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FACTORS INVOLVED IN SOLUBLE SOLIDS ACCUMULATION IN CITRUS FRUITS

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Abstract. **The direction of photosynthate movement among plant organs is determined by "sink strength", a model involving the importation of sucrose (or other sugars) by hydrolysis or sequestration. In most plants, sink strength is determined by the activities of two sucrose-cleaving enzymes: sucrose synthase(SS) and invertase(INV). In addition, activities of sucrose phosphate synthase(SPS), sucrose phosphate phosphatase(SPP), and tonoplast-bound ATP*ase* could affect sink strength. Increased activities of membrane-bound sucrose transporters or decreased vacuolar pH could also enhance accumulation of soluble solids. In fruits of many commercial crops, increases in soluble solids have been recorded during drought conditions. This research investigates the determinants of citrus fruit sink strength through drought stress. Potted Hamlin orange trees were grown under watered or drought-stressed conditions and fruit harvested and analyzed for Brix and acids. Stressed fruits had higher acid content and soluble solids, and lower pH than controls. The following components of fruit sink strength were measured: SS, INV, SPS, SPP, ATP*ase*, PP*ase*. In addition, isolated and purified membranes from fruit were tested for the presence of a sucrose symport at the plasmalemma and an antiport at the tonoplast. Increased sink strength appeared to be the result of SS, since SS activity was higher in drought-stressed versus well-watered fruit. Activities of other enzymes and transporters were not significantly different between control and treated fruit. We concluded that SS is the predominant factor controlling Brix levels in citrus fruit, although the altered pH could have contributed to sink strength by enhancing acid hydrolysis.**

It is well known that some temperate fruits accumulate higher levels of soluble solids during mild drought stress (Behboudian and Mills, 1997). Since fruit quality and production is not compromised by mild late-season drought but rather enhanced, this issue has attracted the interest of many researchers and fruit producers. The mechanism involved is more than concentration by dehydration as there is active accumulation of solids in fruit (Mills et al., 1996; Yakushiji et al., 1996). Increasing fruit soluble solids during drought involves one or more of the following processes, such as sugar movement, accumulation, and/or storage into fruit. However, the precise mechanisms are still unclear.

The direct movement of assimilated carbon into a particular plant organ is determined by its 'sink strength' and by photosynthesis in source tissue. 'Sink strength' is the ability of a particular organ to attract photoassimilates (Ho, 1988). During fruit elongation and expansion, fixed carbon is required to provide growing tissues with energy for metabolism and to provide osmotic solutes to maintain turgor pressure. Fixed carbon is transported through the phloem in the form of sucrose, a disaccharide composed of joined molecules of fructose and glucose. To develop a concentration gradient for adequate sink strength, cells must cleave sucrose, or effectively sequester it into the vacuole as in sugar beets (Getz et al., 1991). Therefore, sink strength is determined by the ability of the sink to metabolize sucrose and/or by its capacity for compartmentation and storage. In plants, there are two specific enzymes capable of cleaving sucrose. The first is invertase (INV), whose unidirectional catalytic action yields fructose and glucose. The second enzyme is sucrose synthase (SS) with a reversible reaction using sucrose and UDP to yield UDP-glucose and fructose. Sequestering sucrose in vacuoles permits the sink cell to maintain a sucrose gradient between itself and the phloem, allowing the continuous movement of sucrose toward the sink.

Many storage organs appear to require the resynthesis and storage of sucrose into the vacuole in a seemingly 'futile' cycle (Ho, 1988). Related sucrose-metabolizing or synthesizing enzymes that may play a role in sink strength are sucrose phosphate synthase (SPS, UDPG + F-6-P↔S-6-P + UDP) and sucrose phosphate phosphatase (SPP, S-6-P→Sucrose + Pi), although their exact functions in a sink organ are uncertain. Other factors such as low vacuolar pH may be involved in sink strength, since low pH can cleave sucrose (Wienen and Shallenberger, 1988). In citrus, the vacuoles of fruit juice cells can be extremely acidic with a pH of 3 or lower (Echeverria and Burns, 1989). Such low pH is capable of hydrolyzing sucrose into glucose and fructose *in vitro* (Wienen and Shallenberger, 1988). That same low pH could automatically cleave sucrose entering the vacuole at a rate dependent on the hydronium ion concentration and temperature. A fruit cell could use a sucrose antiport at the tonoplast to sequester sucrose into the vacuole, utilizing the existing ýpH (Getz et al., 1991).

