northern Cape. This region is designated as semi-arid, with a mean minimum temperature of -5^{o}C during the frost month of August 2012.

7.2.2. Low temperature and fruit quality assessment under field trial

The experiment was conducted in the Northern Cape Province on Saam Farm (S 27° 54' 54.33" E 24° 51' 30.38"). The experiment was conducted in two seasons, May 2011 to September 2012, and September 2012 to May 2013. In the first season A Si uptake

experiment was performed on 12 years old citrus trees of the cultivar ‘Cara-Cara’, a late navel orange (*Citrus sinensis*) grafted onto Carrizo citrange rootstocks, and grown on a Hutton soil with 4-6% clay content with 5 mg kg^{-1} of extractable Si. A commercial product of potassium silicate (K_2SiO_3) was used to supplement Si levels. The Si was applied at three concentrations (0, 100 and 500 mg L^{-1}) sprayed onto the trees every three weeks until runoff to ensure uniform coverage, using a commercial sprayer (Banjo, South Africa). The treatments were allocated respectively to Blocks 25, 26 and 27. These blocks were situated in the most frost-prone area of the farm. In each block, the allocated treatment was applied to three rows. Ten trees randomly selected were sampled in the middle row per treatment to assess Si content and fruit quality. Due to a hailstorm during the harvest season, fruit yield was not assessed during the first season. Leaf samples were taken every two months to assess Si uptake over time. Temperature and relative humidity were recorded during winter to monitor the impact of Si on post-frost effects.

In the second season, a Si uptake experiment was performed on the same blocks. Commercially available potassium silicate (K_2SiO_3) was applied. The concentration of Si applied was calculated according to the product information and applied as a drench, based on the results obtained from Chapter 3, which demonstrated that the root drench application was the best mode of application. The maximum absorption rate determined in Chapter 3 was used to adjust Si application rates in the second season (0, 500 and 1000 mg L^{-1}). The treatments were allocated randomly respectively to Blocks 25, 26 and 27, each block consisted of three rows. To minimize border effect, the middle row was selected to assess Si content, fruit quality and yield. The effect of Si uptake on trees exposed to a cold spell in August 2012 on fruit quality was investigated at harvest in May 2013.

7.2.3. Si analysis procedure

Polypropylene and Teflon containers were used for the preparation and digestion of the samples to minimize risks of contamination. All containers were rinsed with NaOH (10%) prior to use. The microwave digestion method of Haysom & Ostatek-Boczynski (2006) was used. Three mL of 65% HNO_3 (AR grade) was added to dry, ground 500 mg leaf samples or fruit parts. The tubes were capped and left to stand for five minutes to thoroughly wet the sample. Two mL of 30% H_2O_2 (AR grade) were then added and left to stand overnight. The capped Teflon tube (TFM) reaction vessels were placed in the ceramic tube holders of the microwave system (Mars 5, CEM Corporation). The rotor was placed in the cavity of the microwave unit and the acid digestion step conducted. On completion of the cycle, the tubes were cooled, and then NaOH (10%) solution was added to the digestate and returned to the microwave system for the second heating step, which resulted in the solubilisation of amorphous Si. After the final cooling step, the contents of the tubes were neutralized with HNO_3 (2 M) in a plastic beaker, using phenolphthalein as an indicator, and then diluted to a volume of 250 mL in a volumetric flask to reduce the matrix effect. An Inductively Coupled Plasma optical emission spectrometry (ICP-OES) analysis procedure was developed to measure Si concentration. The plasma power was 1000W with a plasma flow rate of 15 L min^{-1} and an integration time of 1 second. The wavelength 251.611 nm was selected as the most sensitive wavelength based on the correlation, accuracy based on relative standard deviation was used for the analysis of both samples and standards. Calibrating standards for the ICP-OES instrument were prepared from a 1000 mg L^{-1} standard (Fluka). The ICP was fitted with a hydrofluoric acid (HF) resistant torch, Sturrman Master spray-chamber and V Groove nebulizer assembly. The Si concentration was determined with ICP-OES (Varian Liberty 200).

7.2.4. Fruit quality

Fruits from ten randomly selected trees from the middle row of each treatment were used to assess fruit quality. Fruit quality was assessed based on brix content, acidity% and juice % at harvest maturity (NDA, 1990).

7.2.5. Brix content

Brix content measures the total soluble solids % (TSS%) in the juice. The soluble solids are primarily sugars: sucrose, fructose, and glucose. Citric acid and minerals in the juice also contribute to the soluble solids. Total soluble sugar (TSS)% of the juice was determined by using a Zeiss hand refractometer (Hanna Instruments, South Africa).

7.2.6. Acidity (%)

This is a measure of the total acid present in a juice. The predominant acid naturally occurring in orange juice is citric acid. There are also traceable amounts of malic acid and tartaric acid present. The amount of acid present in the fruit is reported as a percent of citric acid. The acid content was measured using a titration with sodium hydroxide.

7.2.7. Juice (%)

This test determines the quality of the fruit. The fruit quality affects the eating quality and the revenue that the farmer will receive for their crop. The percentage of juice content is calculated as follows:

% juice content = juice weight net /fruit weightx100.

Phenolphthalein was used as an indicator and titration was complete when the liquid turned pink in colour.

7.2.8. Yield assessment

Harvesting of the fruit was undertaken at the same time as non-selective, commercial picking, running from the beginning of May 2013. Individual tree yields were taken from the

randomly selected trees (as mentioned in Fruit Quality). Yield was determined in kg tree^{-1}, replicated for each separate treatment.

7.2.9. Statistical analysis

The analysis of variance was performed with SAS (version 9.4) to determine treatment mean effects. Differences between treatments were determined using LSD $P \leq (0.05)$ according to the F test.

7.3. Results

7.3.1. Silicon uptake and fruit quality in first season

Silicon uptake in leaves improved significantly with foliar-Si application rates of 100 and 500 mg L^{-1}. The highest Si uptake rate was observed at 500 mg L^{-1} of applied Si (Figure 7.1).

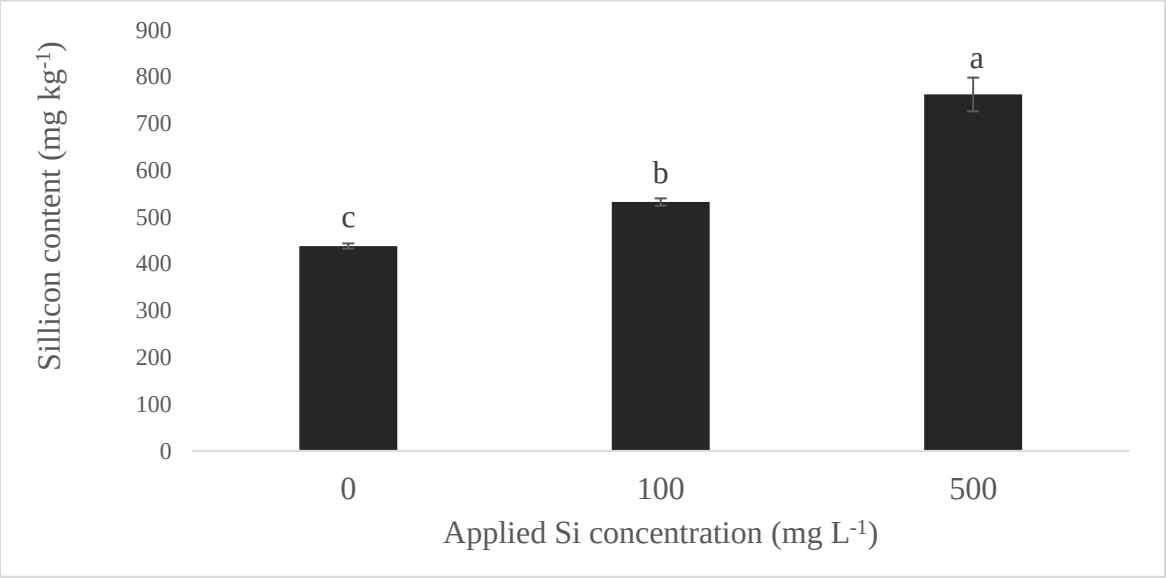
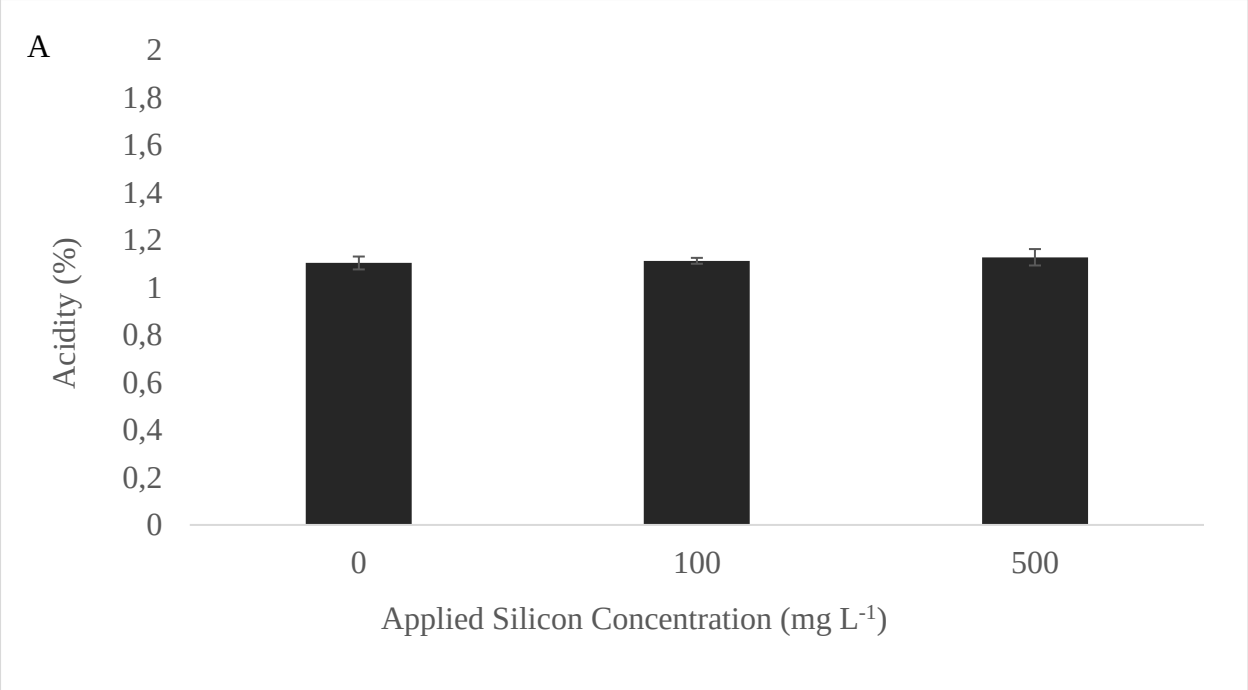
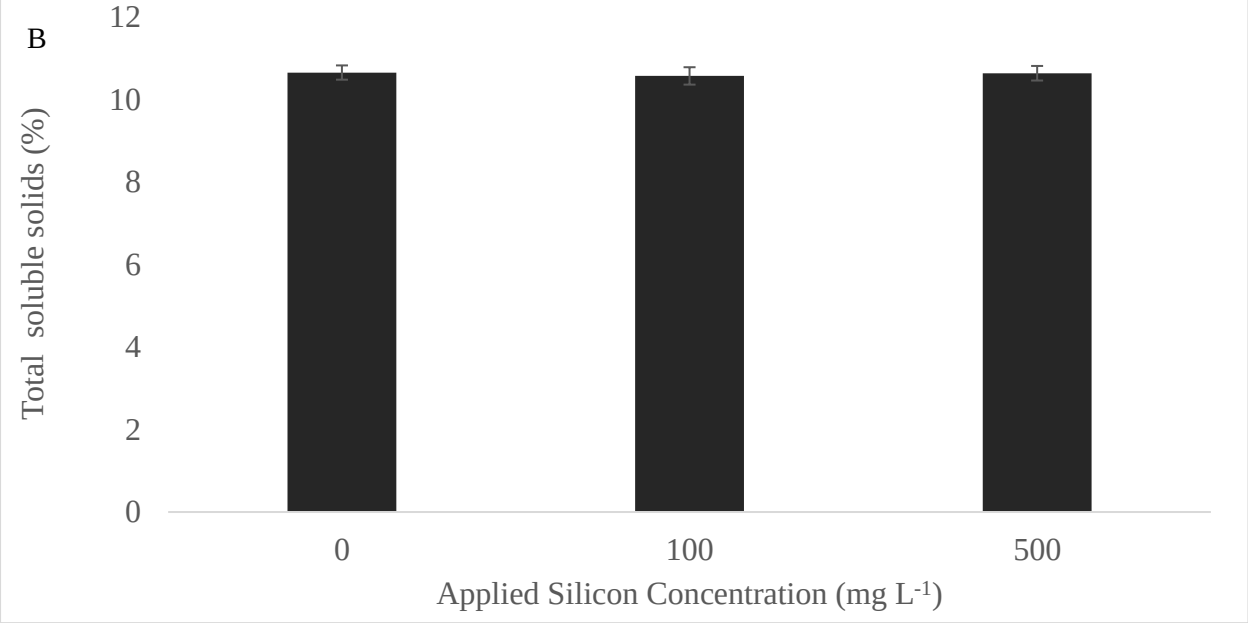


Figure 7. 1 First Season Si content in Navel trees subjected to foliar-Si application. Means followed by the same letter are not significantly different at 5% level of probability according to Fisher's LSD test.

In the evaluation of the impact of Si on fruit quality in navels in the first season, the total soluble sugars parameters acidity (%), TSS% and juice (%) were not influenced by Si fertilization (Figure 7.2).


A
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Acidity (%)
0
100
500
Applied Silicon Concentration (mg L-1)


B
12
10
8
6
4
2
0
Total soluble solids (%)
0
100
500
Applied Silicon Concentration (mg L-1)

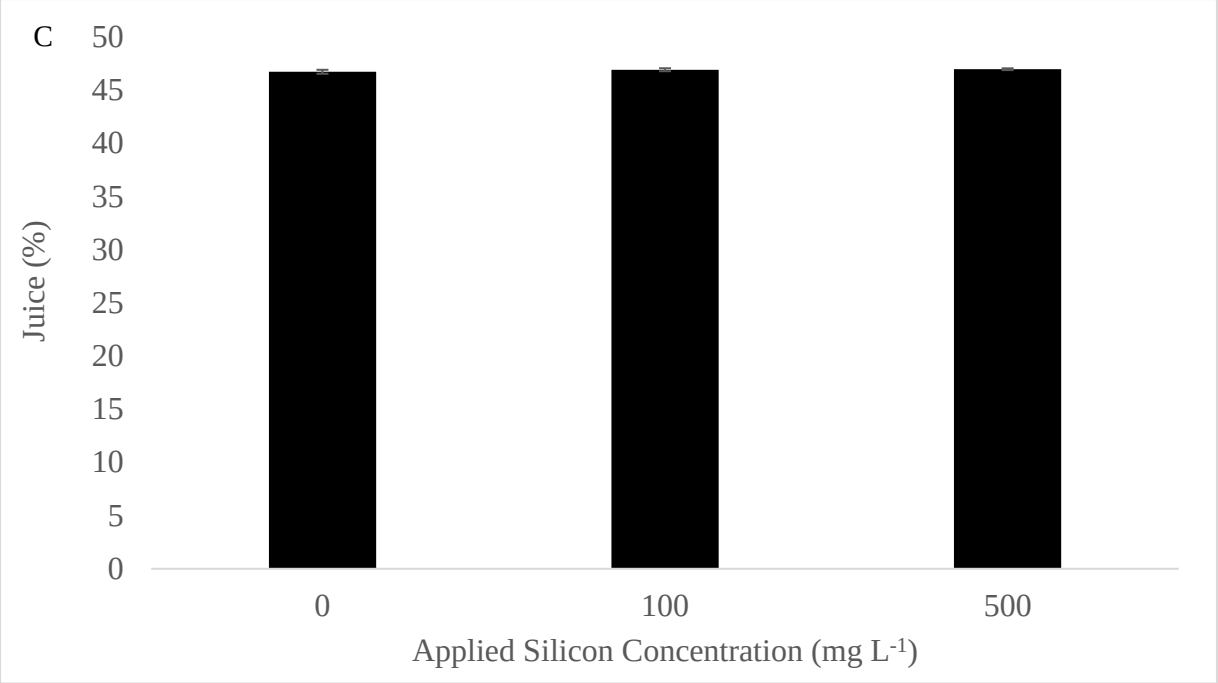


Figure 7.2 The influence of Si fertilization on fruit quality: (a) Acidity %, (b) Total Soluble sugar %, (c) Juice % of Navel trees.

7.3.2. Second season, the impact of Si applications on fruit quality and yield

In the second season, the Si application rates were adjusted based on the determination of the maximum uptake of 1000 mg L^{-1} in the previous study conducted on Si uptake (Chapter 4). Si uptake rate improved significantly in Si-treated plants compared to the control. The highest Si uptake was observed to result from the application of 1000 mg L^{-1} (Figure 7.3). Silicon uptake increased significantly over 2, 4, 6 and 8 months (Figure 7.3). Silicon content was not detectable in fruit.

