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Artigo/Article

Calcium nutrition of orange and its impact on growth, nutrient uptake and leaf cell wall

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SUMMARY

Calcium (Ca) is the most abundant nutrient in orange [*Citrus sinensis* (L.) Osbeck] and has multiple functions in relation to plant growth, cell structure and self-defense. Pot trials were conducted with orange (cv. Navelate grafted on *C. macrophylla* rootstock) to investigate the effect of Ca on growth and nutrient uptake. In the leaves, photosynthetic rate and cell wall material were measured. With increasing Ca supply, shoot and root growth as well as photosynthetic rate increased. Root volume increased from 158 to 246 cm^3/plant and fruit production from 0.95 to 1.14 kg/plant one year after transplanting. Close correlations were observed between Ca content in the leaves and fruit yield (r = 0.94); micronutrient uptake (r = 0.90[Mn], 0.73[Zn], 0.70[Cu], 0.65[B], 0.57[Mo], 0.51[Fe],) and leaf cell wall material content (r = 0.73). The higher content of cell wall material in high Ca leaves indicates higher leaf firmness and probably a lower attractiveness for sucking insects. This could reduce damage caused directly by insects and the risk of infection with vector transmitted diseases such as HLB (citrus greening). We conclude that optimization of Ca nutrition in orange is essential for better growth, optimal nutrient uptake, enhanced pest/pathogen-resistance, and high yield.

Index terms: disease resistance, hydroponic, photosynthesis, root growth, water use efficiency.

Nutrição de cálcio para laranja e seu impacto no crescimento, absorção de nutrientes e parede celular das folhas

RESUMO

O cálcio (Ca) é o nutriente mais abundante nas laranjeiras [*Citrus sinensis* (L.) Osbeck] e tem múltiplas funções em relação ao crescimento da planta, estrutura celular e autodefesa. Os ensaios foram conduzidos em vasos, com laranja (cv. Navelate enxertada em porta-enxerto de *C. macrophylla*) para investigar o efeito do Ca sobre o crescimento e a absorção de nutrientes. Nas folhas, a taxa fotossintética e o material da parede celular foram medidos. Com o aumento do suprimento de Ca, o crescimento dos ramos, raízes, bem como a taxa fotossintética aumentaram. O volume da raiz aumentou de 158 para 246 cm^3/planta e a produção de frutas de 0,95 para 1,14 kg/planta, um ano após o transplante. Foram observadas correlações estreitas entre o teor de

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Ca nas folhas e o rendimento da fruta (r = 0,94); absorção de micronutrientes (r = 0,90 [Mn], 0,73 [Zn], 0,70 [Cu], 0,65 [B], 0,57 [Mo], 0,51 [Fe]) e o conteúdo do material da parede celular da folha (r = 0,73). O maior conteúdo de material da parede celular em folhas com altos teores de Ca, indica uma maior firmeza da folha e provavelmente uma menor atratividade para insetos sugadores. Isso poderia reduzir os danos causados diretamente por insetos e o risco de infecção por doenças transmitidas por vetores, como HLB (*greening* dos citros). Concluímos que a otimização da nutrição do Ca em laranja é essencial para um melhor crescimento, absorção ideal de nutrientes, maior resistência à praga/patógeno e alta produtividade.

Termos de indexação: resistência a doenças, hidropônica, fotossíntese, crescimento radicular, eficiência de uso da água.

INTRODUCTION

Calcium (Ca) is a macro nutrient essential for plant growth. The Ca demand of certain plants particularly of Citrus species is very high. For instance, mature leaves of orange trees contain up to 7% Ca on dry matter basis (Bergmann, 1992). Calcium has several functions for plant growth and development. It plays a structural role in the cell wall and membrane systems. The majority of cellular Ca is found in the apoplast where it serves in cross-linking of pectin molecules and stabilizing the cell wall. The cross-linking function in the middle lamella helps to glue adjacent cells together. Moreover, Ca stabilizes plasma membranes by bridging phosphate and carboxylate groups of phospholipids and proteins. On the other hand cytoplasmic Ca functions as a signal transducer, coordinating cellular responses to developmental cues and environmental challenges. Furthermore, it serves to counterbalance organic and inorganic anions in the vacuoles (White & Broadley, 2003).