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Drought-stress provides a tool to alter sink strength. Why is sink strength actively increased in fruit during drought-stress and by what mechanism does it occur? Understanding these critical factors of sink strength could lead to improved fruit quality and aid in breeding and molecular work with citrus fruit. Understanding the mechanisms at work in sink strength and stress physiology of plants would allow this work to be applicable to other crops.

Materials and Methods

Fifty 3-year old potted 'Hamlin' orange [*Citrus sinensis* (L.) Osbeck] trees grafted on Troyer citrange [*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] in 7-gal pots were purchased in June 1999 from a local nursery. Trees were selected for the presence of 10 to 15 fruit each and were monitored for pest and disease problems throughout the season. All trees were kept outdoors at the University of Florida Citrus Research and Education Center in Lake Alfred, Florida on black landscape tarp to prevent weed growth around the pots. Before experimentation, all trees were trimmed and pruned to develop similar leaf to fruit ratios. In late August and early September, five trees were set aside and monitored for water stress according to specific volumes of water applied each day, ranging between 200 mL and 1000 mL. It was concluded from leaf water potential data and environmental conditions that 1000 mL water per d constituted well-watered plants, while 500 mL or less would be adequate to impose measurable drought stress. Volumes were modified according to environmental fluctuations and rainy periods. A microsprinkler was placed in each pot and trees were deficit-irrigated for development of water stress. On the few rainy occasions, drought-stressed tree pots were covered with plastic bags to prevent entrance of excess water. The drought stress was imposed beginning 25 Oct. 1999 and continued until 24 Dec. 1999. Leaf water potential was measured about every 7-10 days using a pressure bomb chamber and nitrogen gas, in order to monitor drought stress on trees. Two leaves from each of five control or stressed trees were selected for similar canopy position and size, wrapped at dawn in black plastic and aluminum foil, then removed from the trees after 2 hrs and analyzed immediately.

Fruit were harvested every second week beginning in mid-Oct. through the end of Dec., with a follow-up data point in later Jan. Five fruit were randomly selected, one from each of five trees, on the morning that data was to be taken. This occurred consecutively for four days, taking fruit from drought-stress or control trees, repeating each twice using the same trees. After fruit were harvested, trees were removed from the experiment. Fruit were immediately taken to the lab and subjected to fruit quality and enzyme analysis. Each fruit was weighed and cross sectioned at the equator. Thirty mL of a cold, stirring buffer (0.5 M Mops 8.0, 1.5% w/v PVP, 250 mM sucrose, 7.5 mM EDTA, 0.1% BSA, 14 mM mercaptoethanol) was ready in a beaker at a measured volume of 30 mL. Juice was extracted from one half of the fruit using a hand-held juicer and 15 mL of this juice were immediately poured into the cold buffer. Fifteen mL of the remaining juice were placed in a large centrifuge tube. The second half of the fruit was treated similarly and 15 mL were added to the 45 mL of stirring buffer and juice. The remaining juice was treated as with the other half, added to the same test tube. The buffer/juice mixture (60 mL) was poured through a cheesecloth/nylon mesh into a cold beaker on ice. This procedure was repeated with all five oranges, adding the buffer/juice solutions together for a total volume of ~300 mL and stirring well with 1 mL of PMSF (100 mM PMSF in 95% EtOH, 5% 2-Propanol). The buffer/juice extract solution was used for enzyme assays and isolation of cell compartment membranes. Fruit juice was set aside for fruit quality analysis.

A subsample of buffered juice extract (5 mL) was used for enzyme assays. This solution was centrifuged at 30,000 rpm for 30 min. at 4°C. After centrifugation, 2.5 mL were desalted through a Sephadex PD-10 pre-equilibrated column with 10mM Hepes, 2 mM DTT, 1 mM $MgCl_2$ and 3.0 mL were collected for enzyme assays. Untreated juice was filtered through a nylon mesh to remove debris, then °Brix (soluble solids concentration), pH, and % acid were measured individually for each of the 5 fruit juice extracts. Unbuffered juice (15 mL) was used to measure pH and titrated to pH 8.0 for percent acid determination using standard alkali solution (0.3125 N NaOH).