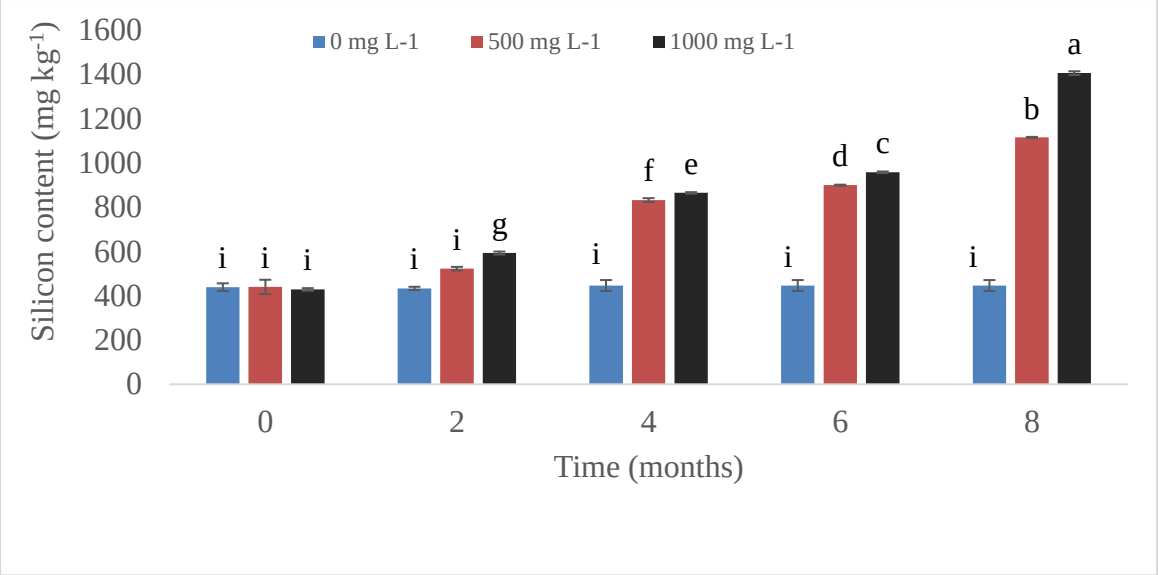
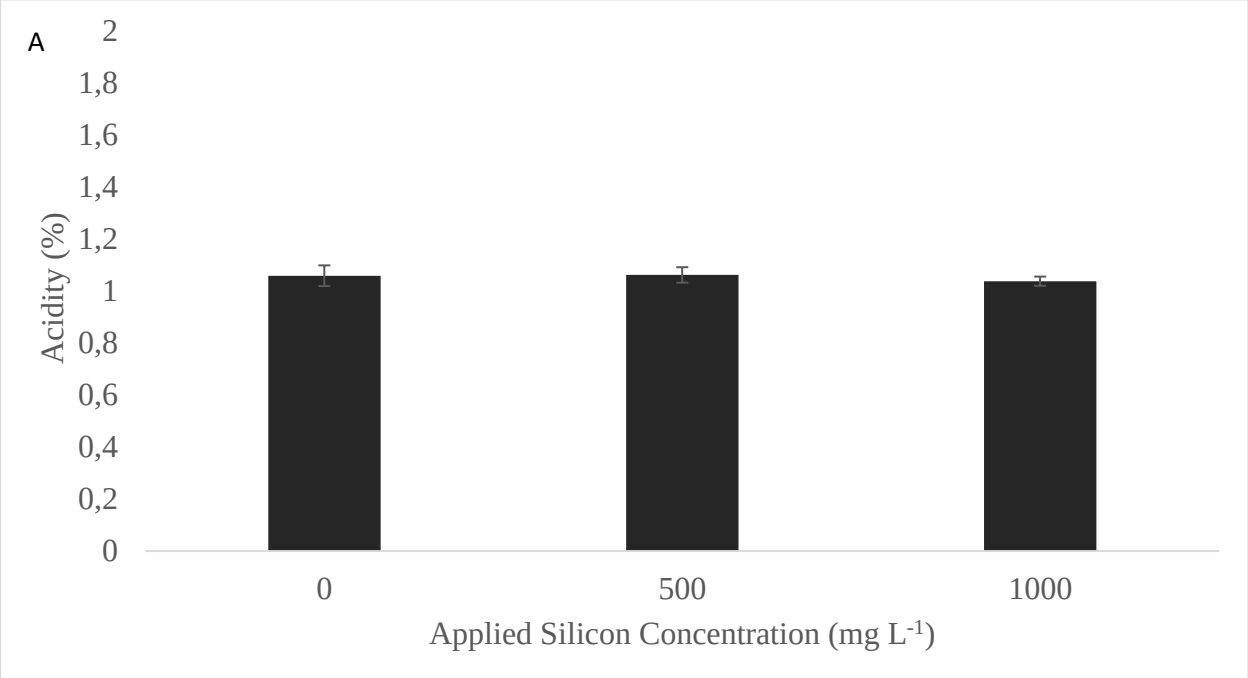
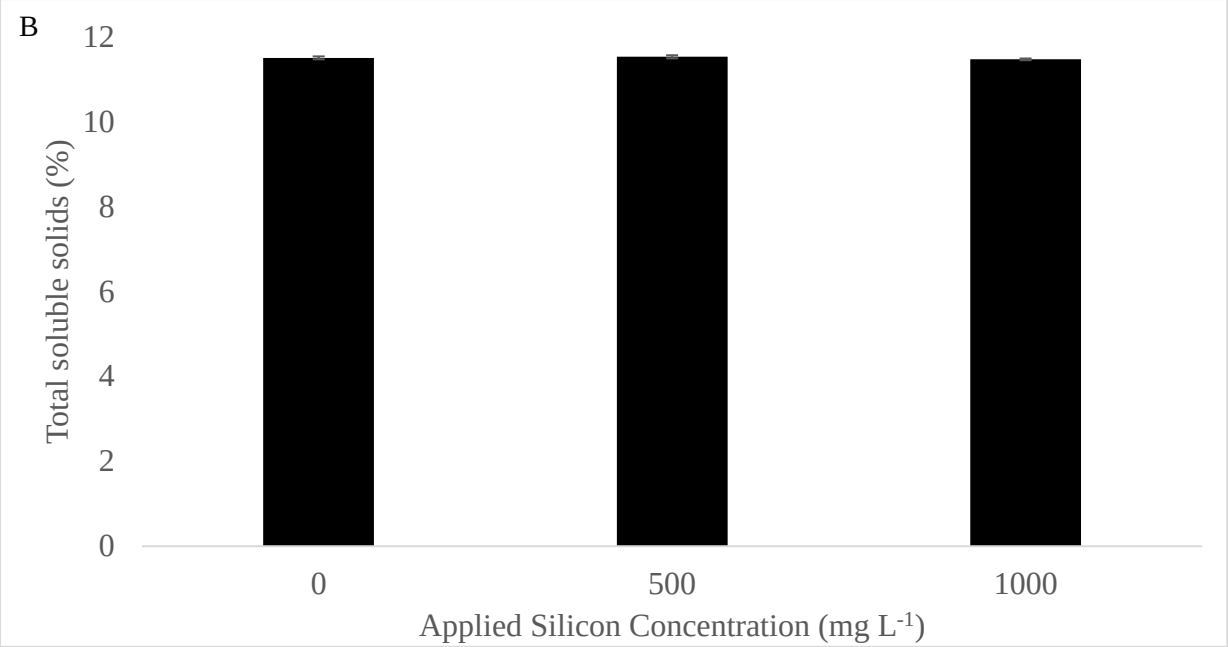


Figure 7. 3 The evaluation on the impact of root drench application of Si on Si uptake in Navel trees over an 8 month period during the second season. Means followed by the same letter are not significantly different at 5% level of probability according to Fisher's LSD test.

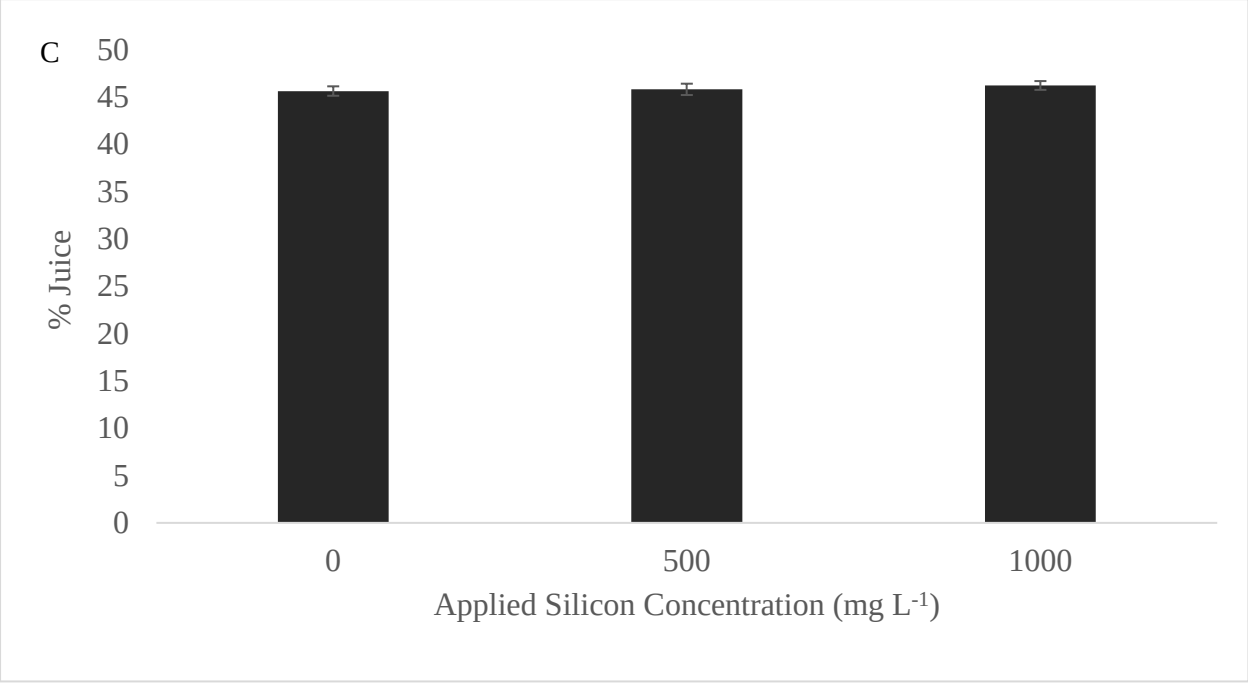
The investigation showed that Si did not impact on fruit quality in navel, and the parameters Total Soluble Sugar (TSS)%, juice (%) and acidity were not influenced by Si fertilization (Figure 7.4). Similarly, Si application played no role with respect to fruit yield and size (Figure 7.4).

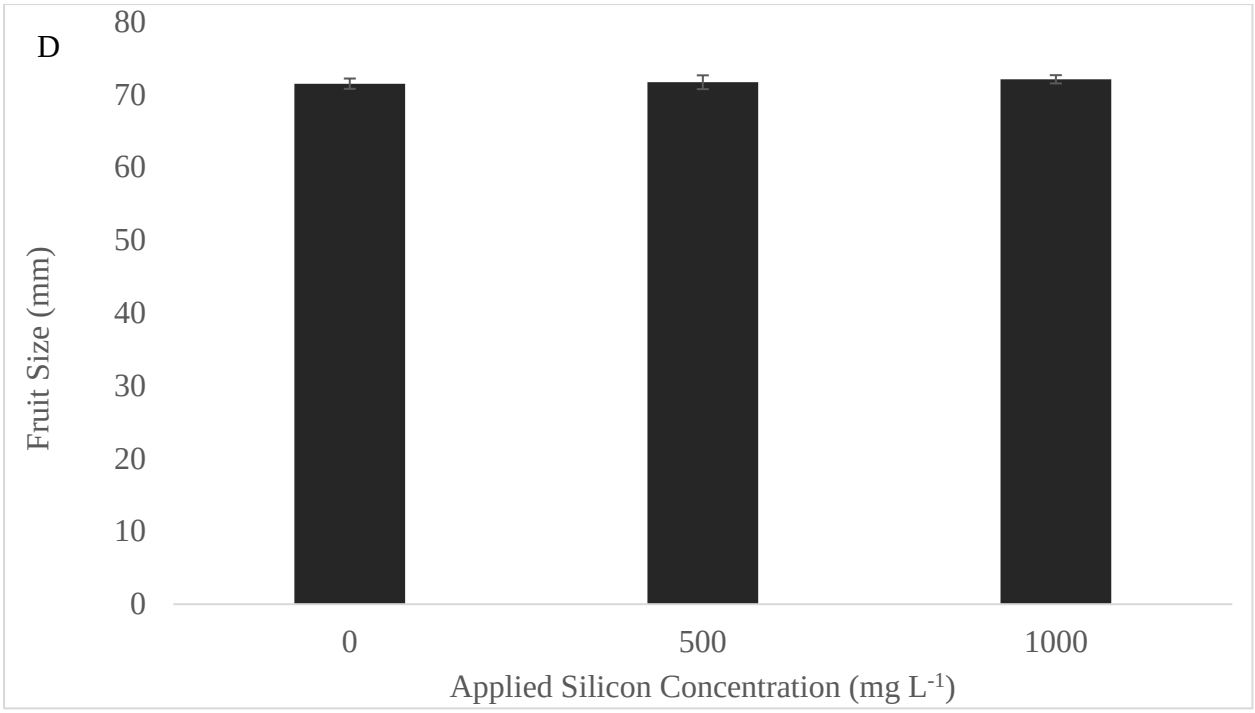


B


12
10
8
6
4
2
0
Total soluble solids (%)
0
500
1000
Applied Silicon Concentration (mg L-1)

C


50
45
40
35
30
25
20
15
10
5
0
% Juice
0
500
1000
Applied Silicon Concentration (mg L-1)



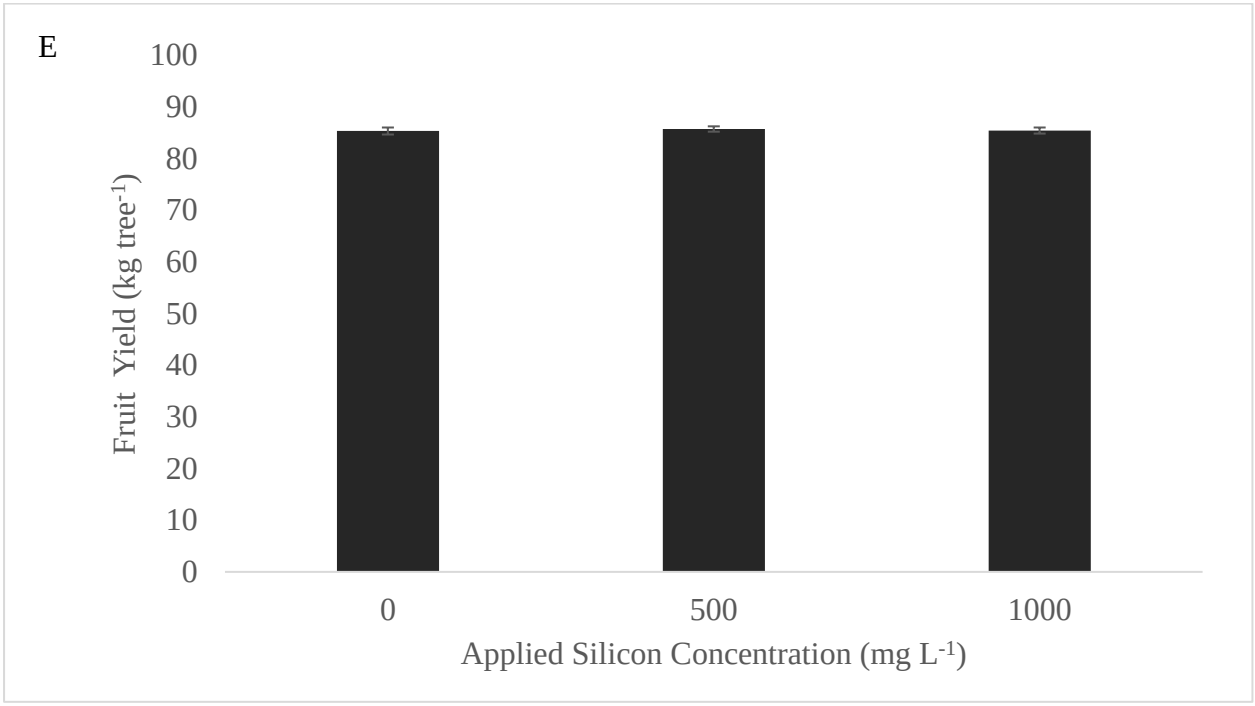


Figure 7.4 Second season assessment of fruit quality parameters: (a) Acidity %, (b) Total Soluble sugar %, (c) Juice % and yield: (d) Fruit size and (e) fruit yield in Navel trees subjected to three Si application rates.

7.4. Discussion

To establish the function of Si in cold protection, in the first season it was essential to increase the Si content in the leaves. Silicon content increased significantly at $P \leq (0.05)$ with foliar applications of silicon at 100 and 500 mg L^{-1}, and the highest uptake was observed at 500 mg L^{-1} (Figure 7.1). This agreed with previous studies that demonstrated Si uptake increased with Si supply in cucumber (Adatia & Besford, 1986); tomato (Marodin *et al.*, 2014); rice (Ma & Yamaji, 2006, Rao & Susmitha, 2017) and citrus (Wutscher, 1989, Mvondo-She & Marais, 2019).