Calcium deficiency occurs in many citrus orchards globally, particularly in acidic and light sandy soils where Ca is leached out by heavy rainfall and irrigation water (Srivastava, 2012). Besides, poor management practices such as continuous use of ammonium containing fertilizers, particularly ammonium sulfate, accelerates Ca loss from the soil (Zekri & Obreza, 2015; Quaggio et al., 2014). Calcium deficiency in citrus can be observed as chlorotic leaf margins and interveinal regions, which may hamper leaf photosynthesis and crop productivity. Under severe Ca deficiency, trees may develop, necrotic shoot meristems, twig dieback and multiple bud growth of new leaves. However, plant growth and fruit yield can be reduced by inadequate Ca supply long before deficiency symptoms become evident (Zekri & Obreza, 2012).

One of the signaling roles of Ca is illustrated in the regulation of stomata closure. Schwartz et al., (1988) observed that the stomata of epidermal cells of *Commelina communis* failed to close properly when apoplastic Ca concentration was reduced. This may have serious consequences on crop water productivity. In the present study, we investigated the role of Ca supply on photosynthesis capacity and water use efficiency at leaf level. In addition, the effect of Ca nutrition on root growth of orange trees was investigated. Furthermore, the significance of Ca for cell wall development was studied and its implication for disease and pest resistance in orange orchards is discussed.

MATERIALS AND METHODS

Plant material and growing conditions

Two-year-old sweet orange plants (*Citrus sinensis* cv. Navelate) grafted on *Citrus macrophylla* rootstock, were grown in a greenhouse at the research center Hanninghof, Germany, to study the effect of Ca supply on growth, nutrient uptake and leaf cell wall characteristics. Young orange plants were transplanted to 10-liter pots filled with washed sand. Pots were watered with a complete nutrient solution containing N (11 mM), P (0.6 mM), K (6.4 mM), Mg (4.1 mM), S (1.4-5.3 mM), Fe (40 µM), Mn (22 µM), Zn (15 µM), Cu (0.5 µM), B (46 µM), Mo (0.5 µM) but treated with different Ca concentrations, namely: 0, 1, 2, 3 and 4 mM, with four replications. The pH of the nutrient solution was adjusted to 6.2. Pots were kept at 70% water holding capacity and were regularly leached out to avoid salt accumulation in the root zone. Plants were grown in a glasshouse at 24-30°C day and 18-22°C night temperature, and 60-70% relative air humidity. Supplemental light of 300 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) with a 14 h light period was used when the natural light became insufficient. Leaf samples were collected periodically for monitoring of Ca status and plant nutrient analysis. After first fruit set and maturity, plants were harvested and separated into leaves,

stems and fruits; and the fresh weight of each fraction was determined. Fruit were divided into two sections: upper and lower hemispheres, and further dissected into outer peel (exocarp), inner peel (mesocarp), segment walls, and fruit pulp (juice sac) to study the distribution of Ca within the fruit. All plant parts were dried in an oven at 65°C for a few days till constant weight was attained. The dried plant materials were finely ground for nutrient analysis.

A separate trial was conducted in a hydroponic culture to investigate the effects of Ca supply on root growth and nutrient uptake. Orange seedlings (*C. sinensis* cv. Navelate, grafted on *C. macrophylla* rootstock) were grown in 30-liter pots filled with continuously aerated nutrient solution containing N (5.5 mM), P (0.3 mM), K (1.9 mM), Mg (1.4 mM), S (0.5-2.4 mM), Fe (20 μM), Mn (11 μM), Zn (7.5 μM), Cu (0.25 μM), B (23 μM), Mo (0.25 μM) but with four Ca supply levels: 0.1, 0.5, 1 and 2 mM, with eight replications. The nutrient solution was changed every fourteen days. The pH of the nutrient solution was maintained between 5.5 and 6.5 by daily monitoring and titrating with diluted acid (H_2SO_4) or base (NaOH). Leaf samples were collected, dried, and finely milled for nutrient analysis.