Isolation and purification of Hamlin vesicle membranes was achieved using the method of Echeverria et al. (1997b). Vesicles were re-suspended in buffer at pH 7.5 in ~500 µL and stored at –80°C. Freeze/thaw cycles were performed as previously described by Echeverria et al. (1997a). Protein concentration in extract and vesicles was determined by the Coomassie Blue method of Bradford (1976).

Enzyme Assays

All assays were performed at 30°C in a water bath as follows:

Sucrose Synthase. The reaction mixture contained 100 µL enzyme extract, 100 mM Hepes (pH 7.2), 2 mM UDPG, 10mM Fructose, and 2 mM $MgCl_2$ in a final volume of 500 µL. Aliquots of 100 µL were taken at 0, 20, 40, and 60 min into 100 µL of 30% KOH and boiled for 10 min. Sucrose was analyzed by the anthrone method of van Handel (1968).

Sucrose Phosphate Synthase, Sucrose Phosphate Phosphatase. Both reactions were assayed as described by Echeverria et al. (1997b).

Invertases. Invertases were assayed in a solution containing 0.5 mL enzyme extract, 100 mM BTP/Mes (pH 7.5) or sodium acetate (pH 5.0), and 100 mM sucrose with a final volume of 2.0 mL. Assays were developed using the glucose oxidase method Kilburn and Taylor (1969).

V-ATPase, V-Pyrophosphatase. Both enzyme activities were determined as in Marsh et al. (2000) and product measured using the method of Chifflett et al. (1988).

Sucrose uptake experiments: Sucrose uptake into tonoplast vesicles was performed as in Echeverria et al. (1997a) with limes.

Results

Drought-stressed trees had lower leaf water potentials (Fig. 1). The only exception occurred on d 21 and 43 during brief periods of high relative humidity and rainy weather. Titratable acids were higher in drought-stressed fruit over the duration of the experiment and maintained this difference until late in maturity (Fig. 2). Similarly, pH remained lower in the drought-stressed fruit (Fig. 3). Juice soluble solids concentrations (SSC) over the course of the season were slightly higher for drought-stressed tree fruit than for well-watered controls (Fig. 4). The largest increase in SSC occurred between the third and fourth weeks after beginning drought stress and was maintained for the rest of experiment. This indicates an increase in sink strength of the drought-stressed fruits since the increase in soluble solids was active and not due to dehydration (Yakushiji et al., 1996).

Data from enzymatic analysis suggest that only one of the tested enzymes played a major role in the change in sink strength. Invertase activity was barely detectable at the initiation of the experiment and dissapeared altogether thereafter (data not shown).

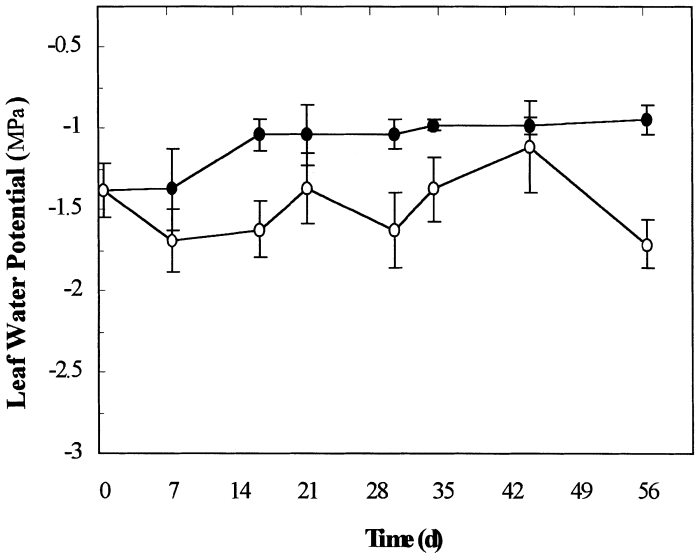

Figure 1. Leaf water potential of control (•) and drought-stressed (o) trees showing decreased values as a result of water stress (n = 10, ±SE).