In the second season, Si was applied to the citrus trees over an eight-month period prior to the onset of cold stress. Silicon uptake increased significantly with the duration and frequency of application at $P \leq (0.05)$ (Figure 7.3). This finding corroborated with a previous study in zucchini (*Cucurbita pepo*) that demonstrated an increase in Si content with the frequency in Si application (Tesfagiorgis & Laing, 2013). Silicon content increased significantly at $P \leq (0.05)$ with silicon application rates (500 and 1000 mg L^{-1}). The highest uptake was observed at 1000 mg L^{-1} (Figure 7.3). Increases in Si uptake in response to Si applications has been confirmed in several studies (Mitani & Ma, 2005, Kidane, 2008, Marschner, 2012, Marodin *et al.*, 2014). Si levels in the fruits were undetectable, which contradicts previous findings by Abraham (2010). The contradictory results could be linked to the prevailing low temperature conditions around flowering and fruit set period during the research, which did not encourage an active Si uptake process and transfer into the fruit, whereas Si was already transferred into the mature leaves during optimal environmental conditions.

The fruit quality parameters were all within export standards (NDA, 1990). Si uptake did not show statistical differences and failed to impact on fruit quality parameters: acidity%, total soluble sugar%, and juice% (Figures 7.2 and 7.4). This corroborated with the findings of

Abraham (2010), where silicon applications failed to improve the total soluble sugar content in citrus fruits. In another study conducted on zucchini, Si treatment had little impact on fruit quality (Tesfagiorgis & Laing, 2013). Similarly, an investigation of the impact of foliar and root applications of Si to pepper (*Capsicum annuum*) demonstrated that fruit quality parameters were not significantly impacted by Si treatments (Jayawardana *et al.*, 2014). In contrast, in a study conducted on tomato grown under heat stress Si applications improved the fruit quality (Toresano-Sánchez *et al.*, 2012). The lack of influence of Si in citrus fruit quality in this study could be explained by the low levels of Si in the fruits and Si polymerization in leaves (Ma & Takahashi, 2002). Fruit quality parameters were non-significantly different in both seasons, implying that Si application method did not impact on fruit quality.

In the current study, the yield was 85 kg tree^{-1} regardless of Si treatment, which is lower than the optimum Navel fruit yield of 96 kg tree^{-1} (de Villiers & Joubert, 2006). This could be explained by the prevailing temperatures, which were below the threshold recommendation of 2-3°C for optimum production (Ribeiro *et al.*, 2004). Si application did not influence the fruit yield. In melon Si fertilization caused an increase in fruit yield, which was linked to the increase in nutrient uptake (N, P, K, Ca, Mg, Fe, Mn and Zn) associated with Si fertilization (do Nascimento *et al.*, 2019). Similarly, in rice, a known Si accumulator, Si uptake improved yield (Detmann *et al.*, 2012, Dorairaj *et al.*, 2017). Navel orange has been classified as moderately tolerant to cold stress, which might have reduced the beneficial role of Si, which is enhanced in very susceptible plants (Liang *et al.*, 2008, Zekri, 2011, Hussain *et al.*, 2018).The results obtained in the current study could also be explained in that the effect of Si application on citrus fruit quality and yield under field conditions could have been masked, due to inherent resistance mechanisms against abiotic stresses expressed in that environment.

Conclusions

Si application to citrus trees that subsequently experienced cold stress was not effective as a tool to enhance fruit quality and production in citrus plants. Si uptake improved with frequency and duration of application and demonstrated a limited role in the fruit quality and yield of Navel orange trees.

The absence of Si in the fruit implied that Si was not relocated within the citrus trees and therefore it had a limited effect on fruit quality and production.

CHAPTER 8

PHYSIOLOGICAL EFFECTS OF SI UPTAKE ON CITRUS SUBJECTED TO COLD STRESS

Abstract

Plant adaptation to cold stress involves increases in levels of osmolytes and osmoprotectants, among other processes. Si application to plants is known to improve cold tolerance by triggering an increase in the levels of proline and sugar in many crops. This study investigated the effect of silicon fertilization on the levels of proline and soluble sugars of cold stressed citrus seedlings under cold room and greenhouse conditions.

Silicon was applied (potassium silicate at 0 and 1000 mg L^{-1}) to the roots of two citrus cultivars, 'Nova' (*Citrus reticulata*) and 'Nules' (*Citrus reticulata*) for three months, and were subjected to cold stress for 8 days at 2.5^{o}C. The Si treatment resulted in increased sucrose, glucose and proline in plants exposed to cold stress. The current findings suggested that Si application could contribute to the improvement in cold adaptation of citrus trees by increasing osmolytes and proline productions under cold stress.

Keywords: Nova, Nules, proline, soluble sugars.

8.1. Introduction

Cold stress is categorised as injury that takes place when plants are exposed to temperature above 0^{o}C (Levitt, 1980, Esmaili & Salehi, 2012). This injury often results in photosynthetic activity reduction, cellular membrane damage and oxidative stress in plants (Suzuki & Mittler, 2006). However, some plants are able to develop tolerance mechanisms for cold and freezing stresses by producing cryoprotective and osmoprotective compounds such as soluble sugars, and by reducing carbohydrate utilization for photosynthesis (Horacio & Martinez-

Noel, 2013). Another important function of sugars is the prevention of crystal formation in the cytoplasm at low temperatures, protecting membrane phospholipids (Levitt, 1980). In a study conducted on zoysiagrass (*Zoysia japonica* Steud.) grown under cold stress an increase in leaf carbohydrates (glucose, fructose, sucrose, trehalose, fructan and starch) was observed with the temperature reduction (Li *et al.,* 2018). Another adaptation to cold induced stress is the accumulation of cytosol compatible solutes such as proline, which serve as osmotic buffers and provide protection against scavenging ROS in plants (Alscher *et al.*, 2002, Xiong & Zhu, 2002). This was evident in a study conducted to assess salt tolerance in five ber (*Zizyphus mauritiana* Lamk) cultivars. The amino acid proline content increased significantly in sensitive cultivars compared to tolerant ones (Verma *et al.*, 2018). Similarly, in another study conducted on Bermuda grass (*Cynodon dactylon* L. Pers.) the proline content increased significantly under cold stressed conditions (Esmaili & Salehi, 2012).

Si application is known to provide tolerance to abiotic stress by reducing proline content in cold stressed banana (*Musa* sp. L.) (Kidane, 2008); water stressed strawberry (*Fragaria ananasa* L., Camarosa) (Dehghanipoodeh *et al.*, 2018) and water-stressed rice (*Oryza sativa* L.) (Mauad *et al.,* 2016). In contrast, Si application to saline stressed gooseberry (*Physalis peruviana* L.) increased proline content compared to untreated Si plants (Renata *et al.*, 2017). With respect to soluble sugar, Si treated banana subjected to cold stress demonstrated an increase in sucrose and raffinose content, which are known as cryoprotectants (Kidane, 2008). Similarly, in another study conducted on salt-stressed plants of sorghum (*Sorghum bicolor* L.) Si uptake was linked with increased levels of sucrose and glucose (Abdel-Latif & El-Demerdash, 2017).

The optimum temperature for citrus leaf photosynthesis is in the range of 20 to 35^{o}C (Ribeiro *et al.,* 2004). However, increased levels of sugars has been observed in citrus species

subjected to cold stress, accompanied by an increase in the proline level (Yelenosky, 1979). In a subsequent cold stress study, proline levels also increased in stressed citrus plants. (Kushad & Yelenosky, 1987). In other studies conducted in salt-stressed citrus plants, proline content increased linearly with salt stress conditions (Mademba-Sy *et al.*, 2003, Abadi *et al.*, 2010). In a study conducted by Mademba-Sy *et al.* (2003) an increase in proline content as a result of stress was linked to inherent ability to have high level proline in leaves rather than salt tolerance. Si fertilization of citrus plants may cause a significant increase in roots and green mass of germinated grapefruit (*Citrus paradisi*), because of the interaction between cold stress and Si fertilization (Matichenkov *et al.*, 2001). However, the underlying biochemical mechanism is not understood. Therefore, this study was aimed to examine the effect of Si applications on the levels of proline and sugars in cold stressed citrus plants.

8.2. Materials and Methods

8.2.1. Plant material

Two citrus cultivars, 'Nules' and 'Nova', were used for the study. These are two widely cultivated commercial citrus cultivars in South Africa that were selected to compare a highly and moderately vigorous mandarin species, respectively (de Villiers & Joubert, 2006).

8.2.2. Evaluating the effect of root applied Si in young citrus trees

The experiment was conducted in a glasshouse at the experimental farm of the University of Pretoria (S25^{o} 44' E28^{o} 15') (January-May 2018). A Si uptake experiment was performed on two-year-old citrus trees 'Nova' and 'Nules' (*Citrus reticulata*) (both grafted onto Swingle rootstocks) that were grown in 10L pots containing an artificial growing medium of Coir-Perlite 60%-40%, supplemented via root applications with fertilizers of N, P and K at the concentrations of 85-115-40 kg ha^{-1}, which represents fertiliser recommendation for two-year citrus trees (FSSA-MVSA., 2007). N-P-K were applied in the form of ammonium nitrate

(NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4) and potassium nitrate (KNO_3). The trees were left to acclimatize for three weeks prior to the Si uptake study.

Commercially available potassium silicate (K_2SiO_3) was applied as drench once a month (0 and 1000mg L^{-1}) with three trees per treatment and four replications. Potassium introduced by K_2SiO_3 was corrected by adding the equivalent level of K_2O to the control trees. The trees were subjected to Si drenches fortnightly, for three months to ensure maximum absorption. Daily climatic variables (temperature and humidity) were measured with data loggers.

8.2.3. Cold stress induction

After three months of Si uptake, the young citrus trees were subjected to cold stress in a chamber at the University of Tshwane's Experimental Farm, which was set at 2.5^{o}C for a period of 0, 2d, 4d and 8d with a 12h photoperiod. Thereafter the trees were moved back into the glasshouse and sampled after 12 and 21d.

8.2.4. Chemical analysis

The microwave-assisted digestion method modified from Haysom & Ostatek-Boczynski (2006) was used to extract Si content. Three mL of 65% HNO_3 (EMSURE) was added to dry, ground 500 mg leaf samples. The tubes were capped and left to stand for five minutes to thoroughly wet the sample. Two mL of 30% H_2O_2 (AR grade) was then added and left to stand overnight. The capped Teflon tube (TFM) reaction vessels were placed in the ceramic tube holders of the microwave system (Mars 5, CEM Corporation). The rotor was placed in the cavity of the microwave unit to the ramping temperature of 180^{o}C for 15min after which the temperature was maintained for 15min and the acid digestion step conducted. On completion of the cycle, the tubes were cooled, and then NaOH (10%) solution was added to the digestate. The tubes were recapped and returned to the microwave system for the second heating step to the ramping temperature of 180^{o}C for 15min after which the temperature was maintained for 15min and the acid digestion step conducted, resulting in the solubilisation of

amorphous Si. After the final cooling step, the contents of the tubes were neutralized with HNO_3 (2 M) in a plastic beaker, using phenolphthalein as an indicator, and then diluted to a volume of 250 mL with type I deionised (DI) water 18MΩ cm^{-1} type I H_2O in a volumetric flask to reduce the matrix effect. The Si concentration was determined with ICP-OES (Varian Liberty 200).

8.2.5. Proline analysis

Proline concentration was determined by means of a rapid colorimetric method, modified from Bates et al. (1973). A 100 mg sample of freeze-dried citrus leaves was homogenized in a test tube containing 10 mL sulphosalicylic acid (3% w/v) for one minute and filtrated through Whatman filter paper #2. Two mL of the filtrate was mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid and incubated at 100^oC for 1h, before the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene, vortexed vigorously for 15-20s and then the toluene phase of the solution was pipetted. The absorbance was read at 520 nm using toluene for a blank. Proline standards were used to generate a calibration curve, and proline concentration was determined from this standard curve as μg proline g^{-1} dry matter (DM).

8.2.6. Soluble sugars

Freeze dried samples (0.1 g) of citrus leaves were extracted with 10mL of 80% ethanol with three replicates per treatment (3 samples/replicate). Thereafter, the mixture was incubated in 80^oC water bath for 60min and kept at 4^oC, the supernatant was filtered through glass wool and taken to dryness in a Savant Vacuum Concentrator (Speedvac, Savant, Holbrook, NY, USA) (Tesfay et al., 2012). Dried samples were re-suspended in 2 mL of ultra-pure water, filtered through 0.45 μm nylon filters. The sugars were analysed according to Liu et al. (2002), using an HPLC equipped with a refractive index detector (RID-10 A, Shimadzu Corporation, Kyoto, Japan). The samples were analysed with an HPLC (LC- 20 AT, Shimadzu Corporation, Kyoto, Japan) and a Rezex RCM-monosaccharide column (300 mm x

7.8 mmm) (8 μm pore size; Phenomenex, Torrance, CA, USA). The concentration of each sugar (mg g^{-1}) was determined from a sugar standard calibration curve.

8.2.7. Statistical analysis

The collected data was subjected to analysis of variance by using Statistical Analysis System software (SAS) version 9.4 (Cary, NC, USA) to determine treatment mean effects. Differences between treatments were determined using Fisher's Least Significant Difference (LSD) at 5% level of significance.

8.3. Results

8.3.1. Visual symptoms of stress

Visual symptoms of stress were observed after 2d of exposure to the cold stress of 2.5^{o}C. Visual symptoms observed were leaf curling and leaf dropping (Figure 8.1).

Figure 8. 1 Effect of cold stress on citrus leaves after 2d of exposure to the temperature of 2.5^{o}C.

Si-treated Nules demonstrated a striking difference compared to the control trees of which most of the leaves were curled and brown (Figure 8.2).

Figure 8. 2 Effect of Si application on Nules clementine observed 21 days post-cold treatment of 2.5ºC. Left: treated with 1000 mg L^{-1} Si. Right: untreated control plant.

8.3.2. Effect of root Si-application on proline content under cold stress of 2.5ºC

Proline levels of citrus cultivars subjected to root Si-application under cold stress of 2.5ºC are shown in Table 8.1. Proline levels of the control trees were significantly higher (365.8 µg ml^{-1}) in non-stressed Nules trees than in trees exposed to cold stress of 2.5ºC for 2d, 4d, 8d, and 12 days after cold stress (DAC). Additionally, the lowest and highest proline contents were observed after 2d cold stress exposure and 21 DAC: 186.49 µg ml^{-1} and 514.96 µg ml^{-1}. In

trees treated with Si drenches, proline levels of trees 21 DAC was significantly higher (514.96 µg ml^{-1}) compared to the levels in trees exposed to other cold stress treatments in the following order: 4d, 2d, 0d, 8d and 12DAC.

Two trends emerged from the interaction between time of exposure to cold stress of 2.5ºC and Si treatments in Nules. Firstly, there was a significant reduction in proline content in trees treated with Si drenches (339.26 µg ml^{-1}) compared to the control trees (365.8 µg ml^{-1}) under non-stressed conditions. A significant reduction was observed in trees treated with Si drenches (184.43) compared to the control trees (309.89) 12d post-cold treatment. Secondly, a significant increase in proline content was observed in trees treated with Si drenches (361.84 µg ml^{-1}) compared to the control trees (186.49 µg ml^{-1}) after 2d of cold stress. Additionally, a significant increase in proline content was observed in trees treated with Si drenches (319.16 µg ml^{-1}) compared to the control trees (374.09 µg ml^{-1}) after 4d of cold stress. There was no significant difference at 21DAC in trees treated with Si drenches compare to the control trees.

In Nova, the proline content of the control trees was significantly higher (451.1 µg ml^{-1}) in non-stressed trees compared to those exposed to cold stress of 2.5ºC, in the order of exposure: 8d, 2d and 4d. Further significant reductions in proline levels were observed in post-stressed periods of 21 DAC (274.9 µg ml^{-1}) and 12 DAC (152.05 µg ml^{-1}), compared to the other cold stress treatments in the control trees.

In trees treated with Si drenches trees, significantly higher levels of proline (447.05 µg ml^{-1}) developed 12d post-cold treatment compared to other cold stress treatments in the following order: 0 d, 8d, 4d, 21DAC, and 2d time of exposure cold stress.

Two trends emerged in the interaction between time of exposure to cold stress of 2.5ºC and Si treatments in Nova. Firstly, a significant reduction in proline content in trees drenched with Si (422.38 µg ml^{-1}) compared to the control trees (451.1 µg ml^{-1}) under non-stressed

conditions. There was a significant reduction in proline in trees drenched with Si (288.96 µg ml^{-1}) compared to the level in the control trees (329.55 µg ml^{-1}) after 2d of cold stress. Additionally, a significant reduction was observed in trees drenched with Si (364.38 µg ml^{-1}) compared to the control trees (415.8 µg ml^{-1}) after 8d of cold stress. Secondly, a significant increase in proline content was observed in trees drenched with Si (331.29 µg ml^{-1}) compared to the control trees (296.5 µg ml^{-1}) after 4d of cold stress. There was a significant increase in proline levels in trees drenched with Si (447.05 µg ml^{-1}) compared to the control trees (152.05 µg ml^{-1}) after a post-stress period of 12 DAC. Additionally, a significant increase in proline content was observed in trees drenched with Si (319.38 µg ml^{-1}) compared to that in the control trees (274.9 µg ml^{-1}) after a post-stress period of 21 DAC.