Root growth measurement

After 90 days of treatment in hydroponic culture, root growth was visually examined and root volume was measured by the displacement method. For nondestructive measurement of root volume, two identical glass cylinders were connected by a tube at the base and half-filled with water. One of the cylinders was placed on a weighing scale but the second one was placed on a box at equal height. Plant root was immersed into the second cylinder which caused displacement of water. The displaced water equally spreads into both cylinders. Thus, change in weight of first cylinder is equal to half of the water displaced. Therefore, root volume was calculated as:

$$Root\ volume\left(cm^3\right)=\frac{2\times change\ in\ weight\ of\ \ first\ cylinder\left(g\right)}{density\ of\ \ water\left(g/cm^3\right)} \quad (1)$$

Leaf gas exchange measurements

Photosynthetic rate, transpiration rate, stomatal conductance and instantaneous water use efficiency of the youngest fully expanded leaves were measured in pot grown plants using the Li-6400 Portable Photosynthesis System (Li-Cor Biosciences, Inc., Lincoln, NE, USA) at a photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1} PAR (400-700 nm) in a leaf chamber Li-6400-02B. Measurements were made after 3 min equilibration in the cuvette between 10:00 am and 3:00 pm with a cuvette temperature of 25°C and the CO_2 concentration of 370 μmol mol^{-1}.

Nutrient analysis

Finely milled plant materials were used for elemental analysis after wet digestion in a microwave digester (MLS 1200 mega; MLS GmbH, Leutkirch, Germany). All micro and macro nutrients (excluding nitrogen) were analyzed using inductively coupled plasma optical emission spectrometry (Perkin-Elmer Optima 3000 ICP-OES; Perkin-Elmer Corp., Nerwalk, CT, USA).

Cell wall analysis

Leaf cell wall analysis was done at the Institute of Horticultural Production Systems, Leibniz Universität Hannover, according to the method described in Harrison et al. (2009). Briefly, freeze-dried leaves were milled and extracted with 50 mM tricine buffer (pH 8.1) containing 1% PVP40 (Polyvinylpyrrolidone, average molecular weight 40000). The sample was vortexed, centrifuged at 12000xg for 5 min and the supernatant was removed. The pellet was re-suspended in a tricine buffer without PVP but containing 1% sodium dodecyl sulfate (SDS), incubated at 90°C for 5 min, then centrifuged at 12000xg for 5 min. This step was repeated, followed by two washes with 0.2 m KOH, two washes with deionized water and finally two washes with ethanol. In the end, the pellet was then oven-dried at 80°C and the remaining dry mass of the pellet was recorded which was assumed to represent the leaf cell wall as insoluble leaf structural material.

RESULTS

Calcium uptake

Calcium uptake and concentration in plant tissue increased with increasing Ca supply (Figure 1). Tissue Ca concentration greatly varied among plant parts. Leaves and stems contain large quantities of Ca compared to

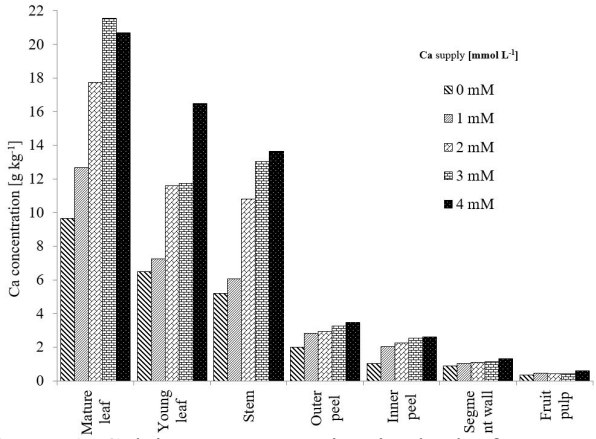

Figure 1. Calcium concentration in the leaf, stem and different fruit parts of orange grown in pot with increasing Ca supply levels. Bars are means of four independent replications.

the fruit. Calcium concentration in mature leaves was about ten times higher than the concentration in the fruit. Within a fruit, calcium concentration was fairly uniform when upper (proximal end) and lower (distal end) fruit hemispheres were compared, hence the data was averaged.

