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Nutrient Status and Root Density of Huanglongbing-Affected Trees: Consequences of Irrigation Water Bicarbonate and Soil pH Mitigation with Acidification

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Abstract: The *Candidatus* Liberibacter asiaticus bacterium, associated with Huanglongbing (HLB) disease of citrus trees, moves downward in the phloem and infects the roots soon after transmission by the Asian Citrus Psyllid (*Diaphorina citri*) vector into shoots. Before canopy symptoms appear, 30–50% of the roots are damaged. Without aggressive management to reduce abiotic and biotic stress, root loss increases to 70–80%. An extensive survey of HLB-affected groves in central and south-central Florida indicated that a greater decline in fibrous root health as well as a greater expression of HLB symptoms is observed where irrigation water is high in bicarbonates (>100 mg L^{-1}) and soil pH is >6.5. Over three seasons of survey, acidification of irrigation water in the central and south-central citrus growing regions of Florida reduced the decline in root density associated with HLB. Irrigation water treatment with sulfuric acid and soil amendment with elemental sulfur for 36 months to establish a soil pH range from 4.0 to 7.0 increased root growth, soil nutrient availability, and the uptake of Ca, Mg, Mn, and Zn in response to a gradual reduction in soil pH in young and mature Valencia orange groves on Swingle citrumelo rootstock. The reduction in soil pH increased yield and soluble solids in fruit and so would improve citrus production.

Keywords: Greening; *Candidatus* Liberibacter asiaticus; citrus; irrigation water acidification; nutrient uptake

1. Introduction

Huanglongbing (HLB), associated with the bacterium *Candidatus* Liberibacter asiaticus (Las), was first detected in Florida in late 2005 [1]. Symptoms of HLB include a distinctive chlorotic mottle on fully expanded leaves. Infected shoots are stunted and branches gradually die back as the symptoms appear in other sectors of the tree canopy. Excavation of the root–soil zone in the irrigated area under the canopy reveals a deficit of fibrous roots compared to that of a healthy tree [2–4]. Yield is reduced directly by the root loss which leads to fruit drop and eventually to tree decline [5]. HLB reduces fruit size, weight, and other fruit quality variables, such as total soluble solids (TSS) content, acidity, and the TSS/acidity ratio [6]. In Florida, HLB is widely distributed throughout all the citrus-growing regions [1].

Symptomatic HLB-infected trees are more affected by extremes of soil moisture than trees without HLB [7,8]. The symptoms exhibited by HLB-infected trees are typical of nutrient and water stress, with reduced vigor and excessive leaf loss followed by premature fruit drop even when trees are managed following good nutritional and irrigation practices recommended to support the health of HLB-affected trees. Stress intolerance of HLB trees is a direct consequence of a greater than 30% loss of fibrous root density compared to nondiseased trees [9–11].

Typically, citrus trees in Florida groves under microjet irrigation concentrate fibrous roots in the wetted zone [4]. Some citrus groves have a history of excessive dolomite liming to manage the availability of high residual soil copper [11]. In recent decades, soils under tree canopies statewide have increased in pH and bicarbonate concentrations due to irrigation with alkaline water from deep wells extending into Florida's limestone aquifers [12]. As soils become more alkaline, some nutrients are less available for uptake (e.g., Ca, Mg, Fe, Mn, Zn, and B) as observed for other crops [13,14].

Hence, there is a need for specific information regarding the effects of HLB, bicarbonates, soil pH, and their interaction for trees in Florida under field conditions. The practical application of these results will be a better understanding of the impact of bicarbonates on the health and productivity of HLB-affected trees and the need for treatment of alkaline irrigation water. Results of two surveys of commercial citrus groves were used as preliminary data on the effect of soil pH on citrus tree root density, leaf nutrient status, and yield. The goals of the research study described in this paper were to: (1) Determine the relationship between soil pH, soil bicarbonate concentration, and fibrous root density; (2) document improved root growth and nutrient uptake of HLB-affected trees with pH moderation; and (3) quantify leaf nutrient concentrations, growth, and yield in response to a reduction in soil pH below 6.5.