The proline content of Nova was higher than in Nules prior to cold stress, irrespective of Si status. After cold stress, Nova proline content was more variable than Nules.

Table 8. 1. Proline content of citrus plants subjected to cold stress of 2.5°C and post cold treatments

		Proline (µg ml^{-1})	
Silicon (mg L^{-1})	**Time (d)**	**Nules**	**Nova**
0	0	365.80^{bc}	451.10^{a}
	2	186.49^{ij}	329.55^{g}
	4	319.16^{f}	296.50^{i}
	8	304.17^{gh}	415.80^{d}
	12 DAC	309.89^{fg}	152.05^{l}
	21 DAC	514.96^{a}	274.90^{k}
1000	0	339.26^{de}	422.38^{c}
	2	361.84^{c}	288.96^{j}
	4	374.09^{b}	331.29^{f}
	8	295.95^{h}	364.38^{e}
	12 DAC	184.43^{j}	447.05^{b}
	21 DAC	514.96^{a}	319.38^{h}
Summary of Analysis of Variance			
Interaction		Time*Silicon <0.001	Time*Silicon <0.001
LSD		11.298	1.746
CV%		10.2	7

Means of the same letter in the same column are not significantly different at *P*≤ 0.05. *DAC: days after exposure to cold treatment.

8.3.3. Effect of root Si-application on sugar content in Nules and Nova cultivars under cold temperature stress of 2.5 °C

Sugar levels of Nules and Nova trees in response to root Si-application and subjected to cold temperature stress of 2.5°C are shown in Tables 8.2-8.4. Fructose content was higher than sucrose and glucose contents, irrespective of cold and Si treatments. In Nules cultivar, Si

treated plants sucrose content was significantly higher in trees drenched with Si (0.212 mg ml^{-1}) compared to the control trees (0.140 mg ml^{-1}) after 2d of cold stress in Table 8.2. A significant increase in sucrose in trees drenched with Si (0.170 mg ml^{-1}) compared to the control trees (0.136 mg ml^{-1}) after 4d of cold stress. Moreover, a significant increase in sucrose content was observed in trees drenched with Si (0.195 mg ml^{-1}) compared to the control trees (0.163 mg ml^{-1}) after 8d of cold stress. In post-cold treatment of 21DAC a significant increase in sucrose content was observed in trees drenched with Si (0.157 mg ml^{-1}) compared to the control trees (0.133 mg ml^{-1}).

The levels of sugars of Nova trees treated with Si drenches and subjected to cold temperature stress of 2.5^{o}C are shown in Table 8.2. A significant increase in sucrose in trees drenched with Si (0.157 mg ml^{-1}) compared to the control trees (0.135 mg ml^{-1}) after 2d of cold stress. A significant increase in sucrose in trees drenched with Si (0.186 mg ml^{-1}) compared to the control trees (0.148 mg ml^{-1}) after 4d of cold stress. Moreover, a significant increase in sucrose content was observed in trees drenched with Si (0.197 mg ml^{-1}) compared to the control trees (0.157 mg ml^{-1}) after 8d of cold stress. In post-cold treatment of 12DAC a significant increase in sucrose content was observed in trees drenched with Si (0.182 mg ml^{-1}) compared to the control trees (0.124 mg ml^{-1}). Similarly, at 21DAC a significant increase in sucrose content was observed in trees drenched with Si (0.188 mg ml^{-1}) compared to the control trees (0.129 mg ml^{-1}).

Table 8. 2. Sucrose content (mg ml^{-1}) in Nules and Nova trees drenched with Si and subjected to cold stress of 2.5ºC and post cold treatments

		Sucrose (µg ml^{-1})	
Silicon (mg L^{-1})	**Time (d)**	**Nules**	**Nova**
0	0	0.165^{cde}	0.168^{bcde}
	2	0.140^{defgh}	0.135^{fghi}
	4	0.136^{fghi}	0.148^{efg}
	8	0.163^{cdef}	0.157^{defg}
	12 DAC	0.186^{bc}	0.124^{i}
	21 DAC	0.133^{i}	0.129^{ghi}
1000	0	0.171^{cd}	0.136^{fghi}
	2	0.212^{a}	0.157^{defg}
	4	0.170^{cd}	0.186^{bc}
	8	0.195^{b}	0.197^{b}
	12 DAC	0.157^{defg}	0.182^{bc}
	21 DAC	0.157^{defg}	0.188^{b}
Summary of Analysis of Variance		P value	LSD
Silicon		0.023	0.02299
CV%		8.4	

Means of the same letter in the same column are not significantly different at P ≤ 0.05. *DAC: days after exposure to cold treatment.

With respect to glucose content, no significant differences were observed with respect to cultivars, cold stress, and Si treatment in Table 8.3.

Table 8. 3. Glucose content (mg ml^{-1}) in Nules and Nova trees drenched with Si trees after cold stress of 2.5ºC and post cold treatments

		Glucose (µg ml^{-1})	
Silicon (mg L^{-1})	**Time (d)**	**Nules**	**Nova**
0	0	0.157	0.1165
	2	0.165	0.1301
	4	0.114	0.129
	8	0.176	0.134
	12 DAC	0.206	0.183
	21 DAC	0.160	0.165
1000	0	0.136	0.124
	2	0.207	0.153
	4	0.175	0.144
	8	0.200	0.188
	12 DAC	0.179	0.196
	21 DAC	0.150	0.189
Summary of Analysis of Variance		P value	LSD
Cultivar		0.241	
Si		0.159	
Time		0.079	
CV%		6.3	

Means of the same letter in the same column are not significantly different at *P*≤ 0.05. *DAC: days after exposure to cold treatment.

With respect to fructose content, Nules was significantly reduced following cold stress in both treated and control Si treatments in Table 8.4.

However, in Si treated plants there was a significant increase in fructose content at 12DAC (0.347 mg ml^{-1}) compared to the Si control (0.278 mg ml^{-1}). This recovery in fructose content was lost at 21 DAC. Additionally, Nules had a significantly higher fructose content compared to Nova under optimum and cold stress conditions.

In Nova, Si control at 21 DAC demonstrated a significantly high fructose content compared to cold stress treatments (2,4, 8 and 12 DAC).

About Si treated trees, there was a significantly higher fructose content in post-stress treatments (12 and 21 DAC) compared to cold stress treatments (0, 2, 4 and 8d). Additionally, Si treated plants showed a significant increase in fructose content at 12DAC (0.293 mg ml^{-1}) compared to the cold stress (0.272 mg ml^{-1}).

Table 8. 4. Fructose content (mg ml^{-1}) in Nules and Nova trees drenched with Si trees after cold stress of 2.5ºC and post cold treatments

		Fructose (µg ml^{-1})	
Silicon (mg L^{-1})	**Time (d)**	**Nules**	**Nova**
0	0	0.385^{a}	0.319^{abc}
	2	0.290^{bcd}	0.232^{d}
	4	0.282^{bcd}	0.129^{f}
	8	0.266^{cd}	0.298^{bcd}
	12 DAC	0.278^{cd}	0.272^{cd}
	21 DAC	0.283^{bcd}	0.390^{a}
1000	0	0.373^{ab}	0.163^{ef}
	2	0.252^{cd}	0.138^{f}
	4	0.246^{d}	0.183^{e}
	8	0.257^{cd}	0.136^{f}
	12 DAC	0.347^{abc}	0.293^{bcd}
	21 DAC	0.248^{d}	0.286^{bcd}
Summary of Analysis of Variance Interaction		P value	LSD
Cultivar		0.006	0.03889
Silicon		0.036	0.03889
Time		0.011	0.06736
Cultivar *Time		0.045	0.09526
CV%		6	

Means of the same letter in the same column are not significantly different at *P*≤ 0.05. *DAC: days after exposure to cold treatment.

8.4. Discussion

Proline accumulation is a physiological response of plants under stress conditions (Verbruggen & Hermans, 2008, Renata *et al.*, 2017, Verma *et al.*, 2018). A significant increase in proline content was observed in trees drenched with Si after 2d, 4d and 8d of exposure to cold stress compared to the control in both cultivars. This implies that Si application under cold stress conditions facilitated an increase in proline content. This concurred with a previous study that demonstrated an increase in proline content in cold stressed plants subjected to Si treatment (Renata *et al.*, 2017). Meanwhile, the reduction in proline accumulation observed in Nules subjected to non-stressed and at 12d post-cold treatment could be explained by a lower stimuli for proline accumulation in normal and post-stressed conditions due to the reduction in protein breakdown (Verbruggen & Hermans, 2008). The increase in proline content observed in trees drenched with Si at 21d post-cold treatment compare to other chilling treatments was due to stimuli for proline accumulation. This was linked to the observed reduced symptomatic stress of trees drenched with Si compared to the control trees after 21d post-cold (Figure 8.2). The significantly higher proline content observed at 21 DAC in both Si root-treated and control trees suggested the strong involvement of the chilling adaptation process in Nules (Yuanyuan *et al.*, 2009).

Regarding control trees of Nova, the proline content was reduced by cold stress and subsequent post-cold treatments (12 and 21 DAC). In comparison, there was a significant increase in proline content because of Si application after cold stress of 4d and 8d, which could be because the plant underwent shock after 2d and started acclimatizing during prolonged stresses. This agreed with prior findings of increases in proline content in cold stressed plants (Renata *et al.*, 2017). In contrast, several previous studies conducted on plants exposed to cold stress and water deficit conditions, Si application resulted in reductions of the levels of proline (Kidane, 2008, Mauad *et al.*, 2016, Dehghanipoodeh *et al.*, 2018). The

acclimatizing effect observed in post-cold treatments (12 and 21 DAC) suggested the continuation in chilling adaptation process (Yuanyuan *et al.*, 2009). The significantly higher and variable proline content observed in Nova compared to Nules was linked to the greater sensitivity of Nova to cold stress than Nules (Verma *et al.*, 2018). Moreover, in Nova trees drenched with Si proline content was reduced significantly at 21 days post-cold stress, whilst in Nules the proline content was significantly increased, a further argument that Nules is colder tolerant than Nova.

Sugars play an important function in membrane stabilization during cold stress (Kidane, 2008, Horacio & Martinez-Noel, 2013). In the current study, an increase in sucrose content was observed in both cultivars drenched with Si and subjected to cold stress, and 21 days post cold stress, compared to the control trees. This corroborates with previous studies that demonstrated an increase in sucrose content in response to Si treatment of plants exposed to abiotic stress (Kidane, 2008, Abdel-Latif & El-Demerdash, 2017). The increased levels of sucrose in cold stressed plants also confirmed their involvement in tolerance to cold stress. This corroborated with a previous study conducted in Valencia orange leaves in which carbohydrate accumulation increased with colder temperatures (Yelenosky & Guy, 1977). This was further substantiated in a study conducted on cabbage that demonstrated increased freezing tolerance during exposure to cold stress. Sucrose content did not increase in trees treated with Si drenches subjected to normal conditions. This agreed with a previous study that reported Si effects are mostly detected in plants grown under abiotic and biotic stress conditions (Li *et al.*, 2007, Perez *et al.*, 2014, Mauad *et al.*, 2016).

Fructose is an important carbohydrate involved in photosynthetic production and cold adaptation (Levitt, 1980, Yuanyuan *et al.*, 2009, Li *et al.*, 2018). In Si treated trees, the increase in fructose content observed in both cultivars during post-cold treatment implied that the plants were acclimatizing to cold stress effect by slowing down their metabolisms

(Sasaki *et al.*, 1996, Kidane, 2008). However, this increase could also be linked to increased senescing in leaves (Yuanyuan *et al.*, 2009).

Silicon treatment did not result in increased levels of glucose content in either cultivars, irrespective of cold stress regime, indicating that this sugar plays no role in cold stress acclimatization linked to Si applications in citrus.

The increased sucrose and fructose contents in cold stressed citrus plants subjected to Si treatment implies that these citrus leaves would have acquired membrane strength, which helps to preserve membrane fluidity and enables the leaves to slowly acclimatize to cold stress (Ma & Yamaji, 2006, Horacio & Martinez-Noel, 2013).

Conclusions

The present study evaluated the impact of cold stress on two citrus cultivars subjected to root-Si treatment, by measuring the levels of proline and three sugars. Proline, sucrose, and fructose levels were positively influenced by Si applications. Additionally, a chilling adaptation mechanism was evident in post-cold treatment. Therefore, this study provided evidence that Si root drenching improved cold stress tolerance in citrus. However, the stress tolerance mechanism of citrus cannot be understood only by the response of proline and sugar under stressed conditions. Many other physiological adaptation mechanisms are involved to enable citrus to survival cold stress conditions.

CHAPTER 9

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Cold stress is categorised as injury that takes place when plants are exposed to temperature above $0^{o}C$ (Levitt, 1980, Esmaili & Salehi, 2012). This injury often results in photosynthetic activity reduction, cellular membrane damage and oxidative stress in plants (Suzuki & Mittler, 2006). However, plants have evolved adaptation mechanisms to chilling and freezing stresses, partly based on producing cryoprotective and osmoprotective compounds such as soluble sugars. Their high accumulation may be explained by the reduction in carbohydrate utilization for photosynthesis (Horacio & Martinez-Noel, 2013). Another important function of sugars is the prevention of crystal formation in plants under cold stress, which leads to the protection of membrane phospholipids (Levitt, 1980). Another adaptation to cold induced stress is the increased levels of cytosol compatible solutes such as proline, which serves as an osmotic buffer, and provides protection against scavenging ROS in plants (Alscher *et al.*, 2002, Xiong & Zhu, 2002).

Global climate changes may result in increased incidences of spring frost (Habibi, 2016). Low spring temperatures could result in a major reduction in citrus yield and quality (Xin & Browse, 2000, Ladaniya, 2008). Barthel *et al.* (2014) observed that a sudden decrease in temperature from 25 to $10^{o}C$ delayed plant carbon transport and increased carbon lost via respiration process in preference to involvement in growth and storage.

Under cold stress, a reduction in stomatal conductance has been reported in citrus (Ribeiro & Machado, 2007), which may impair photosynthetic activity by decreasing CO_2 availability to Rubisco (Machado *et al.*, 2002, Medina *et al.*, 2002). Cold stress is also known to increase proline activity in citrus, which protects membrane integrity by reducing water loss from the cell (Kushad & Yelenosky, 1987, Krasensky & Jonak, 2012, Habibi, 2015, 2016). This

consequently affects flower induction, resulting in reduced fruit yield and quality (Ribeiro & Machado, 2007), and therefore revenues (Ladaniya, 2008).

Despite being ubiquitous in nature, Si is not classified as an essential element in higher plants (Epstein, 1999). Silicon uptake is known to impact positively on plant yield and quality (Matichenkov *et al.*, 2001, Henriet *et al.*, 2006, Barker & Pilbeam, 2015, Tubana *et al.*, 2016, Dorairaj *et al.*, 2017). The beneficial effects of Si uptake are more evident in plants growing under abiotic and biotic stress conditions such as drought, cold stress, metal toxicity, nutrient imbalance, diseases and pests (Ma, 2004, Gong *et al.*, 2005, Liang *et al.*, 2008, Liu *et al.*, 2009, Zhu & Gong, 2014, Liang *et al.*, 2015, Debona *et al.*, 2017).

In the conclusion, important findings of this study and the future perspective are presented.

Optimization of Si quantification in horticulture crops

Quantifying Si accumulated in plant tissue remains the most common approach for routine monitoring of the Si status of crops (Kraska & Breitenbeck, 2010). Many extraction methods have been developed for Si extraction from plant material such as gravimetric, autoclave induced digestion (AID), HCl-HF digestion and microwave assisted digestion using nitric acid, hydrogen peroxide and sodium hydroxide (Van der Vorm, 1987, Elliott & Snyder, 1991, Haysom & Ostatek-Boczynski, 2006). However, some of these extraction procedures are not reliable for horticultural crops due to their small Si content. There is limited information on the validation of these methods for many important horticultural crops. Method validation is important due to the contrasting theories pertaining to plant Si quantification linked to the extractant used, and that the extraction and analytical methods used impact on the levels of extractable Si. The aim of this experiment was to validate silicon measurement by means of inductively coupled plasma optical emission spectrometry (ICP-OES) against the conventional spectrophotometer method of analysis. The ICP-OES analysis method proved to

be a more sensitive and rapid method for Si analysis in horticulture crops. Results from this work confirmed that the removal of foliar bound Si prior to extraction procedure reduced surface bound Si. The ICP-OES analysis agreed with the conventional spectrophotometer (colorimetric analysis) method rate. However, the ICP-OES analysis showed a better detection limit, which was 10 times lower than the lowest Si concentration in the leaf tissues, implying the method is more sensitive, precise, and faster than the colorimetric analysis. This suggests that the ICP-OES with microwave assisted digestion could be used for routine analysis of lower chemical element concentration in plant nutrition studies.