Leaf photosynthesis and tree productivity

Calcium supply had a significant effect on leaf gas exchange. Leaf photosynthesis rate, stomata conductance and transpiration rate increased with increasing Ca supply (Figure 2 a, b, c). Instantaneous water use efficiency which was measured as a ratio of photosynthesis to transpiration, was not affected by Ca supply (Figure 2d). Enhanced leaf photosynthetic activity further contributed to better fruit

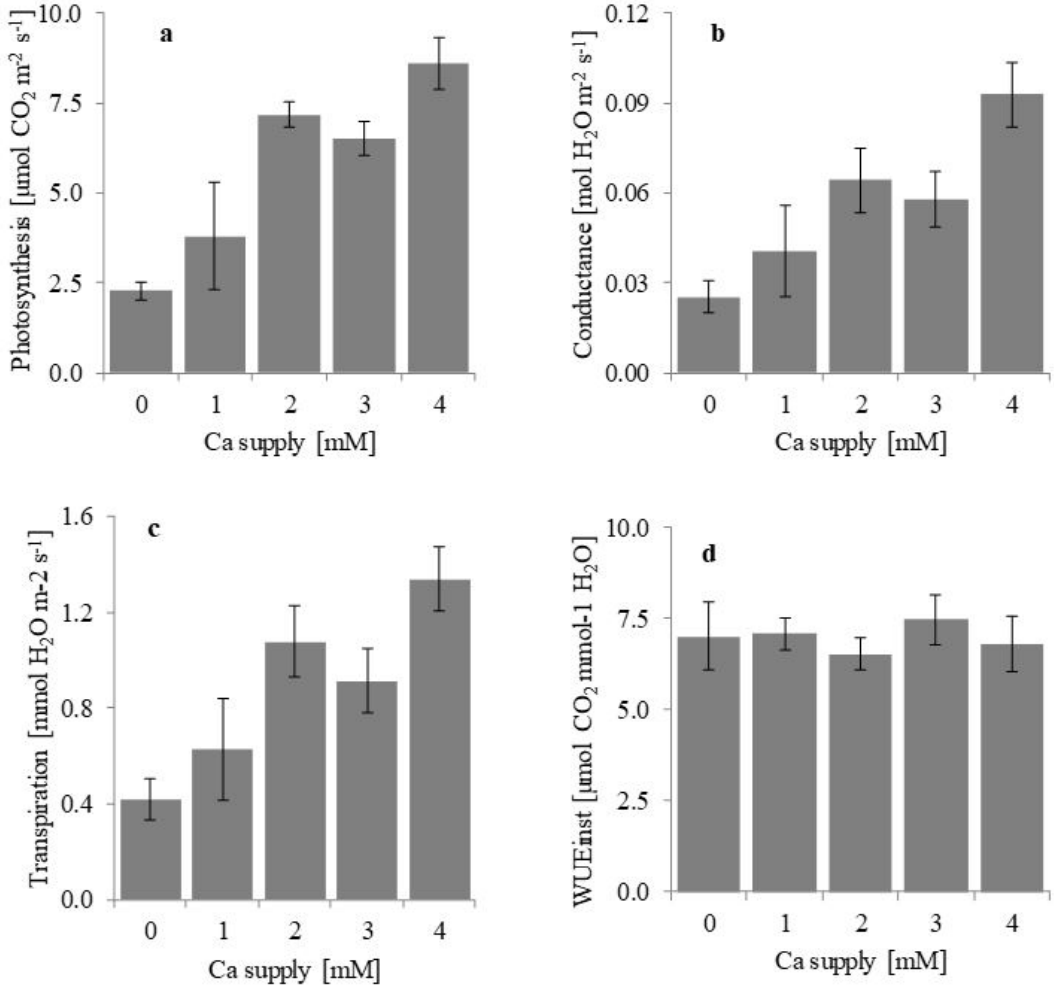

Figure 2. Photosynthetic rate (a), stomata conductance (b), transpiration rate (c), and instantaneous water use efficiency (d) of orange plants grown in pot at different levels of calcium nutrition. Bars are means ± SE of four independent replications.