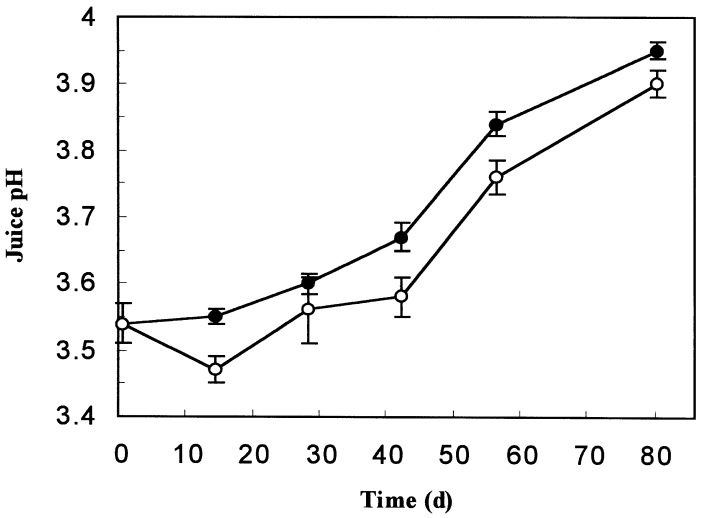

Figure 3. Seasonal changes in pH of juice (vacuolar pH) with drought-stress fruits (o) maintaining lower pH than control fruits (•) (n = 10, ±SE).

Sucrose synthase activity increased after imposition of water stress (Fig. 5), while SS from well-watered fruit decreased. SPS activities differed very little and followed no interpretable pattern (data not shown). In contrast, SPP activity was consistent and increasing in both extracts as the season progressed (data not shown). V-ATP*ase* and V-PP*ase* activities revealed no treatment effects. However, PP*ase* activity did show a seasonal increase in activity per mg protein, but not when expressed per gfw. Neither ATP*ase* nor PP*ase* activities showed treatment differences and therefore are not shown here.

Membrane analysis for the presence of sucrose transporters suggests the presence of two types of active transporters for sucrose within juice cells. Firstly, a sucrose symport appears to be present at the plasmalemma. The second transport (antiport) can be found at the tonoplast. Research on both active transporters suggests that activities of either are not significantly altered as a result of the treatment. However, their existence, regulation, location, and function is critical to the sink strength of citrus fruit and its ability to import carbohydrates.

Discussion

The present study indicates that sink strength in citrus fruits can be altered by the imposition of a mild water stress. Juice quality data were found to be in concurrence with previously published data in apples (Ebel et al., 1993; Mills et al., 1996), kiwifruit (Miller et al., 1998), strawberry (Pomper and Breen, 1997), and mandarins (Yakushiji et al., 1996, 1998), where mild, late-season drought stress upregulates osmoregulation in fruit. Consequently, fruit osmoregulation and changes in sink strength increase juice quality indices. Comparable to previous work by Yakushiji et al. (1996, 1998), reduced irrigation led to enhancement of fruit quality indices and decreased rates of acid metabolism. Total soluble solids, measured as °Brix, increased over the course of fruit maturation, with drought-stressed fruits maintaining slightly higher levels of soluble solids after establishment of water stress. These results are in accordance with Yakushiji et al. (1996, 1998) working with 'Satsuma' mandarins. Regulated deficit irrigation has been shown to significantly increase fruit soluble solids without jeopardizing

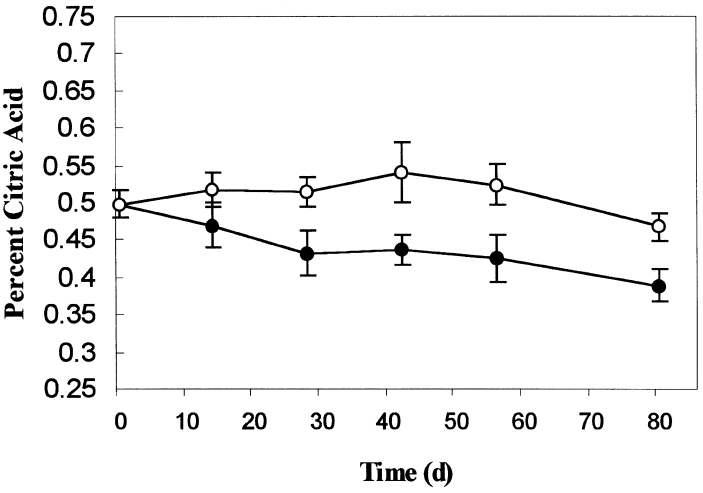

Figure 2. Titratable acids in Hamlin fruit from control (•) and drought-stressed (o) fruits indicating maintenance of acids during stress ±SE (n = 10).