Si uptake and dry matter production in citrus

The influence of Si uptake in abiotic stress has been reported in several studies (Ma *et al.*, 2004, Zhu *et al.*, 2006, Liang *et al.*, 2007, Zhu & Gong, 2014, Liang *et al.*, 2015). Extensive research on Si uptake and influence in abiotic stress have been conducted in monocotyledons (Rafi *et al.*, 1997, Ma *et al.*, 2011). Therefore, to establish the impact of Si in citrus under cold stress it was important to determine the factors that influence Si uptake in citrus and the maximum absorption rate. This study demonstrated that Si uptake in citrus increased with the duration of application, leaf age and concentration applied, with 1000 mg L^{-1} as the optimum concentration. Root drenching of trees with Si was the most consistent way to apply Si. The season influenced the uptake of Si, with more Si accumulating in summer months than in winter. Dry matter production was positively influenced by Si application. This implies that, specific aspects of growth or development may be positively affected when the Si is supplied in large amounts. This study provides a basis for application of Si fertilizer in citrus production.

Silicon deposition and localization in citrus

There are two general mechanisms for Si uptake and transport (active and passive uptake) co-existing in a plant, with their relative contribution being dependent much upon the plant species, external Si concentration and transporters (Liang *et al.*, 2005, Ma & Yamaji, 2006, Ma *et al.*, 2011, Mitani *et al.*, 2011, Yamaji *et al.*, 2012). In dicots, the coexistence of both passive and active transport mechanisms is dependent upon the external Si supply as demonstrated in cucumber (Liang *et al.*, 2005). In another study, a passive uptake mechanism was observed in cucumber and tomato, based on their roots' ability, and their inefficient radial transporters that enable them to take up less Si than rice due to transporter for xylem loading in the latter (Mitani & Ma, 2005). Silicon deposition was found in the upper and lower epidermis of most leaves as silica bodies that eventually constituted a cuticle-silica double layer (Kaufman *et al.*, 1985, Motomura *et al.*, 2000, Ma & Takahashi, 2002, Richmond & Sussman, 2003, Motomura *et al.*, 2006). Moreover, Si deposition was found around the cell wall regions, leaf blades and inflorescence bracts tissues (Motomura *et al.*, 2004, de Melo *et al.*, 2010).

Once we determined the uptake factors in citrus the area of deposition and localization was examined, to understand the uptake mechanism. Through electron microscopy studies we demonstrated that Si accumulated in upper epidermal cell regions and constituted a double Si layer. Si distribution in citrus was in order of young leaves<mature leaves<roots. The high Si content observed in roots highlights the need to reclassify uptake mode based on Si content in all plant organs. The lack of Si accumulation in the fruits in this study is explained by the low temperature observed during flower induction and subsequent fruit set, this demonstrates the involvement of an active uptake depending on the prevailing temperature conditions.

There has been limited research into transporters in dicots with focus mainly on the *Cucurbitaceae, Urticaceae,* and *Asteraceae* families (Hodson *et al.*, 2005, Mitani *et al.*,

2011). It is crucial for future studies in dicots to investigate transport mechanisms in order to manipulate the transporters by introducing an appropriate mutant that will improve the Si uptake ability in the existing cultivars to enhance tolerance of abiotic and biotic stress.

Effects of silicon on cold stress, and fruit quality and yield

There have been several reports on the effect of Si on cold stress on graminaceous crops through various mechanisms (Gong *et al.*, 2005, Zhu *et al.*, 2006, Kidane, 2008, Liang *et al.*, 2008, Liang *et al.*, 2015). Despite being inefficient in taking up large amounts of Si from soil solution, and translocating this element to shoots, some dicots species are able to benefit from Si application (Matichenkov *et al.*, 1999, Matichenkov *et al.*, 2001, Hattori *et al.*, 2008, Marodin *et al.*, 2014). However, there are few reports of the effects of Si fertilization on photosynthesis, sugars and proline content in dicotyledonous species (Hattori *et al.*, 2008, Kidane, 2008, Zhang *et al.*, 2018). There are several recognized mechanisms involved in stress alleviation during photosynthesis. The role of sugars and proline accumulation in stress tolerance is also well recognized. Plants treated with Si, and subsequently exposed to cold stress develop enhanced levels of proline and osmoregulatory sugars, which balance osmosis, and protect membrane integrity (Krasensky & Jonak, 2012, Habibi, 2015, 2016). Relatively normal water content and plant growth are therefore preserved.

In this study, the underlying mechanisms of Si in cold stress alleviation are the improvement in osmotic balance provided by the sugar content (sucrose and fructose), and an enhanced proline content, which improves membrane rigidity during stress conditions. The improvement in photochemical efficiency and water use efficiency because of Si applications have been confirmed in both citrus cultivars. The photosynthetic rate improved mainly in Nules, suggesting the existence of a specific physiological and biochemical response under stress conditions in each citrus species. The increased water use efficiency in cold stressed

citrus subjected to Si fertilization may have been due, in part, to the increased proline content, which improves the osmoregulation of plants. Meanwhile a reduction in transpiration and stomatal conductance observed may be due to the deposition of Si on the epidermal surface, which serves as a barrier against water loss and strengthens the cell wall region. It should be noted that many other physiological adaptations must take place to allow citrus to survive cold stress. Fully integrated stress tolerance mechanism cannot be fully understood by only looking at physiological parameters. Future studies on the use of untargeted transcriptomics and metabolomics will more likely provide further details into the mechanisms of stress alleviation.

The observed improvement in photosynthetic rate and water use efficiency of trees exposed to cold stress conditions after Si fertilization in greenhouse did not correlate with fruit yield and quality in the harvest following frost stress that occurred during flowering induction stage. This suggests that the field trials may have been confounded by interactions between crop growth and the prevailing environment. Additionally, citrus crop quality and yield may have been limited under cold stressed conditions due the reduction in stomatal conductance. This study established an understanding of the physiological responses of citrus directly impacted at flower induction.

Silicon application improvement in chilling stressed plants tolerance has also been demonstrated through an increase in antioxidant activities and reduction in free radicals in maize (Habibi, 2016, Moradtalab et al., 2018); cucumber (Liu et al., 2009); Banana (Kidane, 2008). Therefore, future studies could investigate the antioxidant response in citrus subjected to chilling stress and abiotic stress conditions in general.

In conclusion, considering the research findings on the active uptake of Si and its involvement in the improvement in dry matter production and cold stress tolerance which

caused an improvement in the physiological and biochemical activities in citrus. For the citrus industry and citrus farmers, the incorporation of Si at the optimum application of 1000 mg L^{-1} through root drenching from the nursery stage to the field stage could be considered as an effective integrated fertilizer and abiotic stress management strategy. The improvement in WUE of cold stressed citrus trees subjected to Si fertilization, and the reduction in WUE as a primary outcome during heat and drought stress. These results present many opportunities for further research into the impact of Si fertilisation on citrus being grown in cold, heat and drought prone regions.

References

ABADI, F.G., MOSTAFAVI, M., EBOUTALEBI, A., SAMAVAT, S. & EBADI, A. 2010. Biomass accumulation and proline content of six citrus rootstocks as influenced by long-term salinity. *Research Journal of Agricultural Sciences* 4: 158-165.

ABD-ALKARIM, E., BAYOUMI, Y., METWALLY, E. & RAKHA, M. 2017. Silicon supplements affect yield and fruit quality of cucumber (*Cucumis sativus L*.) grown in net houses. *African Journal of Agriculture Research* 12: 2518-2523.

ABDEL-LATIF, A. & EL-DEMERDASH, F.M. 2017. The Ameliorative Effects of Silicon on Salt-Stressed Sorghum Seedlings and Its Influence on the Activities of Sucrose Synthase and PEP Carboxylase. *Journal of Plant Physiology and Pathology* 5 (2):1-8.

ABRAHAM, A.O. 2010. Integrated use of yeast, hot water and potassium silicate treatments for the control of postharvest green mould of citrus and litchi. Kwazulu-Natal, Pietermaritzburg, doctoral dissertation.

ADAMS, W.W. & DEMMIG-ADAMS, B. 1994. Carotenoid composition and down regulation of photosystem II in three conifer species during the winter. *Physiologia Plantarum* 92: 451-458.

ADATIA, M. & BESFORD, R. 1986. The effects of silicon on cucumber plants grown in recirculating nutrient solution. *Annals of Botany* 58: 343-351.

AHMAD, R., ZAHEER, S.H. & ISMAIL, S. 1992. Role of silicon in salt tolerance of wheat (*Triticum aestivum L.*). *Plant Science* 85: 43-50.

AL-AGHABARY, K., ZHU, Z. & SHI, Q. 2005. Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. *Journal of Plant Physiology* 27: 2101-2115.

AL-GHANNAM, S. & AL-OLYAN, A. 2008. Spectrofluorometric determination of nicardipine, nifedipine and isradipine in pharmaceutical preparations and biological fluids. *Open Chemistry* 6: 222-228.

ALLEN, D.J. & ORT, D.R. 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science* 6: 36-42.

ALSCHER, R.G., ERTURK, N. & HEATH, L.S. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany* 53: 1331-1341.

ANJUM, S.A., TANVEER, M., HUSSAIN, S., ASHRAF, U., KHAN, I. & WANG, L. 2017. Alteration in growth, leaf gas exchange, and photosynthetic pigments of maize plants under combined cadmium and arsenic stress. *Water Air Soil Pollution* 228: 13.

ARNON, D.I. & STOUT, P. 1939. The essentiality of certain elements in minute quantity for plants with special reference to copper. *Plant Physiology* 14: 371.

ARPAIA, M.L. 1994. Preharvest factors influencing postharvest quality of tropical and subtropical fruit. *HortScience* 29: 982-985.

ARSENAULT-LABRECQUE, G., MENZIES, J.G. & BÉLANGER, R.R. 2012. Effect of silicon absorption on soybean resistance to *Phakopsora pachyrhizi* in different cultivars. *Plant disease* 96: 37-42.

ASHRAF, M. & FOOLAD, M. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental Experimental Botany* 59: 206-216.

AUCIQUE-PÉREZ, C.E., DE MENEZES SILVA, P.E., MOREIRA, W.R., DAMATTA, F.M. & RODRIGUES, F.Á. 2017. Photosynthesis impairments and excitation energy dissipation on wheat plants supplied with silicon and infected with Pyricularia oryzae. *Plant Physiology and Biochemistry* 121: 196-205.

AZEEM, S., LI, Z., ZHENG, H., LIN, W., ARAFAT, Y., ZHANG, Z., LIN, X. & LIN, W. 2016. Quantitative proteomics study on Lsi1 in regulation of rice (*Oryza sativa L*.) cold resistance. *Plant growth regulation* 78: 307-323.

BAKER, N. 1996. Photosynthesis and the Environment. Dordrecht: Kluwer. Academic.

BAKER, N.R. & ROSENQVIST, E. 2004. Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany* 55: 1607-1621.

BAKER, N.R. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Review of Plant Biology* 59: 89-113.

BARKER, A.V. & PILBEAM, D.J. 2015. Handbook of Plant Nutrition. 2nd ed. CRC press.
BARTHEL, M., CIERAAD, E., ZAKHAROVA, A. & HUNT, J. 2014. Sudden cold temperature delays plant carbon transport and shifts allocation from growth to respiratory demand. *Biogeosciences* 11: 9.
BIRCHALL, J. 1995. The essentiality of silicon in biology. *Chemical Society Reviews* 24: 351-357.
BUCK, G.B., KORNDÖRFER, G.H., NOLLA, A. & COELHO, L. 2008. Potassium silicate as foliar spray and rice blast control. *Journal of Plant Nutrition* 31: 231-237.
CASEY, W., KINRADE, S., KNIGHT, C., RAINS, D. & EPSTEIN, E. 2004. Aqueous silicate complexes in wheat, Triticum aestivum L. *Plant, Cell and Environment* 27: 51-54.
CHEN, D., WANG, S., YIN, L. & DENG, X. 2018. How does silicon mediate plant water uptake and loss under water deficiency? *Frontiers in Plant Science* 9: 281.
CHIBA, Y., MITANI, N., YAMAJI, N. & MA, J.F. 2009. HvLsi1 is a silicon influx transporter in barley. *Plant Journal* 57: 810-818.
CORADIN, T. & LOPEZ, P.J. 2003. Biogenic silica patterning: simple chemistry or subtle biology? *ChemBioChem* 4: 251-259.
CROOKS, R. & PRENTICE, P. 2017. Extensive investigation into field based responses to a silica fertiliser. *Silicon* 9: 301-304.
CRUSCIOL, C.A.C., DE ARRUDA, D.P., FERNANDES, A.M., ANTONANGELO, J.A., ALLEONI, L.R.F., FERNANDES, D.M. & MCCRAY, J.M. 2018. Evaluation of soil extractants for silicon availability for sugarcane. *Journal of Plant Nutrition* 41: 2241-2255.
CUONG, T.X., ULLAH, H., DATTA, A. & HANH, T.C. 2017. Effects of silicon-based fertilizer on growth, yield and nutrient uptake of rice in tropical zone of Vietnam. *Rice Science* 24: 283-290.
DA CUNHA, K.P.V. & DO NASCIMENTO, C.W.A. 2009. Silicon effects on metal tolerance and structural changes in maize (*Zea mays L.*) grown on a cadmium and zinc enriched soil. *Water Air Soil Pollution* 197: 323.
DA CUNHA, P.V., KARINA, WILLIAMS ARAÚJO DO NASCIMENTO, C. & JOSÉ DA SILVA, A. 2008. Silicon alleviates the toxicity of cadmium and zinc for maize (*Zea mays L.*) grown on a contaminated soil. *Journal of Plant Nutrition and Soil Science* 171: 849-853.
DATNOFF, L.E., SNYDER, G.H. & KORNDÖRFER, G.H. 2001. Silicon in agriculture, Elsevier.
DE MELO, S.P., MONTEIRO, F.A. & DE BONA, F.D. 2010. Silicon distribution and accumulation in shoot tissue of the tropical forage grass (*Brachiaria brizantha*). *Plant and Soil* 336: 241-249.
DE VILLIERS, E.A. & JOUBERT, P.H. 2006. The Cultivation of Citrus. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa.
DEBONA, D., RODRIGUES, F.Á., RIOS, J.A., MARTINS, S.C.V., PEREIRA, L.F. & DAMATTA, F.M. 2014. Limitations to photosynthesis in leaves of wheat plants infected by *Pyricularia oryzae*. *Phytopathology* 104: 34-39.
DEBONA, D., RODRIGUES, F.A. & DATNOFF, L.E. 2017. Silicon's role in abiotic and biotic plant stresses. *Annual Review of Phytopathology* 55: 85-107.
DEHGHANIPOODEH, S., GHOBADI, C., BANINASAB, B., GHEYSARI, M. & SHIRANIBIDABADI, S. 2018. Effect of Silicon on Growth and Development of Strawberry under Water Deficit Conditions. *Horticultural Plant Journal* 4: 226-232.
DEMMIG-ADAMS, B. & ADAMS III, W.W. 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in Plant Science* 1: 21-26.
DEMMIG-ADAMS, B. & ADAMS III, W.W. 2006. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytologist* 172: 11-21.
DETMANN, K.C., ARAÚJO, W.L., MARTINS, S.C., SANGLARD, L.M., REIS, J.V., DETMANN, E., RODRIGUES, F.Á., NUNES-NESI, A., FERNIE, A.R. & DAMATTA, F.M. 2012. Silicon nutrition increases grain yield, which, in turn, exerts a feed-forward stimulation of photosynthetic rates via enhanced mesophyll conductance and alters primary metabolism in rice. *New Phytologist* 196: 752-762.
DETMANN, K.C., ARAÚJO, W.L., MARTINS, S.C.V., FERNIE, A.R. & DAMATTA, F.M. 2013. Metabolic alterations triggered by silicon nutrition. *Plant signaling and behavior* 22523: 71.

DO NASCIMENTO, C.W.A., DE SOUZA NUNES, G.H., PRESTON, H.A.F., DA SILVA, F.B.V., PRESTON, W. & LOUREIRO, F.L.C. 2019. Influence of Silicon Fertilization on Nutrient Accumulation, Yield and Fruit Quality of Melon Grown in North eastern Brazil. *Silicon*: 1-7.

DORAIRAJ, D., ISMAIL, M.R., SINNIAH, U.R. & KAR BAN, T. 2017. Influence of silicon on growth, yield, and lodging resistance of MR219, a lowland rice of Malaysia. *Journal of Plant Nutrition* 40: 1111-1124.

ELAWAD, S., STREET, J. & GASCHO, G. 1982. Response of Sugarcane to Silicate Source and Rate. II. Leaf Freckling and Nutrient Content 1. *Agronomy Journal* 74: 484-487.

ELLIOTT, C. & SNYDER, G.H. 1991. Autoclave-induced digestion for the colorimetric determination of silicon in rice straw. *J. Agric. Food. Chem* 39: 1118-1119.

EMICH, F., SCHNEIDER, F.L. & FEIGL, F. 1932. Microchemical laboratory manual.

ENEJI, A.E., INANAGA, S., MURANAKA, S., LI, J., HATTORI, T., AN, P. & TSUJI, W. 2008. Growth and nutrient use in four grasses under drought stress as mediated by silicon fertilizers. *Journal of Plant Nutrition* 31: 355-365.

EPSTEIN, E. 1994. The anomaly of silicon in plant biology. *Proceedings of National Academy of Sciences* 91: 11-17.

EPSTEIN, E. 1999. Silicon. *Annual Review of Plant Biology* 50: 641-664.