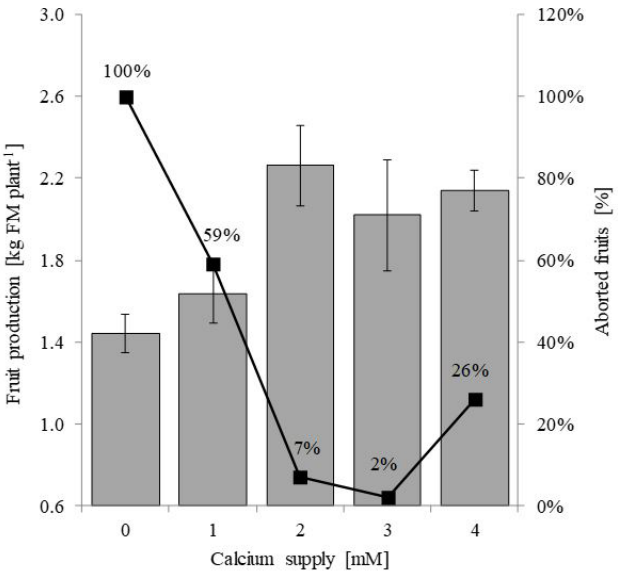

Figure 3. Total fruit production in young orange trees grown in pot at different Ca supply levels. Fruits aborted before harvest were collected, weighed and percentage of aborted fruits was calculated as ratio of fruits aborted before harvest date to the total fruit produced per plant. Data are means ± SE of four independent replications. FM = fresh mass of orange fruits.

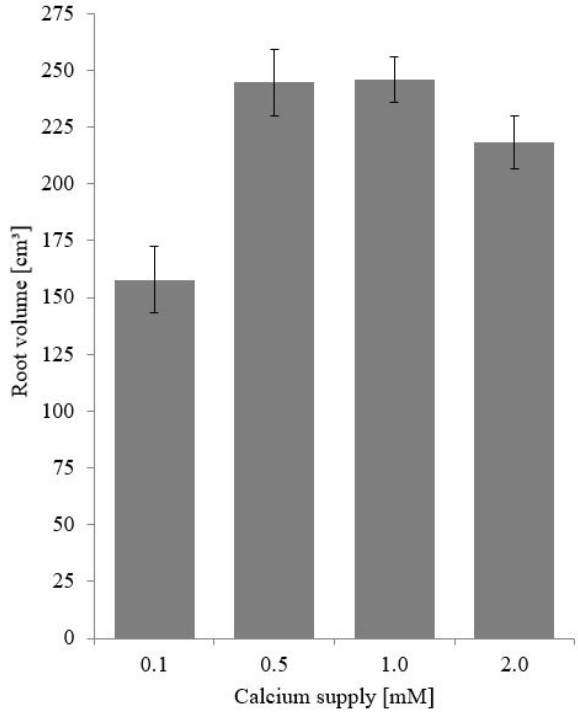

Figure 4. Root growth of orange grown in hydroponic solution at different Ca supply levels. Bars are means ± SE of eight independent replications.

production (Figure 3). At lower Ca-treatments, a larger percentage of fruits were dropped before harvest. A higher rate of premature fruit drop was thus an additional reason for lower yield of Ca deficient trees.

Root growth and nutrient uptake

Non-destructive root volume was measurement to evaluate the effect of Ca supply on root growth of orange in a hydroponic trial (Figure 4). Root growth sensitively responded to Ca level in the nutrient solution. Calcium deficiency resulted in a higher root tip mortality and decaying of the root system. In contrast, healthy and prolific root formation was observed in plants that received Ca treatment. After 8 weeks of treatment duration, the root growth of Ca deficient plants was only about two-thirds of well-supplied plants. Not only the root mass but also the root activity, specifically the uptake micronutrients was affected by Ca supply. Micronutrient concentration in the leaves positively correlated with Ca content (Figure 5). The correlation coefficient was higher for Mn than for other micronutrients analyzed. On the other hand, no effect of Ca supply on leaf tissue concentration of macronutrients such as N, P, and K was observed. However, Mg concentration in the leaf tissue was reduced with increasing Ca supply.