2. Materials and Methods

2.1. Survey I

Fibrous root dry weight density and change in fruit production for groves irrigated with low vs. high bicarbonate concentrations in well water. Thirty-six groves of 8- to 12-year-old Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) trees on Swingle citrumelo (*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.) or Carrizo citrange (*C. sinensis* × *Poncirus trifoliata* (L.) Raf.) rootstocks irrigated from wells with varying bicarbonate levels were surveyed in May 2013. Groves were located in the central ridge with well-drained deep fine sand soils (Entisols) (Highlands County, FL, USA) and south-central flatwoods with poorly drained sandy soils with spodic horizons (Spodosols) (Hardee and Desoto Counties, FL, USA). Bicarbonates were measured in water samples from wells in each grove location collected by the grove manager and submitted for analysis to a commercial testing laboratory (Waters Agricultural Laboratories, Camilla, GA, USA). To estimate root dry weight density in each grove site, 8 trees located in 6 row middles were sampled. Four trees of a similar canopy size were selected from each side of the middle at 5–10 tree intervals down the rows. For root sampling, 1 soil core (2.4 cm diameter × 15 cm depth, 67.8 cm^3 per core) per tree was taken from under the canopy in the wetted zone of the microsprinkler pattern. Soil cores from trees in each row middle were combined into 1 composite sample. Samples were placed in a shaded cooler and returned immediately to the laboratory for processing. After the composite sample was thoroughly mixed, a subsample of 20 cm^3 volume was taken to assess soil pH using a saturated water past method [15]. The volume of remaining soil was estimated before washing through a mesh screen with 2 mm openings. Citrus fibrous roots recovered on the screen were separated from weed roots and organic debris, the roots were dried at 70 °C for 24 h and the root dry weight was measured. Root mass density per cm^3 soil was calculated by dividing the dry weight of the roots by the volume of soil in the composite sample. Root mass density per grove was calculated as the mean of the 6 samples collected at each grove location. The relationship between well-water bicarbonate and soil pH or root mass density for the 36 grove locations was calculated using regression analysis in SigmaPlot v14 (Systat Software, Inc, San Jose, CA, USA).

Grove locations were classified according to bicarbonate status of the well water (low (<100 mg L^{-1}) or high (>100 mg L^{-1})) and location (flatwoods or central ridge). Fruit production data from 2011 to

2013 obtained from the citrus producer (Davis Citrus Management) was used to estimate change in tree productivity during the time period when the incidence of HLB was increasing exponentially in Florida citrus groves [1]. Change in fruit production (%) was calculated as (1—the product of production (40 kg boxesha^{-1}) in 2013) divided by (production in 2011 × 100 for each grove). The mean of the root mass density and % change in production was estimated for 10 to 14 grove sites in each region/soil pH classification.

2.2. Survey II

Fibrous root density response to acidification in the central ridge and flatwoods groves irrigated with low or high bicarbonate concentrations in well water. From 2014 to 2016, a survey of central ridge (Highlands County, FL, USA) and flatwoods (Hardee County, FL, USA) groves of Valencia trees (8 to 12 years old) on Swingle citrumelo or Carrizo citrange rootstock irrigated with well water low (<100 mg L^{-1}) or high (>100 mg L^{-1}) in bicarbonates was conducted to evaluate the root dry weight density response to acidification of the irrigation water. In both the central ridge and flatwoods groves, 4 low-bicarbonate groves without acidification of the irrigation water were compared with 4 high-bicarbonate groves treated by sulfuric acid (40%) injection into the irrigation system to target a pH of 6.2 in the rhizosphere. Root mass and soil pH sampling and measurement was conducted using the same methods as in Survey I on 3 dates in 2014 beginning in May and 5 dates in 2015 and 2016. The leaf nutrient status of macro- and micronutrients was measured in November 2016 in each location by collecting leaves from the spring flush for analysis (Waters Agricultural Labs, Camilla, GA, USA).

2.3. Acidification Field Trials

Two groves, 1 with trees planted in 2013 (young, ~5 years old), and a second planted in 2006 (mature, ~10 years old), were evaluated for tree response to acidified irrigation water. Trees at both locations were Valencia on Swingle rootstock and planted on flatwoods soils. The grove of young trees was planted on Malabar fine sand (Loamy, siliceous, active, hyperthermic Grossarenic Endoaqualfs) in Desoto County, Florida, and the grove of mature trees was planted on Electra fine sand (Sandy, siliceous, hyperthermic Oxyaquic Alorthods) in Lee County, Florida. The sites were chosen for high irrigation water bicarbonate corresponding to high soil pH (≥7.3), soil calcium (≥250 mg L^{-1}), and irrigation water bicarbonate (≥100 mg L^{-1}). Each of the flatwoods groves had a nontreated control, acid injection, and elemental sulfur (S) amendment. To establish irrigation water pH treatments for 4.0, 5.0, and 6.0, sulfuric acid (40%) was injected by hydraulic powered proportioning pumps located at the irrigation riser of each treatment row. Irrigation water samples were taken monthly and tested for pH using electrodes (14). Plots with the 3 rates of acid injection and the nontreated control were split randomly with 1 of the split plots receiving S in a controlled release form (Tiger 90CR; Tiger-Sul Products, LLC, Atmore, AL, USA) at 560 kg per ha 2 times per year. The splitting of plots resulted in 8 treatment combinations of 4 acid rates (including the control) with or without S.