EPSTEIN, E. & BLOOM, A. 2005. Mineral nutrition of plants: principles and perspectives 2nd ed. Sinauer Associates, Oxford Uiversity.

EPSTEIN, E. 2009. Silicon: its manifold roles in plants. *Annals of Applied Biology* 155: 155-160.

ESMAILI, S. & SALEHI, H. 2012. Effects of temperature and photoperiod on postponing bermudagrass (Cynodon dactylon [L.] Pers.) turf dormancy. *Journal of Plant Physiology* 169: 851-858.

EVERT, R.F. & EICHHORN, S.E. 2013. Raven Biology of Plants. 8th ed. Peter Marshall, United States of America.

EXPORTERS, F.P. 2019. Growing in harmony with nature. *Fresh Produce Exporters Forum*: 156.

FAO. 2015. Citrus fruit statistics 2015. FAO, Rome.

FAOSTAT. 2015. http://fao.org/faostat/es/#data/QC.

FARQUHAR, G.D. & SHARKEY, T.D. 1982. Stomatal Conductance and Photosynthesis. *Annual Review of Plant Physiology* 33: 317-345.

FAWE, A., ABOU-ZAID, M., MENZIES, J. & BÉLANGER, R. 1998. Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. *Phytopathology* 88: 396-401.

FLEXAS, J., ESCALONA, J.M., EVAIN, S., GULÍAS, J., MOYA, I., OSMOND, C.B. & MEDRANO, H. 2002. Steady-state chlorophyll fluorescence (Fs) measurements as a tool to follow variations of net CO_2 assimilation and stomatal conductance during water-stress in C3 plants. *Physiologia Plantarum* 114: 231-240.

FOX, R.L., SILVA, J.A., PLUCKNETT, D.L. & TERANISHI, D.Y. 1969. Soluble and total silicon in sugar cane. *Plant and Soil* 30: 81-92.

FRANTZ, J.M., LOCKE, J.C., DATNOFF, L., OMER, M., WIDRIG, A., STURTZ, D., HORST, L. & KRAUSE, C.R. 2008. Detection, distribution, and quantification of silicon in floricultural crops utilizing three distinct analytical methods. *Communication of Soil Science and Plant Analysis* 39: 2734-2751.

FRENKEL, A.W. 1995. Photosynthetic phosphorylation. *Photosynthesis Research* 46: 73-77.

FROES, R.E., NETO, W.B., NAVEIRA, R.L., SILVA, N.C., NASCENTES, C.C. & DA SILVA, J.B. 2009. Exploratory analysis and inductively coupled plasma optical emission spectrometry (ICP-OES) applied in the determination of metals in soft drinks. *Microchemical Journal* 92: 68-72.

FRYER, M.J., ANDREWS, J.R., OXBOROUGH, K., BLOWERS, D.A. & BAKER, N.R. 1998. Relationship between CO_2 assimilation, photosynthetic electron transport, and active O_2 metabolism in leaves of maize in the field during periods of low temperature. *Plant Physiology* 116: 571-580.

FSSA-MVSA. 2007. fertiliser handbook of south africa. 6th ed. The Fertilizer Society of South Africa, Lynnwood Ridge, South Africa.

FU, J. & HUANG, B. 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. *Environmental Experiment of Botany* 45: 105-114.

GAO, X., ZOU, C., WANG, L. & ZHANG, F. 2005. Silicon improves water use efficiency in maize plants. *Journal of Plant Nutrition* 27: 1457-1470.

GAO, X., ZOU, C., WANG, L. & ZHANG, F. 2006. Silicon decreases transpiration rate and conductance from stomata of maize plants. *Journal of Plant Nutrition* 29: 1637-1647.

GENTY, B., BRIANTAIS, J.-M. & BAKER, N.R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA)-General Subjects* 990: 87-92.

GEORGE, S., STEINBERG, S. & HODGE, V. 2000. The concentration, apparent molecular weight and chemical reactivity of silica from groundwater in Southern Nevada. *Chemosphere* 40: 57-63.

GILMORE, A.M. & YAMAMOTO, H.Y. 1992. Dark induction of zeaxanthin-dependent nonphotochemical fluorescence quenching mediated by ATP. *Proceedings of National Academy of Science* 89: 1899-1903.

GONG, H., CHEN, K.M., CHEN, G.C., WANG, S.M. & ZHANG, C.L. 2003. Effects of silicon on growth of wheat under drought. *Journal of Plant Nutrition* 26: 1055-1063.

GONG, H., ZHU, X., CHEN, K., WANG, S. & ZHANG, C. 2005. Silicon alleviates oxidative damage of wheat plants in pots under drought. *Plant Science* 169: 313-321.

GONG, H. & CHEN, K. 2012. The regulatory role of silicon on water relations, photosynthetic gas exchange, and carboxylation activities of wheat leaves in field drought conditions. *Acta Physiologiae Plantarum* 34: 1589-1594.

GONZÁLEZ, A.G. & HERRADOR, M.Á. 2007. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends in Analytical Chemistry* 26: 227-238.

GUNES, A., INAL, A., BAGCI, E., COBAN, S. & PILBEAM, D. 2007. Silicon mediates changes to some physiological and enzymatic parameters symptomatic for oxidative stress in spinach *(Spinacia oleracea L*.) grown under B toxicity. *Scientia Horticulturae* 113: 113-119.

GUNES, A., INAL, A., BAGCI, E.G. & PILBEAM, D.J. 2007. Silicon-mediated changes of some physiological and enzymatic parameters symptomatic for oxidative stress in spinach and tomato grown in sodic-B toxic soil. *Plant and Soil* 290: 103-114.

GUNES, A., KADIOGLU, Y.K., PILBEAM, D.J., INAL, A., COBAN, S. & AKSU, A. 2008. Influence of silicon on sunflower cultivars under drought stress, II: essential and nonessential element uptake determined by polarized energy dispersive X-ray fluorescence. *Communication of Soil Science and Plant Analysis* 39: 1904-1927.

HABIBI, G. 2015. Exogenous silicon leads to increased antioxidant capacity in freezing-stressed pistachio leaves. *Acta agriculturae Slovenica* 105: 43-52.

HABIBI, G. 2016. Effect of foliar-applied silicon on photochemistry, antioxidant capacity and growth in maize plants subjected to chilling stress. *Acta agriculturae Slovenica* 107: 33-43.

HAMM, C.E., MERKEL, R., SPRINGER, O., JURKOJC, P., MAIER, C., PRECHTEL, K. & SMETACEK, V. 2003. Architecture and material properties of diatom shells provide effective mechanical protection. *Nature* 421: 841.

HATTORI, T., INANAGA, S., ARAKI, H., AN, P., MORITA, S., LUXOVÁ, M. & LUX, A. 2005. Application of silicon enhanced drought tolerance in Sorghum bicolor. *Physiologia Plantarum* 123: 459-466.

HATTORI, T., SONOBE, K., INANAGA, S., AN, P. & MORITA, S. 2008. Effects of silicon on photosynthesis of young cucumber seedlings under osmotic stress. *Journal of Plant Nutrition* 31: 1046-1058.

HAYNES, R.J. 2014. A contemporary overview of silicon availability in agricultural soils. *Journal of Plant Nutrition and Soil Science* 177: 831-844.

HAYSOM, M.B. & OSTATEK-BOCZYNSKI, Z.A. 2006. Rapid, Wet Oxidation Procedure for the Estimation of Silicon in Plant Tissue. *Communications in Soil Science and Plant Analysis* 37: 2299-2306.

HAZRATI, S., TAHMASEBI-SARVESTANI, Z., MODARRES-SANAVY, S.A.M., MOKHTASSI-BIDGOLI, A. & NICOLA, S. 2016. Effects of water stress and light intensity on chlorophyll fluorescence parameters and pigments of Aloe vera L. *Plant Physiology and Biochemistry* 106: 141-148.

HE, D. & EDWARDS, G.E. 1996. Evaluation of the potential to measure photosynthetic rates in C_3 plants (Flaveria pringlei and Oryza sativa) by combining chlorophyll fluorescence analysis and a stomatal conductance model. *Plant Cell and Environment* 19: 1272-1280.

HE, Y., XIAO, H., WANG, H., CHEN, Y. & YU, M. 2010. Effect of silicon on chilling-induced changes of solutes, antioxidants, and membrane stability in seashore paspalum turfgrass. *Acta Physiologiae Plantarum* 32: 487-494.

HECKMAN, J. 2013. Silicon: A beneficial substance. *Better crops* 97: 14-16.

HEINE, G. 2005. Silicon Nutrition and Resistance Against Pythium Aphanidermatum of *Lycopersicon Esculentum* and *Mormodica Charantia*. University of Hannover, Germany, doctoral dissertation.

HEINE, G., TIKUM, G. & HORST, W.J. 2006. The effect of silicon on the infection by and spread of *Pythium aphanidermatum* in single roots of tomato and bitter gourd. *Journal of Experimental Botany* 58: 569-577.

HENRIET, C., DRAYE, X., OPPITZ, I., SWENNEN, R. & DELVAUX, B. 2006. Effects, distribution and uptake of silicon in banana (Musa spp.) under controlled conditions. *Plant Soil* 287: 359-374.

HODSON, M. & SANGSTER, A. 1989. Silica deposition in the inflorescence bracts of wheat (*Triticum aestivum*). II. X-ray microanalysis and backscattered electron imaging. *Canadian Journal of Botany* 67: 281-287.

HODSON, M., WHITE, P., MEAD, A. & BROADLEY, M. 2005. Phylogenetic variation in the silicon composition of plants. *Annals of Botany* 96: 1027-1046.

HODSON, M.J. & EVANS, D.E. 1995. Aluminium/silicon interactions in higher plants. *Journal of Experimental Botany* 46: 161-171.

HOPKINS, W.G. & HÜNER, N.P. 2008. Introduction to Plant Physiology 4th ed., United States of America.

HORACIO, P. & MARTINEZ-NOEL, G. 2013. Sucrose signaling in plants: a world yet to be explored. *Plant Signal Behaviour* 8: e23316.

HORIGUCHI, T. 1988. Mechanism of manganese toxicity and tolerance of plants: IV. Effects of silicon on alleviation of manganese toxicity of rice plants. *Soil Science and Plant Nutrition* 34: 65-73.

HORST, W.J., FECHT, M., NAUMANN, A., WISSEMEIER, A.H. & MAIER, P. 1999. Physiology of manganese toxicity and tolerance in *Vigna unguiculata* (L.) Walp. *Journal of Plant Nutrition and Soil Science* 162: 263-274.

HOU, X., AMAIS, R.S., JONES, B.T. & DONATI, G.L. 2006. Inductively coupled plasma optical emission spectrometry. *Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation*: 1-25.

HUNER, N.P., ÖQUIST, G. & SARHAN, F. 1998. Energy balance and acclimation to light and cold. *Trends in Plant Science* 3: 224-230.

HUSSAIN, H.A., HUSSAIN, S., KHALIQ, A., ASHRAF, U., ANJUM, S.A., MEN, S. & WANG, L. 2018. Chilling and drought stresses in crop plants: implications, cross talk, and potential management opportunities. *Frontiers in Plant Science* 9: 393.

INANAGA, S., OKASAKA, A. & TANAKA, S. 1995. Does silicon exist in association with organic compounds in rice plant? *Soil Science and Plant Nutrition* 41: 111-117.

JAYAWARDANA, H., WEERAHEWA, H. & SAPARAMADU, M. 2014. Effect of root or foliar application of soluble silicon on plant growth, fruit quality and anthracnose development of capsicum. *Tropical Agriculture Research* 26: 8.

JONES, L. & HANDRECK, K. 1965. Studies of silica in the oat plant: III. Uptake of silica from soils by the plant. *Plant Soil*: 79-96.

JONES, L. & HANDRECK, K. 1967. Silica in soils, plants, and animals. *Advance in Agronomy*. Academic press. p. 107-149.

KALAJI, H.M., BĄBA, W., GEDIGA, K., GOLTSEV, V., SAMBORSKA, I.A., CETNER, M.D., DIMITROVA, S., PISZCZ, U., BIELECKI, K. & KARMOWSKA, K. 2018. Chlorophyll fluorescence as a tool for nutrient status identification in rapeseed plants. *Photosynthetic Research* 136: 329-343.

KALLAS, T. 1994. The Cytochrome b6f complexKluwer Academic, Netherlands.

KAPLAN, F. & GUY, C.L. 2005. RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *Plant Journal* 44: 730-743.

KAUFMAN, P.B., DAYANANDAN, P., FRANKLIN, C. & TAKEOKA, Y. 1985. Structure and function of silica bodies in the epidermal system of grass shoots. *Annals of Botany* 55: 487-507.

KIDANE, E.G. 2008. Management of Fusarium wilt diseases using non-pathogenic Fusarium Oxysporum and silicon. Kwazulu-Natal, South Africa, doctoral dissertation.

KIM, S.G., KIM, K.W., PARK, E.W. & CHOI, D. 2002. Silicon-induced cell wall fortification of rice leaves: a possible cellular mechanism of enhanced host resistance to blast. *Phytopathology* 92: 1095-1103.

KIM, Y.-H., KHAN, A.L., WAQAS, M. & LEE, I.-J. 2017. Silicon regulates antioxidant activities of crop plants under abiotic-induced oxidative stress: a review. *Frontiers in Plant Science* 8: 510.

KING, E., STACY, B., HOLT, P., YATES, D.M. & PICKLES, D. 1955. The colorimetric determination of silicon in the micro-analysis of biological material and mineral dusts. *Analyst* 80: 441-453.

KRASENSKY, J. & JONAK, C. 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany* 63: 1593-1608.

KRASKA, J.E. 2009. Assessing the silicon status of rice (*Oryza sativa*). Louisiana State University, Louisiana, United States of America, Masters dissertation.

KRASKA, J.E. & BREITENBECK, G.A. 2010. Simple, robust method for quantifying silicon in plant tissue. *Communication of Soil Science and Plant Analysis* 41: 2075-2085.

KRAUSE, G.H. 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. *Physiologia Plantarum* 74: 566-574.

KUSHAD, M.M. & YELENOSKY, G. 1987. Evaluation of polyamine and proline levels during low temperature acclimation of citrus. *Plant Physiology* 84: 692-695.

LADANIYA, M. 2008. Citrus Fruit Biology, Technology and Evaluation. Academic Press, USA.

LADO, J., GAMBETTA, G. & ZACARIAS, L. 2018. Key determinants of citrus fruit quality: Metabolites and main changes during maturation. *Scientia Horticulturae* 233: 238-248.

LAÎNÉ, P., HADDAD, C., ARKOUN, M., YVIN, J.-C. & ETIENNE, P. 2019. Silicon Promotes Agronomic Performance in Brassica napus Cultivated under Field Conditions with Two Nitrogen Fertilizer Inputs. *Plants* 8: 137.

LAWLOR, D. 2004. Mengel, K. and Kirkby, EA Principles of plant nutrition. Oxford University Press.

LEVITT, J. 1980. Responses of Plants to Environmental Stress, Volume 1: Chilling, Freezing, and High Temperature Stresses. Academic Press., New York.

LI, H., ZHU, Y., HU, Y., HAN, W. & GONG, H. 2015. Beneficial effects of silicon in alleviating salinity stress of tomato seedlings grown under sand culture. *Acta Physiologiae Plantarum* 37: 71.

LI, Q., MA, C. & SHANG, Q. 2007. Effects of silicon on photosynthesis and antioxidative enzymes of maize under drought stress. *Ying yong sheng tai xue bao= The journal of applied ecology* 18: 531-536.

LI, S., YANG, Y., ZHANG, Q., LIU, N., XU, Q. & HU, L. 2018. Differential physiological and metabolic response to low temperature in two zoysiagrass genotypes native to high and low latitude. *Plos One* 13: 20.

LI, Y., ALVA, A. & SUMNER, M. 1989. Response of cotton cultivars to aluminum in solutions with varying silicon concentrations. *Journal of Plant Nutrition* 12: 881-892.

LIANG, W., WANG, M. & AI, X. 2009. The role of calcium in regulating photosynthesis and related physiological indexes of cucumber seedlings under low light intensity and suboptimal temperature stress. *Scientia Horticulturae* 123: 34-38.

LIANG, Y., SHEN, Q., SHEN, Z. & MA, T. 1996. Effects of silicon on salinity tolerance of two barley cultivars. *Journal of Plant Nutrition* 19: 173-183.

LIANG, Y., SI, J. & RÖMHELD, V. 2005. Silicon uptake and transport is an active process in *Cucumis sativus*. *New Phytologist* 167: 797-804.

LIANG, Y., WONG, J. & WEI, L. 2005. Silicon-mediated enhancement of cadmium tolerance in maize (*Zea mays L*.) grown in cadmium contaminated soil. *Chemosphere* 58: 475-483.

LIANG, Y., HUA, H., ZHU, Y.G., ZHANG, J., CHENG, C. & RÖMHELD, V. 2006. Importance of plant species and external silicon concentration to active silicon uptake and transport. *New Phytologist* 172: 63-72.

LIANG, Y., SUN, W., ZHU, Y.-G. & CHRISTIE, P. 2007. Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: a review. *Environmental Pollution* 147: 422-428.