Calcium nutrition and the cell wall

The largest proportion of Ca taken up by the plant goes to the leaf (Figure 1) where it serves as the integral part of leaf cell wall. Therefore, leaf cell wall substance was isolated and its weight was determined to study how Ca supply influences cell wall development in oranges. A close linear correlation was observed between leaf Ca content and cell wall dry matter (Figure 6).

DISCUSSION

Calcium requirement of citrus trees is relatively high compared to other tree crops. An orange grove with a standing biomass of about 28 ton DW ha^{-1} requires more

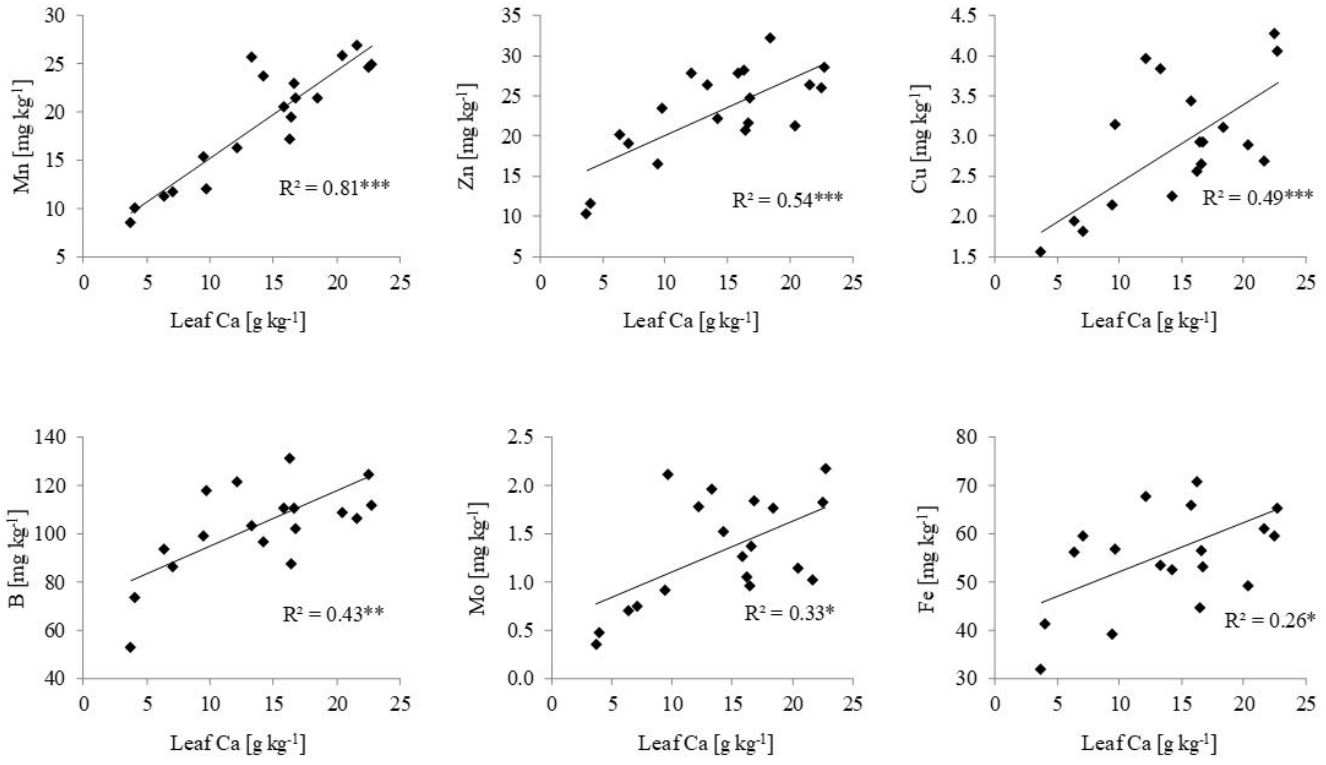

Figure 5. Relationship between plant Ca status and micronutrient concentration in the leaves of orange plants grown in hydroponic solution at different Ca supply levels. *, **, ***denote significant correlation at $p < 0.05$, 0.01, 0.001, respectively.