To determine nutrient uptake, leaf samples (a total of 20 leaves from 4 randomly sampled trees per plot) were collected from each subplot monthly using the procedures outlined by Obreza and Morgan [11]. Leaves were dried at 60 °C for 72 h and then ground and mixed thoroughly. Ground samples were passed through a 2 mm mesh screen and stored at room temperature in a desiccator until being weighed for analysis [15,16]. Tissue nutrient concentrations were determined using the dry ash combustion digestion method [17]. A 1.5 g sample of dried leaf material was dry-ashed at 500 °C for 16 h [15]. The ash was equilibrated with 15 mL of 0.5 molar HCl at room temperature for 0.5 h. The solution was decanted into 15 ml plastic disposable tubes and placed in a refrigerator at ≤4 °C [16,18] until being analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) [19]. Leaf nutrient concentrations were compared with critical levels for Florida citrus at both sites [11,20].

Soil samples were collected monthly to evaluate soil pH and bicarbonate concentrations using saturated water paste and bicarbonate evolution methods [15]. Mehlich-3 extraction, a procedure recommended for soils with low organic matter and pH < 6.5, was used for the determination of soil nutrient concentrations [21]. Air-dried soil samples weighing 5.0 g (2 mm screened) were placed into extraction bottles and 20 ml of Mehlich-3 extraction solution was added to each sample and shaken at high speed for 5 min at room temperature (25 ± 2 °C) and allowed to settle for 15 min. The extracts were filtered (Whatman filter paper #42) and the supernatant was collected in labeled plastic vials and placed in a refrigerator at ≤4 °C until analyses [17,18] by ICP-AES [19,22,23].

Root density was determined in each field site using a minirhizotron (model CI-600 In-Situ Root Imager, CID-Bioscience, Camas, WA, USA) (18). Cellulose acetate butyrate observation tubes 0.5 m in length were inserted into the soil 30 cm and 150 cm from the tree trunk in the tree row of the 5-year-old and 10-year-old trees, respectively. Digital video images were taken monthly from each tube to measure root length and density surrounding the observation tubes. Minirhizotron measurements were calibrated using destructively sampled fibrous roots to estimate root length density (RLD) at soil depths of 0–45 cm at 15 cm increments. Soil cores were stored at ≤4 °C until analysis. Fibrous roots were collected and stored as described above for the surveys. Roots were rehydrated for 15 min and the RLD was estimated using the grid-line intersect method [12] by counting the number of root intersections on a 1.0 × 1.0 cm grid which was multiplied by 11/14 and divided by the volume of the corer to give root length density in cm per cm^3 soil [24].

Tree growth for both young tree and mature tree blocks were determined by measuring tree canopy volume. Five representative trees from each irrigation/sulfur treatment subplot were measured in the east-west and north-south directions and canopy height. Then, using the formula for a prolate spheroid: (4/3)(π)(tree height/2)(mean canopy radius), canopy volume was calculated [25].

Leaves from each tree used for tissue were sampled quarterly for HLB diagnosis using real-time polymerase chain reaction (PCR) analysis according to Li et al. [26]. Fruit yield weight was estimated prior to commercial harvest for each of the 10 sample trees per plot by fruit count and weight of 10 fruit per tree representing the size range of fruit on each tree. Results for soil pH and bicarbonate concentrations were analyzed by one-way analysis of variance as was tree growth, root density, nutrient status, yield, and fruit drop.

The data collected on soil and leaf nutrient concentrations, RLD, canopy volumes, and yields were analyzed using Mixed Model procedures using Statistical Analysis System 9.3 (SAS, 2011). Means were separated using Tukey's procedure.