LIANG, Y., ZHU, J., LI, Z., CHU, G., DING, Y., ZHANG, J. & SUN, W. 2008. Role of silicon in enhancing resistance to freezing stress in two contrasting winter wheat cultivars. *Environmental and Experimental Botany* 64: 286-294.

LIANG, Y., NIKOLIC, M., BÉLANGER, R., GONG, H. & SONG, A. 2015. Silicon-mediated Tolerance to Drought and Low-temperature stress. Silicon in Agriculture. Springer. p. 143-159.

LICHTE, F., HOPPER, S. & OSBORN, T. 1980. Determination of silicon and aluminum in biological matrixes by inductively coupled plasma emission spectrometry. *Analytical Chemistry* 52: 120-124.

LIU, B., SOUNDARARAJAN, P. & MANIVANNAN, A. 2019. Mechanisms of Silicon-Mediated Amelioration of Salt Stress in Plants. *Plants* 8: 307.

LIU, J.-J., LIN, S.-H., XU, P.-L., WANG, X.-J. & BAI, J.-G. 2009. Effects of exogenous silicon on the activities of antioxidant enzymes and lipid peroxidation in chilling-stressed cucumber leaves. *Agricultural Sciences in China* 8: 1075-1086.

LIU, P., YIN, L., DENG, X., WANG, S., TANAKA, K. & ZHANG, S. 2014. Aquaporin-mediated increase in root hydraulic conductance is involved in silicon-induced improved root water uptake under osmotic stress in *Sorghum bicolor L*. *Journal of Experimental Botany* 65: 4747-4756.

LIU, Y., HEYING, E. & TANUMIHARDJO, S.A. 2012. History, global distribution, and nutritional importance of citrus fruits. *Comprehensive Reviews in Food Science and Food Safety* 11: 530-545.

LOBATO, A., LUZ, L., COSTA, R., SANTOS FILHO, B., MEIRELLES, A., OLIVEIRA NETO, C., LAUGHINGHOUSE, I., NETO, M., ALVES, G. & LOPES, M. 2009. Silicon exercises influence on nitrogen compounds in pepper subjected to water deficit. *Research Journal of Biological Sciences* 4: 1048-1055.

LOBATO, A.K.S., COIMBRA, G.K., NETO, M.A.M., COSTA, R.C.L., SANTOS FILHO, B.G., OLIVEIRA NETO, C.F., LUZ, L.M., BARRETO, A.G.T., PEREIRA, B.W.F. & ALVES, G.A.R. 2009. Protective action of silicon on water relations and photosynthetic pigments in pepper plants induced to water deficit. *Research Journal of Biological Sciences* 4: 617-623.

LOVELOCK, C.E. & WINTER, K. 1996. Oxygen-dependent electron transport and protection from photoinhibition in leaves of tropical tree species. *Planta* 198: 580-587.

LUX, A.L., M; ABE, J; TANIMOTO, E; HATTORI, T; INANAGA, S. 2003. The dynamics of silicon deposition in the sorghum root endodermis. *New Phytologist* 158: 5.

LYONS, J.M. 1973. Chilling injury in plants. *Annual Review of Plant Physiology* 24: 445-466.

MA, C.C., LI, Q.F., GAO, Y.B. & XIN, T.R. 2004. Effects of silicon application on drought resistance of cucumber plants. *Soil Science and Plant Nutrition* 50: 623-632.

MA, F.J. & TAKAHASHI, E. 1993. Interaction between calcium and silicon in water-cultured rice plants. *Plant and Soil* 148: 107-113.

MA, J.F., MIYAKE, Y. & TAKAHASHI, E. 2001. Silicon as a beneficial element for crop plants. *Studies in plant Science*. Elsevier. p. 17-39.

MA, J.F. & TAKAHASHI, E. 2002. Soil, fertilizer, and plant silicon research in Japan. 1st ed. Elsevier, Amsterdam, Netherlands.

MA, J.F. 2004. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. *Soil Science and Plant Nutrition* 50: 11-18.

MA, J.F., MITANI, N., NAGAO, S., KONISHI, S., TAMAI, K., IWASHITA, T. & YANO, M. 2004. Characterization of the silicon uptake system and molecular mapping of the silicon transporter gene in rice. *Plant Physiology* 136: 3284-3289.

MA, J.F. & YAMAJI, N. 2006. Silicon uptake and accumulation in higher plants. *Trends in Plant Science* 11: 392-397.

MA, J.F. & YAMAJI, N. 2008. Functions and transport of silicon in plants. *Cellular Molecular and Life Sciences* 65: 3049-3057.

MA, J.F., YAMAJI, N. & MITANI-UENO, N. 2011. Transport of silicon from roots to panicles in plants. *Proceedings of the Japan Academy Series B* 87: 377-385.

MA, J.F. & YAMAJI, N. 2015. A cooperative system of silicon transport in plants. *Trends in Plant Science* 20: 435-442.

MACHADO, E.C., MEDINA, C.L., GOMES, M.D.M.D.A. & HABERMANN, G. 2002. Seasonal variation of photosynthetic rates, stomatal conductance and leaf water potential in'Valencia'orange trees. *Scientia Agricola* 59: 53-58.

MADEMBA-SY, F., BOUCHEREAU, A. & LARHER, F. 2003. Proline accumulation in cultivated citrus and its relationship with salt tolerance. *Journal of Horticultural Science and Biotechnology* 78: 617-623.

MAGHSOUDI, K., EMAM, Y. & ASHRAF, M. 2015. Influence of foliar application of silicon on chlorophyll fluorescence, photosynthetic pigments, and growth in water-stressed wheat cultivars differing in drought tolerance. *Turkish Journal of Botany* 39: 625-634.

MARKERT, B. 1996. Instrumental Element and Multi-element Analysis of Plant Samples: Methods and Applications, Wiley.

MARODIN, J.C., RESENDE, J.T., MORALES, R.G., SILVA, M.L., GALVÃO, A.G. & ZANIN, D.S. 2014. Yield of tomato fruits in relation to silicon sources and rates. *Horticultura Brasileira* 32: 220-224.

MARSCHNER, H. 2012. Marschner's mineral nutrition of higher plants. 3rd ed. Academic press, UK.

MARTIN, B. & RUIZ-TORRES, N.A. 1992. Effects of water-deficit stress on photosynthesis, its components and component limitations, and on water use efficiency in wheat (*Triticum aestivum L.*). *Plant Physiology* 100: 733-739.

MARTINEZ, S.E., HUANG, D., SZCZEPANIAK, A., CRAMER, W.A. & SMITH, J.L. 1994. Crystal structure of chloroplast cytochrome freveals a novel cytochrome fold and unexpected heme ligation. *Structure* 2: 95-105.

MASHILO, J. 2016. Pre-breeding of bottle gourd [*Lagenaria siceraria* (molina) standl.]. Kwazulu-Natal, Pietermaritzburg, doctoral dissertation.

MASHILO, J., ODINDO, A.O., SHIMELIS, H.A., MUSENGE, P., TESFAY, S.Z. & MAGWAZA, L.S. 2017. Drought tolerance of selected bottle gourd [*Lagenaria siceraria* (Molina) Standl.] landraces assessed by leaf gas exchange and photosynthetic efficiency. *Plant Physiology and Biochemistry* 120: 75-87.

MATICHENKOV, V., CALVERT, D. & SNYDER, G. 1999. Silicon fertilizers for citrus in Florida. *Proceedings of the Florida State Horticultural Society* 112: 5-8.

MATICHENKOV, V., BOCHARNIKOVA, E. & CALVERT, D. 2001. Response of citrus to silicon soil amendments. *Proceedings of the Florida State Horticultural Society* 114: 94-97.

MATICHENKOV, V. & CALVERT, D. 2002. Silicon as a beneficial element for sugarcane. *Journal American Society of Sugarcane Technologists* 22: 21-30.

MATOH, T., KAIRUSMEE, P. & TAKAHASHI, E. 1986. Salt-induced damage to rice plants and alleviation effect of silicate. *Soil Science and Plant Nutrition* 32: 295-304.

MATOH, T., MURATA, S. & TAKAHASHI, E. 1991. Effect of silicate application on photosynthesis of rice *[Oryza sativa*] plants. *Japanese Journal of Soil Science and Plant Nutrition* 62: 248-251.

MAUAD, M., CRUSCIOL, C.A.C., NASCENTE, A.S., GRASSI FILHO, H. & LIMA, G.P.P. 2016. Effects of silicon and drought stress on biochemical characteristics of leaves of upland rice cultivars. *Revista Ciência Agronômica* 47: 532-539.

MAUREL, C., BOURSIAC, Y., LUU, D.-T., SANTONI, V., SHAHZAD, Z. & VERDOUCQ, L. 2015. Aquaporins in plants. *Physiological Reviews* 95: 1321-1358.

MAXWELL, K. & JOHNSON, G.N. 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* 51: 659-668.

MECFEL, J., HINKE, S., GOEDEL, W.A., MARX, G., FEHLHABER, R., BÄUCKER, E. & WIENHAUS, O. 2007. Effect of silicon fertilizers on silicon accumulation in wheat. *Journal of Plant Nutrition and Soil Science* 170: 769-772.

MEDINA, C.L., SOUZA, R.R.P., MACHADO, E.C., RIBEIRO, R.V. & SILVA, J.A.B. 2002. Photosynthetic response of citrus grown under reflective aluminized polypropylene shading nets. *Scientia Horticulturae* 96: 115-125.

MINORSKY, P. 1989. Temperature sensing by plants: a review and hypothesis. *Plant Cell and Environment* 12: 119-135.

MITANI, N., MA, J. & IWASHITA, T. 2005. Identification of silicon form in the xylem of rice (*Oryza sativa L.*). *Plant Cell Physiology* 46: 279-283.

MITANI, N. & MA, J.F. 2005. Uptake system of silicon in different plant species. *Journal of Experimental Botany* 56: 1255-1261.

MITANI, N., YAMAJI, N. & MA, J.F. 2008. Identification of maize silicon influx transporters. *Plant Cell Physiology* 50: 5-12.

MITANI, N., CHIBA, Y., YAMAJI, N. & MA, J.F. 2009. Identification and characterization of maize and barley Lsi2-like silicon efflux transporters reveals a distinct silicon uptake system from that in rice. *Plant Cell* 21: 2133-2142.

MITANI, N., YAMAJI, N., AGO, Y., IWASAKI, K. & MA, J.F. 2011. Isolation and functional characterization of an influx silicon transporter in two pumpkin cultivars contrasting in silicon accumulation. *Plant Journal* 66: 231-240.

MITANI-UENO, N., YAMAJI, N. & MA, J.F. 2016. High silicon accumulation in the shoot is required for down-regulating the expression of Si transporter genes in rice. *Plant Cell Physiology*: pcw163.

MIYAKE, Y. & TAKAHASHI, E. 1978. Silicon deficiency of tomato plant. *Soil Science and Plant Nutrition* 24: 175-189.

MIYAKE, Y. & TAKAHASHI, E. 1983. Effect of silicon on the growth of solution-cultured cucumber plant. *Soil Science and Plant Nutrition* 29: 71-83.

MONTPETIT, J., VIVANCOS, J., MITANI-UENO, N., YAMAJI, N., RÉMUS-BOREL, W., BELZILE, F., MA, J.F. & BÉLANGER, R.R. 2012. Cloning, functional characterization and heterologous expression of TaLsi1, a wheat silicon transporter gene. *Plant Molecular Biology* 79: 35-46.

MORADTALAB, N., WEINMANN, M., WALKER, F., HÖGLINGER, B., LUDEWIG, U. & NEUMANN, G. 2018. Silicon improves chilling tolerance during early growth of maize by effects on micronutrient homeostasis and hormonal balances. *Frontiers in plant science* 9 (420): 1-17.

MOSER, C.C., KESKE, J.M., WARNCKE, K., FARID, R.S. & DUTTON, P.L. 1992. Nature of biological electron transfer. *Nature* 355: 796.

MOTOMURA, H., FUJII, T. & SUZUKI, M. 2000. Distribution of silicified cells in the leaf blades of *Pleioblastus chino* (Franchet et Savatier) Makino (*Bambusoideae)*. *Annals of Botany* 85: 751-757.

MOTOMURA, H., MITA, N. & SUZUKI, M. 2002. Silica accumulation in long-lived leaves of *Sasa veitchii* (Carrière) Rehder (P*oaceae–Bambusoideae*). *Annals of Botany* 90: 149-152.

MOTOMURA, H., FUJII, T. & SUZUKI, M. 2004. Silica deposition in relation to ageing of leaf tissues in Sasa veitchii (Carriere) Rehder (Poaceae: Bambusoideae). *Annals of Botany* 93: 235-248.

MOTOMURA, H., FUJII, T. & SUZUKI, M. 2006. Silica deposition in abaxial epidermis before the opening of leaf blades of *Pleioblastus chino (Poaceae*, *Bambusoideae*). *Annals of Botany* 97: 513-519.

MURCHIE, E.H. & LAWSON, T. 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany* 64: 3983-3998.

MVONDO-SHE, M.A. & MARAIS, D. 2019. The Investigation of Silicon Localization and Accumulation in Citrus. *Plants* 8: 12.

NABLE, R.O., LANCE, R.C. & CARTWRIGHT, B. 1990. Uptake of boron and silicon by barley genotypes with differing susceptibilities to boron toxicity. *Annals of Botany* 66: 83-90.

NAGAO, M., MINAMI, A., ARAKAWA, K., FUJIKAWA, S. & TAKEZAWA, D. 2005. Rapid degradation of starch in chloroplasts and concomitant accumulation of soluble sugars associated with ABA-induced freezing tolerance in the moss *Physcomitrella patens*. *Journal of Plant Physiology* 162: 169-180.

NAIDOO, P., MCFARLANE, S., KEEPING, M. & CALDWELL, P. 2009. Deposition of silicon in leaves of sugarcane (*Saccharum spp*. hybrids) and its effect on the severity of brown rust caused by *Puccinia melanocephala*. *Proceedings of the Annual Congress-South African Sugar Technologists' Association*: 542-546.

NATIONAL DEPARTMENT AGRICULTURE. 1990. Standards and requirements regarding control of export of citrus fruit. National Department of Agriculture, South Africa. Department of Agriculture, Pretoria.

NOVOZAMSKY, I., VAN ECK, R. & HOUBA, V. 1984. A rapid determination of silicon in plant material. *Communication of Soil Science and Plant Analysis* 15: 205-211.

OSTATEK-BOCZYNSKI, Z. & HAYSOM, M. 2003. The determination of silicon in sugarcane tissue using a microwave-assisted acid and alkali digestion procedure. In: M. University, editor Tools for nutrient and pollution management Palmerston North. p. 419-424.

PAPAGEORGIOU, G. 1975. Chlorophyll fluorescence: an intrinsic probe of photosynthesis. *Bioenergetics of Photosynthesis*: 319-371.

PARVEEN, N. & ASHRAF, M. 2010. Role of silicon in mitigating the adverse effects of salt stress on growth and photosynthetic attributes of two maize (*Zea mays L*.) cultivars grown hydroponically. *Pakistan Journal of Botany* 42: 1675-1684.

PEREIRA, T.S., DA SILVA LOBATO, A.K., TAN, D.K.Y., DA COSTA, D.V., UCHOA, E.B., DO NASCIMENTO FERREIRA, R., DOS SANTOS PEREIRA, E., AVILA, F.W., MARQUES, D.J. & SILVA GUEDES, E.M. 2013. Positive interference of silicon on water relations, nitrogen metabolism, and osmotic adjustment in two pepper ('*Capsicum annuum*') cultivars under water deficit. *Australian Journal of Crop Science* 7: 1064.

PEREZ, C.E.A., RODRIGUES, F.Á., MOREIRA, W.R. & DAMATTA, F.M. 2014. Leaf gas exchange and chlorophyll a fluorescence in wheat plants supplied with silicon and infected with *Pyricularia oryzae*. *Phytopathology* 104: 143-149.

PETERHANSEL, C., HORST, I., NIESSEN, M., BLUME, C., KEBEISH, R., KÜRKCÜOGLU, S. & KREUZALER, F. 2010. Photorespiration. *The Arabidopsis book/American Society of Plant Biologists* 8.

PIETRZYKOWSKA, M. 2015. The roles of Lhcb1 and Lhcb2 in regulation of photosynthetic light harvesting Umea University, *Umea, Sweden,* doctoral dissertation.

PORCAR-CASTELL, A., JUUROLA, E., NIKINMAA, E., BERNINGER, F., ENSMINGER, I. & HARI, P. 2008. Seasonal acclimation of photosystem II in *Pinus sylvestri*s. I. Estimating the rate constants of sustained thermal energy dissipation and photochemistry. *Tree Physiology* 28: 1475-1482.

PRAKASH, N., CHANDRASHEKAR, N., MAHENDRA, C., PATIL, S., THIPPESHAPPA, G. & LAANE, H. 2011. Effect of foliar spray of soluble silicic acid on growth and yield parameters of wetland rice in hilly and coastal zone soils of Karnataka, South India. *Journal of Plant Nutrition* 34: 1883-1893.

RAFI, M.M., EPSTEIN, E. & FALK, R.H. 1997. Silicon deprivation causes physical abnormalities in wheat (*Triticum aestivum L*.). *Journal of Plant Physiology* 151: 497-501.

RAO, G.B. & SUSMITHA, P. 2017. Silicon uptake, transportation and accumulation in Rice. *Journal of Pharmacognosy and Phytochemistry* 6: 290-293.