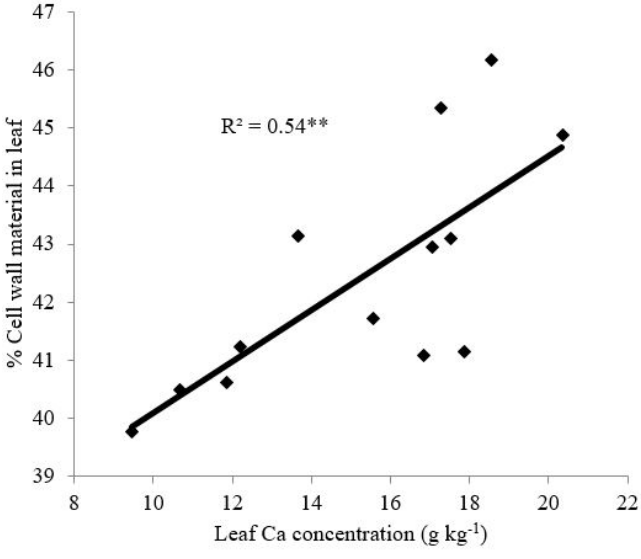

Figure 6. Relationship between leaf Ca status and leaf cell wall dry weight of orange plants grown in pot at different Ca supply levels. **denotes significant correlation at $p < 0.01$.

than 600 kg/ha Ca (Roccuzzo et al., 2012). About two thirds of the total Ca taken up by the tree is located in the leaf. For optimal growth and fruit production, mature orange leaves are expected to contain 3.0 to 4.9% Ca in the dry weight (Koo, 1984). However, leaf Ca content measured in the current trial was slightly lower than in field-grown orange trees. This could be attributed to the relatively low transpiration of trees grown in glasshouse. The uptake of Ca is closely linked to transpiration (Gilliham et al., 2011) as it is evident from higher Ca contents in the transpiring plant organs, particularly the leaves, as compared to less transpiring organs such as the fruits.

Calcium has multiple functions in the plant tissue structure and physiological processes. Consequently, many growth disorders appear as a result of Ca deficiency. Citrus leaves with Ca deficiency exhibit low chlorophyll content (Lavon et al., 1999) which is in line with the chlorotic appearance of the Ca-deficient leaves. Moreover, Ca is required for proper functioning of the photosynthetic enzyme, Rubisco. Dolatabadian et al. (2013) observed

that Ca supply hindered Rubisco degradation under stress conditions. Similarly, Lavon et al. (1999) measured a lower level of Rubisco and its enzyme subunits in Ca-deficient citrus leaves. Thus, low photosynthesis capacity of Ca-deficient orange leaves was the consequence of abnormalities in the photosynthetic apparatus of the leaf.

The signaling role of Ca in controlling stomata movement is well documented (Schroeder & Hagiwara, 1989; Gilroy et al., 1991; Lemtiri-Chlieh & MacRobbie, 1994, McAinsh & Pittman, 2009). For a steady state opening of the stomata, Ca must be excluded from the cytoplasm of guard cells, but stomata closure is stimulated by high Ca in the cytosol. Opening of the stomata is necessary to take in CO_2 from the atmosphere for photosynthesis. However, the uptake of CO_2 is coupled with a loss of large quantity of water from the plant. Therefore, opening and closing of the stomata is finely regulated in terrestrial plant species. In order to prevent water loss, the stomata are normally closed under dark conditions when photosynthesis is not taking place. Deprivation of Ca from guard cells results in failure of stomata closure (Schwartz et al., 1988). This may lead to unproductive loss of water and consequently low water use efficiency of crops suffering from Ca deficiency.