3. Results and Discussion

3.1. Survey I

We hypothesized that groves irrigated with well water high in bicarbonates would express more severe symptoms of HLB. To confirm this relationship, we surveyed 36 groves of Valencia trees on Swingle or Carrizo rootstocks in the central ridge (Highlands County, FL, USA) irrigated with deep and shallow well water and in the flatwoods (Hardee and Desoto Counties, FL, USA) irrigated with deep well water. Water quality for deep and shallow wells was distinctly different in pH and bicarbonate status (Figure 1). Shallow well water ranged from pH 4.0 to 6.3 and from 0 to 32 mg L^{-1} bicarbonate compared with deep well water which ranged from pH 6.7 to 7.8 and from 97 to 218 mg L^{-1} bicarbonate.

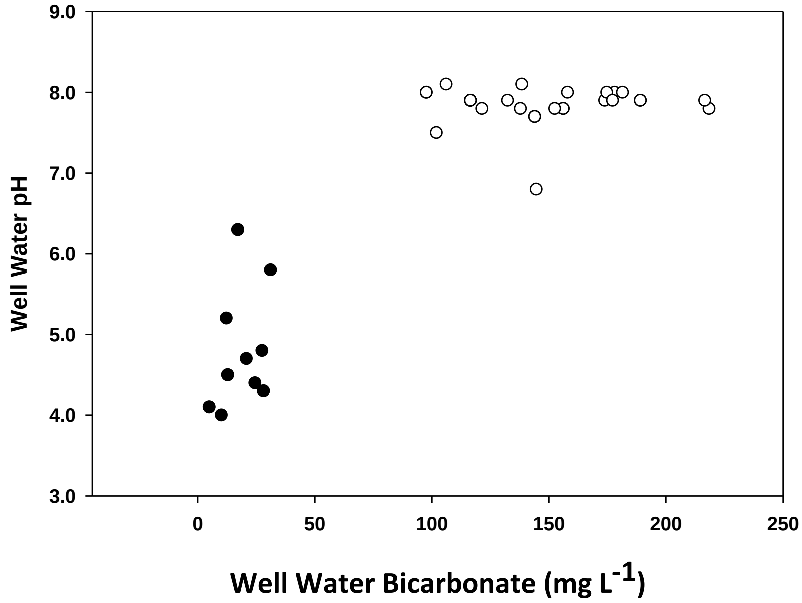

Figure 1. Relationship between well water pH and bicarbonate concentrations at 29 grove locations in the Highlands, Hardee, and Desoto Counties, FL with shallow wells (filled circles, pH < 6.5 and <100 mg L^{-1} bicarbonates) or deep wells (open circles, pH > 6.5 and >100 mg L^{-1} bicarbonates).

Using regression analysis, root mass density was negatively correlated with well water pH (r^2 = 0.53), bicarbonates (r^2 = 0.47), and soil pH (r^2 = 0.26) in the root zone wetted by the microsprinkler (Figure 2). Analysis of the change in yield from the groves under pH stress from high water bicarbonate from 2011–2013 declined 6% and 20% in central ridge and flatwoods groves, respectively, while production in central ridge groves with low bicarbonate stress increased 6% (Table 1). The yield losses in our survey of 36 groves irrigated with well water low (<100 mg L^{-1}) or high (>100 mg L^{-1}) in bicarbonates were related to lower fibrous root mass density, which reduces root system capacity for water and nutrient uptake.