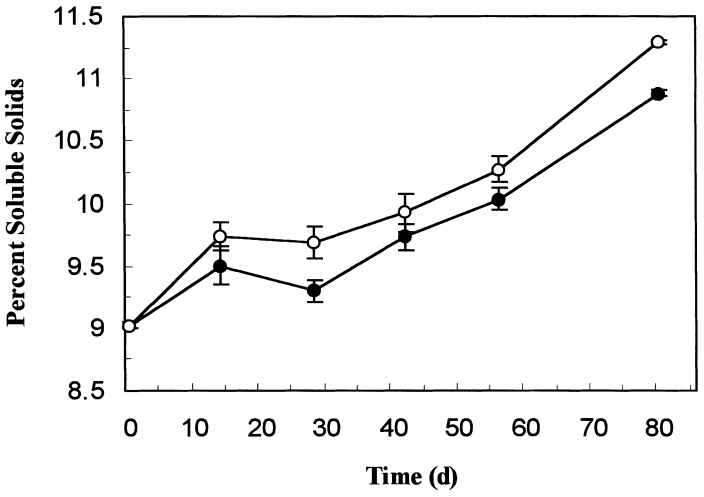

Figure 4. Soluble solids concentration as measured by °Brix in control (•) and drought-stress (o) tree fruit measured during ripening (n = 10, ±SE).

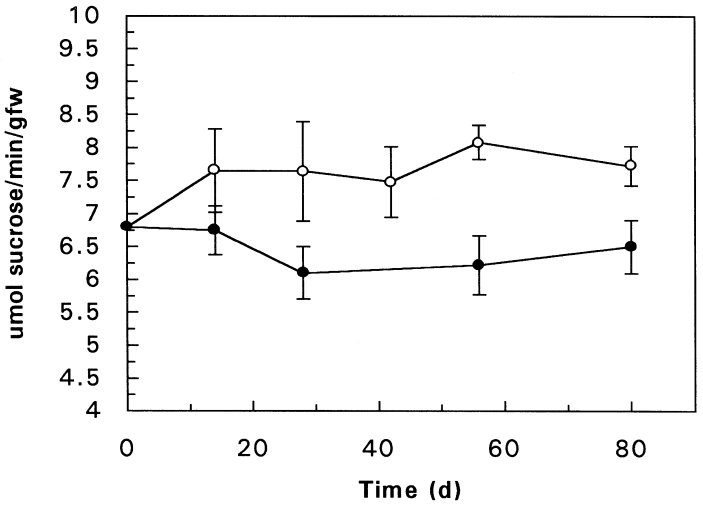

Figure 5. Sucrose synthase activity from control (•) and drought-stressed (o) Hamlin orange fruit. Data are the mean of four assays per treatment, ±SE.

fruit size and juice content if drying conditions are imposed towards the end of fruit expansion in citrus (Yakushiji et al., 1996; 1998) as well as in other fruits (Caspari et al., 1994; Mills et al., 1996). Consequently, the flavor and maturity qualities of citrus fruit are positively altered by mild drought stress.

Of the potential enzymes thought to be involved in sink strength, differences were only measured for SS. Maximal activities of other enzymes did not apparently affect the sink's ability to attract photoassimilates. The negligible activity of acid invertase agrees with previous research showing it decreases during the course of fruit maturation (Kato and Kubota, 1978; Lowell et al., 1989), the point at which our research began. Neutral invertase never yielded measurable activity. Therefore, enzymatic cleavage of sucrose by INV does not seem to be involved in increases in sink strength. SPS activity varied slightly during the experiment but followed no particular pattern and showed no treatment differences. SPP, on the contrary, increased in activity as the fruit matured. Therefore, INV, SPS, and SPP seem to have little effect on increased sink strength during drought stress, or a major role in this stage of ripening.

Sucrose synthase, the other major determinant of sink strength (Ho, 1988), showed an immediate increase in activity in drought-stressed trees. The change in SS activity could have a direct effect on sink strength of fruit by cleaving incoming sucrose from the phloem and maintaining a gradient of disaccharide import. Since all trees were provided with the same quantity of water before the start of the experiment, it is possible that SS functions in osmoregulation. SS activity remained more or less constant in well-watered trees as fruit matured (Fig. 5).