The effect of Ca on crop water use efficiency is not limited to stomata regulation. Calcium is also necessary for healthy root development. Root growth is severely inhibited under Ca deficiency (Marschner & Richter, 1974) and the root tip may stop growth under severe Ca deficiency, resulting in poor root system. Poor and shallow growing roots cannot effectively take up water from the soil leading to drainage losses and low water use efficiency.

In addition to water uptake, the root is also responsible for nutrient uptake. Calcium supply increased the uptake of micronutrients as observed in this study. Similarly, López-Lefebre et al. (2001) found a positive effect of Ca supply on micronutrient uptake. The uptake of nutrients is affected by root mass and root activity both of which are affected by Ca supply. Calcium promotes vigorous root growth which provides more surface area for nutrient absorption. In addition, it also controls the activity of ion channels which are responsible for the uptake and transport of nutrients in the cell (Cox, 2011). Thus, the positive effect of Ca on the uptake of micronutrients could be due to increased root biomass and/or improved activity of ion channels which transport nutrient ions into the cell.

The majority of plant tissue Ca resides in the cell wall and provides structural stability. In contrast, Ca deficiency causes disintegration of the cell wall and collapse of tissues, such as petioles, shoot apices and fruits (Shear, 1975; Ho & White, 2005), hence, a higher percentage of fruit drop of oranges grown at low Ca supply. In this study, a close positive correlation was observed between leaf Ca content and cell wall dry matter of orange leaves. Moreover, electron microscopic investigations indicated a higher cell wall thickness of citrus leaves which are sufficiently supplied with Ca (Quaggio, 2016)[1]. Taken together, these underline the crucial role of Ca in cell wall strength and tissue stability of citrus.

Calcium plays a significant role in plant disease resistance (plant self-defense). Plant pathogens first degrade the cell wall before entering into the cell. Degradation of the cell wall is mediated by polygalacturonase, an enzyme which is released by the pathogen and digests the pectin of the cell wall. Calcium strongly inhibits this enzyme (Wehr et al., 2004) conferring disease resistance to the plant. This has been observed in a number of studies. Adequate soil Ca was needed to protect peanut pods from infections by *Rhizoctonia* and *Pythium* and application of Ca to the soil diminished the occurrence of these diseases (Huber, 1980). Calcium confers resistance against *Pythium*, *Sclerotinia*, *Botrytis* and *Fusarium* (Graham, 1983). Springer et al. (2007) also observed that Ca supply strongly reduced rust fungus (*Melampsora lini*) infection rate of flax plants grown on Ca deficient soils of serpentine ecosystem. These observations indicate the significance of Ca nutrition for better crop health.

Huanglongbing disease (HLB), commonly called citrus greening, devastates many orange plantations worldwide (Gottwald et al., 2007). It is caused by phloem-limited bacteria *Candidatus* Liberibacter ssp and transmitted by insect vector, citrus psyllid (*Diaphorina citri*) which prefers to feed on soft leaves (Grafton-Cardwell, 2016). Symptoms of HLB affected trees include chlorosis/mottling of the leaves, yellow shoot, vein corking, stunted growth, suppression of new root growth, and most economically important, low yield, also the fruits are smaller, green, deformed, with aborted seeds (Brlansky et al., 2010). Some of the symptoms described above were similar to Ca deficiency observed in our trials. Spann & Schumann (2009) found low Ca, Mg and B content in the leaves of trees affected by HLB as compared to healthy trees. In view of its cell wall stabilizing effect, Ca nutrition promotes the formation of firm leaves and may contribute to resistance against psyllids infestation. Besides, Ca may

[1] Quaggio JA (2016) Personal communication March 2016.

counteract the adverse effect of HLB on root growth in infected trees. There is increasing evidence that balance nutrition can mitigate HLB symptoms and prolong the tree health and productivity and we hypothesize that Ca nutrition specifically plays a significant role.

In summary, we observed that Ca supply increased root and shoot growth, nutrient uptake, leaf cell wall stability, and fruit production of orange trees and thus conclude that optimal Ca nutrition is crucial for better crop health and high productivity of orange orchards.

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