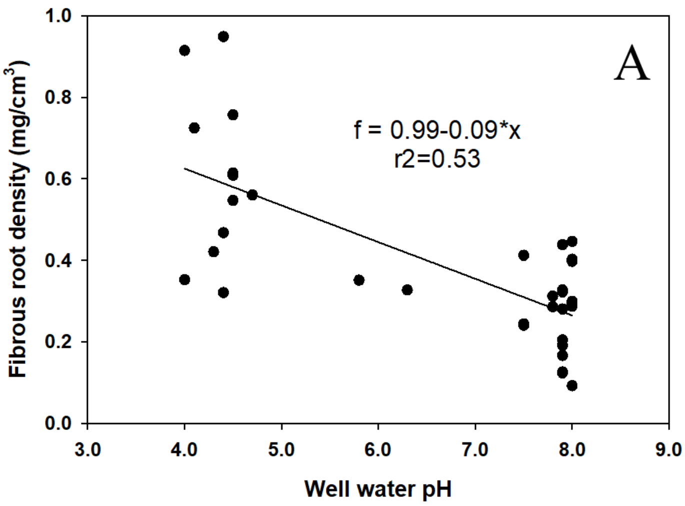

Figure 2. *Cont.*

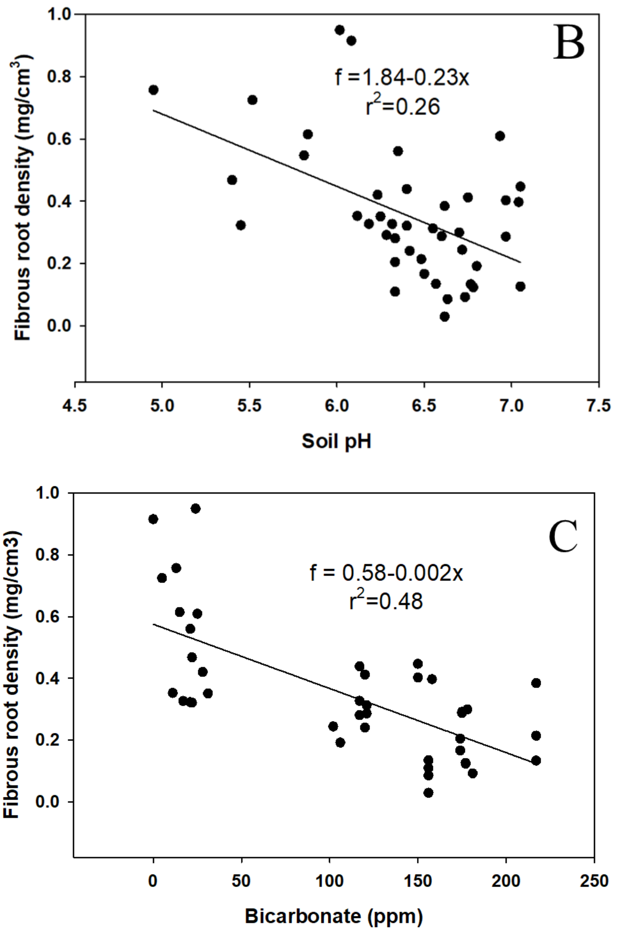

Figure 2. Relationship between fibrous root mass density and well water pH, soil pH, or bicarbonates for Valencia trees on Swingle citrumelo or Carrizo citrange in 36 groves in central ridge and flatwoods locations in FL. R^2 for the linear equations for (**A**) well water pH (0.53), (**B**) soil pH (0.26), and (**C**) bicarbonates (0.47) are significant at $p < 0.001$.

Table 1. Relationship between bicarbonate status, root mass density, and change in yield from 2011 to 2013 †.

Grove Status	No. of Blocks Surveyed	Root Mass Density (mg/cm^3)	Change in Block Yield from 2011–2013
Low pH stress Ridge	14	0.64	Increased 6%
High pH stress Ridge	10	0.34	Decreased 6%
High pH stress Flatwoods	13	0.18	Decreased 20%

† Yield data kindly provided by Davis Citrus Management.

3.2. Survey II

In central ridge groves (Highlands Co.) irrigated with high bicarbonate irrigation water treated by acidification with sulfuric acid, soil in the root zone was maintained in a similar pH range as groves irrigated with low bicarbonate water (Figure 3A). Two of the four ridge groves irrigated with low bicarbonate water (Hugh and Ruvin) and no acidification had greater root density than the acidified high bicarbonate water irrigated groves at the beginning of the survey (Figure 3B). The root densities of all groves were not significantly different at the end of the survey (two years after acidification was initiated).