Although increased SS activity is, in effect, a change in sink strength, we find it difficult to explain why elevated SS activity would increase total soluble solids in stressed fruit vacuoles without a parallel or subsequent increase in other metabolic pathways. Increases in SS activity in a sucrose-storing tissue would consequently require a cycle with no apparent advantages to the plant, instead requiring greater energy input. SPS activity was unchanged as a result of the treatment and remained very low, suggesting resynthesis of sucrose by SPS and SPP is not a major factor. While increasing SS is certainly a feasible pathway in starch or oil storing cells as suggested by Ho (1988), it is not likely to be operative in citrus fruit.

The decrease in vacuolar pH in juice cells (Fig. 3) has a large effect on the cleavage of sucrose and its import from the phloem and into the vacuole. Sugar analysis data (unpublished) on drought-stressed 'Valencia' orange fruit show no difference in sucrose concentration when compared to control fruit. However, glucose and fructose concentrations both increased in drought-stressed fruit in a 1:1 ratio. Consequently, hydrolysis of sucrose must be occurring, leading to the higher °Brix and to the equivalent values of the two hexoses. Acid-mediated hydrolysis of sucrose occurs at pH 3.0. Increased cleavage or hydrolysis of sucrose would increase the osmoticum of the vacuole, maintaining water pressure while simultaneously increasing sink strength. In addition, increasing the ýpH between compartments enhances the catalytic efficiency of sucrose transporters. Therefore, it appears that both pH and SS are intimately involved in increasing fruit sink strength. Decreasing vacuolar pH requires either increased activity of vacuolar ATP*ase* or higher concentrations of ATP. We did not observe increases or changes in ATP*ase* or PP*ase* activity. However, the increased SS activity in drought-stressed fruit could be a pathway used by the cell to upregulate metabolic pathways for ATP production.

Enzymatic data, in conjunction with analysis of other fruit compositional data (pH and acids), lead us to conclude that a decrease in vacuolar pH observed in drought-stressed fruit is the main factor in increased soluble solids concentrations when compared to controls. Higher levels of SS are required for the metabolic production of ATP which in itself energizes H^+ pumps at the tonoplast.

In conclusion, a potentially novel method for osmoregulation and increased sink strength is apparently present in citrus. The importance of SS in sink strength is supported by our research, confirming its critical role in sugar metabolism. However, although SS continues to be a major component in sink determination, we conclude that other factors must be included in understanding sucrose movement. This is especially true in the case of accumulating plant organs where a large ýpH is present across the tonoplast, although certainly other vacuolar characteristics may play as yet unrecognized roles.

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EVALUATION OF COMMERCIAL POTTING MIXES FOR OPTIMIZING GROWTH OF CITRUS IN CONTAINERS

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Abstract. **Optimal plant growth is crucial in research experiments as well as in nursery operations. Two major factors that influence plant growth are soil mix and fertilizer. Use of commercial soil mixes would be desirable for smaller operations which often lack facilities for mixing and sterilization. Several commercial potting mixes were compared to the UC (University of California) mix for growing citrus plants in our greenhouses at the Citrus Research and Education Center in Lake Alfred, FL. The UC mix is part of the UC system that uses a peat moss based soil mix and fertigation for nutrient supply. Commercial mixes were compared against the original UC mix using the UC system. A fertilizer formulation was used that was previously adjusted for Ca content in the irrigation water. Plant growth and soil pH were measured, and plants were monitored for nutrient deficiencies. Two mixes were found to be comparable to the UC mix in terms of plant growth and lack of micronutrient deficiency. No commercial mixes were found to give better plant growth or fewer deficiency symptoms than the original UC mix. The choice of mix appeared to affect mostly the soil pH and development of deficiency symptoms.**

Good plant growth and freedom from micronutrient deficiencies are crucial in greenhouse experiments in which differences among treatments are shown in plant growth and disease symptoms many times show as patterns on the leaves (Nauer et al., 1967b). Micronutrient deficiencies can mask or otherwise interfere with the development and observation of these symptoms. It is therefore very important to develop a system for optimal plant growth free of micronutrient deficiencies.

Also, in nursery operations, optimal plant growth is essential for maximization of productivity. Healthy plants without deficiencies will ensure optimal graft take, will sell at better prices, and will grow into more healthy and vigorous trees once they are planted in the field.

The University of California (UC) system for growing plants in containers was developed in the 1940s by the University of California to meet the need for more efficient production of ornamental plants in nurseries (Baker, 1957). The resulting system addressed the major issues found to limit nursery production: diseases, salinity, and toxicity. Answers were found in a system using a peat moss-based soil mixture that was easily replicated, fertigation and sanitary measures.

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