RAVEN, J.A. 1983. The transport and function of silicon in plants. *Biological Reviews* 58: 179-207.

RAVEN, J.A. 2003. Cycling silicon–the role of accumulation in plants. *New Phytologist* 158: 419-421.

REEKSTING, B.J., TAYLOR, N. & VAN DEN BERG, N. 2014. Flooding and *Phytophthora cinnamomi*: Effects on photosynthesis and chlorophyll fluorescence in shoots of non-grafted *Persea americana* (Mill.) rootstocks differing in tolerance to Phytophthora root rot. *South African Journal of Botany* 95: 40-53.

RENATA, A.L.S.R., RODRIGUES SOARES, J.D., DOS SANTOS, H.O., PASQUAL, M., BRAGA, R.A., REIS, R.O., RODRIGUES, F.A. & RAMOS, J.D. 2017. Effects of silicon on antioxidant enzymes, CO_2, proline and biological activity of in vitro-grown cape gooseberry under salinity stress. *Australian Journal of Crop Science* 11: 438.

REZENDE, D., RODRIGUES, F., CARRÉ-MISSIO, V., SCHURT, D., KAWAMURA, I. & KORNDÖRFER, G. 2009. Effect of root and foliar applications of silicon on brown spot development in rice. *Australasian Plant Pathology* 38: 67-73.

RIBEIRO, R.V., MACHADO, E.C. & OLIVEIRA, R.F.D. 2004. Growth-and leaf-temperature effects on photosynthesis of sweet orange seedlings infected with *Xylella fastidiosa*. *Plant Pathology* 53: 334-340.

RIBEIRO, R.V. & MACHADO, E.C. 2007. Some aspects of citrus ecophysiology in subtropical climates: re-visiting photosynthesis under natural conditions. *Brazilian Journal of Plant Physiology* 19: 393-411.

RIBEIRO, R., MACHADO, E., SANTOS, M. & OLIVEIRA, R. 2009. Photosynthesis and water relations of well-watered orange plants as affected by winter and summer conditions. *Photosynthetica* 47: 215-222.

RICHMOND, K.E. & SUSSMAN, M. 2003. Got silicon? The non-essential beneficial plant nutrient. *Current Opinion in Plant Biology* 6: 268-272.

RIOS, J.J., MARTÍNEZ-BALLESTA, M.C., RUIZ, J.M., BLASCO, B. & CARVAJAL, M. 2017. Silicon-mediated improvement in plant salinity tolerance: the role of aquaporins. *Frontiers in Plant Science* 8: 948.

ROBERT, B., BERTONI, G., SAYAG, D. & MASSON, P. 1996. Assessment of mineral nutrition of cork oak through foliar analysis. *Communication in Soil Science and Plant Analysis* 27: 2091-2109.

ROGALLA, H. & RÖMHELD, V. 2002. Role of leaf apoplast in silicon-mediated manganese tolerance of *Cucumis sativus L*. *Plant Cell and Environment* 25: 549-555.

ROHÁČEK, K. & BARTÁK, M. 1999. Technique of the modulated chlorophyll fluorescence: basic concepts, useful parameters, and some applications. *Photosynthetica* 37: 339.

ROMERO-ARANDA, M.R., JURADO, O. & CUARTERO, J. 2006. Silicon alleviates the deleterious salt effect on tomato plant growth by improving plant water status. *Journal of Plant Physiology* 163: 847-855.

ROSSINI OLIVA, S. & RAITIO, H. 2003. Review of cleanning techniques and their effects on the chemical composition of foliar samples. *Boreal Environment Research* 8: 263-272.

RUELLAND, E., VAULTIER, M.-N., ZACHOWSKI, A. & HURRY, V. 2009. Cold signalling and cold acclimation in plants. *Advances in Botanical Research* 49: 35-150.

RUELLAND, E. & COLLIN, S. 2012. *Plant Stress Physiology.* 1st ed. CABI.

SAKAI, W.S. & SANFORD, W. 1984. A developmental study of silicification in the abaxial epidermal cells of sugarcane leaf blades using scanning electron microscopy and energy dispersive X-ray analysis. *American Journal of Botany* 71: 1315-1322.

SAKR, N. 2016. The role of silicon (Si) in increasing plant resistance against fungal diseases. *Hellenic Plant Protection Journal* 9: 1-15.

SAMUELS, A., GLASS, A., EHRET, D. & MENZIES, J. 1991. Mobility and deposition of silicon in cucumber plants. *Plant Cell and Environment* 14: 485-492.

SANTINI, J., GIANNETTINI, J., HERBETTE, S., PAILLY, O., OLLITRAULT, P., LURO, F. & BERTI, L. 2012. Physiological and biochemical response to photooxidative stress of the fundamental citrus species. *Scientia Horticulturae* 147: 126-135.

SASAKI, H., ICHIMURA, K. & ODA, M. 1996. Changes in sugar content during cold acclimation and deacclimation of cabbage seedlings. *Annals of Botany* 78: 365-369.

SATTAR, A., CHEEMA, M., ABBAS, T., SHER, A., IJAZ, M. & HUSSAIN, M. 2017. Separate and combined effects of silicon and selenium on salt tolerance of wheat plants. *Russian Journal of Plant Physiology* 64: 341-348.

SAUD, S., LI, X., CHEN, Y., ZHANG, L., FAHAD, S., HUSSAIN, S., SADIQ, A. & CHEN, Y. 2014. Silicon application increases drought tolerance of Kentucky bluegrass by improving plant water relations and morphophysiological functions. *The Scientific World Journal*. p. 1-10.

SAUNT, J. 2000. Citrus Varieties of the World. 2nd ed. Sinclair International Limited, Norwich, England.

SAVANT, N.K., KORNDÖRFER, G.H., DATNOFF, L.E. & SNYDER, G.H. 1999. Silicon nutrition and sugarcane production: a review. *Journal of Plant Nutrition* 22: 1853-1903.

SAYED, O. 2003. Chlorophyll fluorescence as a tool in cereal crop research. *Photosynthetica : International Journal for Photosynthesis Research* 41: 10.

SCHULZE, R. & SCHÜTTE, S. 2016. Citrus fruit production in South Africa and Climate change. In: R. Schulze, editor Handbook for farmers, officials and other stakeholders on adaptation to climate change in the Agriculture sector within South Africa.

SCHURT, D.A., CRUZ, M.F., NASCIMENTO, K.J., FILIPPI, M.C. & RODRIGUES, F.A. 2014. Silicon potentiates the activities of defense enzymes in the leaf sheaths of rice plants infected by *Rhizoctonia solani*. *Tropical Plant Pathology* 39: 457-463.

SEATON, G.G. & WALKER, D.A. 1990. Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. *Proceedings of the Royal Society London Series B: Biological Sciences* 242: 29-35.

SHEN, X., ZHOU, Y., DUAN, L., LI, Z., ENEJI, A.E. & LI, J. 2010. Silicon effects on photosynthesis and antioxidant parameters of soybean seedlings under drought and ultraviolet-B radiation. *Journal of Plant Physiology* 167: 1248-1252.

SHETTY, K. 2004. Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: a review. *Process Biochemistry* 39: 789-804.

SHI, Q., BAO, Z., ZHU, Z., HE, Y., QIAN, Q. & YU, J. 2005. Silicon-mediated alleviation of Mn toxicity in Cucumis sativus in relation to activities of superoxide dismutase and ascorbate peroxidase. *Phytochemistry* 66: 1551-1559.

SHI, Z., ZHANG, D., HAYASHI, M., OGATA, H., JI, H. & FUJIIE, W. 2008. Influences of sulfate and nitrate on the hygroscopic behaviour of coarse dust particles. *Atmospheric Environment* 42: 822-827.

SHRESTHA, R.P. & HILDEBRAND, M. 2015. Evidence for a Regulatory Role of Diatom Silicon Transporters in Cellular Silicon Responses. *Eukaryotic Cell* 14: 12.

SILVA, M.L.S., DE RESENDE, J.T.V., TREVIZAM, A.R., FIGUEIREDO, A.S.T. & SCHWARZ, K. 2013. Influence of silicon on production and fruit quality of strawberry. *Semina: Ciências Agrárias* 34: 3411-3424.

SIVAKUMAR, V., ERNYEI, L. & OBENAUF, R.H. 2006. Guide for determining ICP/ICP-MS method detection limits and instrument performance. *Atomic Spectroscopy* 21: 13.

SLATYER, R. & BIERHUIZEN, J. 1964. The effect of several foliar sprays on transpiration and water use efficiency of cotton plants. *Agricultural Meteorology* 1: 42-53.

SMILLIE, R.M. & HETHERINGTON, S.E. 1983. Stress tolerance and stress-induced injury in crop plants measured by chlorophyll fluorescence in vivo: chilling, freezing, ice cover, heat, and high light. *Plant Physiology* 72: 1043-1050.

SOIL & COUNCIL, P.A. 1992. *Handbook on Reference Methods for Soil Analysis.* Council on Soil Testing and Plant Analysis.

SOIL CLASSIFICATION WORKING GROUP. 1991. Soil classification. A taxonomic system for South Africa Department of Agricultural Development, Pretoria.

SPOSITO, G. 2008. The Chemistry of Soils. 2nd ed. Oxford university press.

SUZUKI, N. & MITTLER, R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum* 126: 45-51.

TABER, H.G., SHOGREN, D. & LU, G. 2002. Extraction of silicon from plant tissue with dilute HCl and HF and measurement by modified inductive coupled argon plasma procedures. *Communication in Soil Science and Plant Analysis* 33: 1661-1670.

TAKAHASHI, E., MA, J. & MIYAKE, Y. 1990. The possibility of silicon as an essential element for higher plants. *Comments on Agricultural and Food Chemistry* 2: 99-102.

TAKAHASHI, N., KATO, Y., ISOGAI, A. & KURATA, K. 2006. Silica distribution on the husk epidermis at different parts of the panicle in rice (*Oryza sativa L.*) determined by X-ray microanalysis. *Plant Production Science* 9: 168-171.

TAMAI, K. & MA, J.F. 2003. Characterization of silicon uptake by rice roots. *New Phytologist* 158: 431-436.

TAMAI, K. & MA, J.F. 2008. Reexamination of silicon effects on rice growth and production under field conditions using a low silicon mutant. *Plant and Soil* 307: 21-27.

TARANOVSKAIA, V. 1939. The silicication of subtropic greenhouse and plantations. *Soviet Subtropics* 7: 32-37.

TARANOVSKAIA, V. 1940. The role of silicication for citrus, tunga and sider-ates. *Soviet Subtropics* 5: 38-43.

TESFAGIORGIS, H. & LAING, M. 2013. The effects of silicon level in nutrient solution on the uptake and distribution of silicon in zucchini and zinnia, and its interaction with the uptake of selected elements. *African Journal of Biotechnolology* 12 (14): 1617-1623

TORESANO-SÁNCHEZ, F., VALVERDE-GARCÍA, A. & CAMACHO-FERRE, F. 2012. Effect of the application of silicon hydroxide on yield and quality of cherry tomato. *Journal of Plant Nutrition* 35: 567-590.

TREDER, W. & CIESLINSKI, G. 2005. Effect of silicon application on cadmium uptake and distribution in strawberry plants grown on contaminated soils. *Journal of Plant Nutrition* 28: 917-929.

TREDER, W. & CIESLINSKI, G. 2005. Effect of silicon application on cadmium uptake and distribution in strawberry plants grown on contaminated soils. *Journal of Plant Nutrition* 28: 917-929.

TUBANA, B.S., BABU, T. & DATNOFF, L.E. 2016. A review of silicon in soils and plants and its role in US agriculture: history and future perspectives. *Soil Science* 181: 393-411.

VAN DER VORM, P. 1987. Dry ashing of plant material and dissolution of the ash in HF for the colorimetric determination of silicon. *Communication in Soil Science and Plant Analysis* 18: 1181-1189.

VERBRUGGEN, N. & HERMANS, C. 2008. Proline accumulation in plants: a review. *Amino acids* 35: 753-759.

VERMA, S., VERMA, R., VERMA, S., YADAV, A. & VERMA, A. 2018. Impact of salt stress on plant establishment, chlorophyll and total free amino acid content of ber (*Zizyphus mauritiana Lamk*.) cultivars. *Journal of Pharmacognosy and Phytochemistry* 7: 556-559.

WANG, S., LIU, P., CHEN, D., YIN, L., LI, H. & DENG, X. 2015. Silicon enhanced salt tolerance by improving the root water uptake and decreasing the ion toxicity in cucumber. *Frontiers in Plant Science* 6: 759.

WHITMARSH, J. 1986. Mobile electron carriers in thylakoids. *Encyclopedia of Plant Physiology New Series* 19: 508-527.

WILLIAMS, D.E. & VLAMIS, J. 1957. The effect of silicon on yield and manganese-54 uptake and distribution in the leaves of barley plants grown in culture solutions. *Plant Physiology* 32: 404-409.

WINSLOW, M.D. 1992. Silicon, disease resistance, and yield of rice genotypes under upland cultural conditions. *Crop Science* 32: 1208-1213.

WUTSCHER, H. 1989. Growth and mineral nutrition of young orange trees grown with high levels of silicon. *HortScience (USA)* 24: 3.

XIN, Z. & BROWSE, J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell Environment* 23: 893-902.

XIONG, L. & ZHU, J.K. 2002. Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell and Environment* 25: 131-139.

XU, G., ZHAN, X., LI, C., BAO, S., LIU, X. & CHU, T. 2001. Assessing methods of available silicon in calcareous soils. *Communication of Soil Science and Plant Analysis* 32: 787-801.

YAMAGUCHI-SHINOZAKI, K. & SHINOZAKI, K. 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review and Plant Biology* 57: 781-803.

YAMAJI, N., MITATNI, N. & MA, J.F. 2008. A transporter regulating silicon distribution in rice shoots. *The Plant Cell* 20: 1381-1389.

YAMAJI, N. & MA, J.F. 2009. A transporter at the node responsible for intervascular transfer of silicon in rice. *The Plant Cell* 21: 2878-2883.

YAMAJI, N., CHIBA, Y., MITANI-UENO, N. & MA, J.F. 2012. Functional characterization of a silicon transporter gene implicated in silicon distribution in barley. *Plant Physiology* 160: 1491-1497.

YAMAJI, N., SAKURAI, G., MITANI-UENO, N. & MA, J.F. 2015. Orchestration of three transporters and distinct vascular structures in node for intervascular transfer of silicon in rice. *Proceedings of the National Academy of Sciences* 112: 11401-11406.

YAMAUCHI, M. & WINSLOW, M. 1989. Effect of silica and magnesium on yield of upland rice in the humid tropics. *Plant and Soil* 113: 265-269.

YAN, G.-C., NIKOLIC, M., YE, M.-J., XIAO, Z.-X. & LIANG, Y.-C. 2018. Silicon acquisition and accumulation in plant and its significance for agriculture. *Journal of Integrative Agriculture* 17: 2138-2150.

YELENOSKY, G. & GUY, C. 1977. Carbohydrate accumulation in leaves and stems of'Valencia'orange at progressively colder temperatures. *Botanical Gazette* 138: 13-17.

YELENOSKY, G. 1979. Accumulation of free proline in citrus leaves during cold hardening of young trees in controlled temperature regimes. *Plant Physiology* 64: 425-427.

YIN, L., WANG, S., LI, J., TANAKA, K. & OKA, M. 2013. Application of silicon improves salt tolerance through ameliorating osmotic and ionic stresses in the seedling of *Sorghum bicolor*. *Acta Physiologiae Plantarum* 35: 3099-3107.

YUANYUAN, M., YALI, Z., JIANG, L. & HONGBO, S. 2009. Roles of plant soluble sugars and their responses to plant cold stress. *African Journal Biotechnology* 8 (10): 2004-2010.

ZEKRI, M. 2011. Factors affecting citrus production and quality. *Citrus Industry*. University of Florida, Florida. p. 6-9.

ZHANG, C., WANG, L., ZHANG, W. & ZHANG, F. 2013. Do lignification and silicification of the cell wall precede silicon deposition in the silica cell of the rice (Oryza sativa L.) leaf epidermis? *Plant Soil* 372: 137-149.

ZHANG, Y., YU, S., GONG, H.-J., ZHAO, H.-L., LI, H.-L., HU, Y.-H. & WANG, Y.-C. 2018. Beneficial effects of silicon on photosynthesis of tomato seedlings under water stress. *Journal of Integrative Agriculture* 17: 2151-2159.

ZHU, J., LIANG, Y., DING, Y. & LI, Z. 2006. Effect of silicon on photosynthesis and its related physiological parameters in two winter wheat cultivars under cold stress. *Zhongguo nongye kexue* 39: 1780-1788.

ZHU, Y. & GONG, H. 2014. Beneficial effects of silicon on salt and drought tolerance in plants. *Agronomy for Sustainable of Development* 34: 455-472.

ZHU, Y.-X., GONG, H.-J. & YIN, J.-L. 2019. Role of silicon in mediating salt tolerance in plants: a review. *Plants* 8: 147.

ZUCCARINI, P. 2008. Effects of silicon on photosynthesis, water relations and nutrient uptake of *Phaseolus vulgaris L*. under Na Cl stress. *Biologia Plantarum* 52 (1): 157-160.