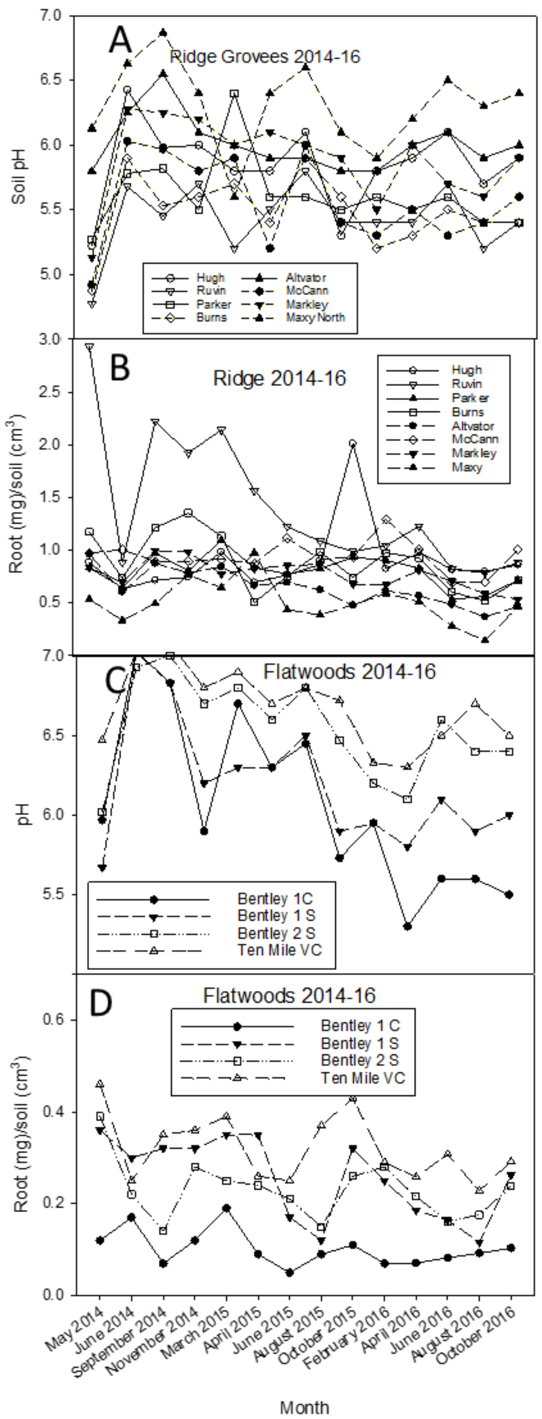

Figure 3. Soil pH and fibrous root density for Valencia trees on Swingle citrumelo and Carrizo citrange rootstocks in eight central ridge groves (**A**,**B**) treated with (filled symbols) or without (open symbols) acidification of irrigation water and four flatwoods groves (**C**,**D**) all treated with acidification from April 2013 to November 2016.

In flatwoods groves (Hardee Co.) irrigated with high bicarbonate well water, the root zone was maintained below pH 6.5 during the irrigation season (spring–early summer) but rebounded when irrigation was less frequent during the summer rainy season (Figure 3C). The root densities of trees grown on flatwoods soils, with higher water and nutrient holding capacities, are typically lower than for trees grown on the central ridge [27]. The root densities at the four flatwoods groves irrigated with acidified high bicarbonate water were not significantly different during the survey (Figure 3D). Leaf analysis in November 2016 after three seasons of acidification (2013 to 2016) indicated that the nutrient status of groves with acidification was in the sufficiency range for macro- and micronutrients and similar to the groves without acidification (Table 2).

Table 2. Leaf nutrient concentrations in Valencia trees on Swingle citrumelo and Carrizo citrange rootstocks in the central ridge and flatwoods groves treated with acidification (D) or without acidification (S) of the irrigation water from April 2013 to November 2016.

Location	Irrigation Water Acidified	Grove Site	N	P	K	Mg	Ca	S	B	Zn	Mn	Fe	Cu
						%					mg kg^{-1}		
Ridge	Without	Hugh	2.5	0.18	1.1	0.39	3.8	0.37	85	66	63	58	10
Ridge	Without	Ruvin	2.4	0.17	1.0	0.40	3.7	0.41	79	55	55	51	19
Ridge	Without	Parker	2.5	0.16	1.0	0.31	3.8	0.35	78	39	43	53	13
Ridge	Without	Burns	2.4	0.16	1.1	0.30	3.9	0.38	86	68	76	54	15
Ridge	With	Altvator	2.4	0.16	0.9	0.31	3.8	0.35	72	47	48	47	14
Ridge	With	McCann	2.5	0.16	1.1	0.26	3.7	0.36	80	48	55	50	11
Ridge	With	Markley	2.5	0.16	1.2	0.27	3.9	0.37	85	81	81	60	15
Ridge	With	Maxy	2.6	0.16	1.2	0.27	3.5	0.36	92	88	84	62	13
Flatwoods	With	Bentley 1C	2.4	0.17	1.1	0.39	3.2	0.29	68	60	60	60	36
Flatwoods	With	Bentley 1S	2.5	0.18	1.1	0.40	3.2	0.31	82	83	77	59	21
Flatwoods	With	Bentley 2S	2.4	0.19	1.4	0.40	3.7	0.34	97	67	58	62	10
Flatwoods	With	Ten Mile VC	2.4	0.18	1.2	0.35	3.1	0.33	77	54	48	56	13
Significance			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

A seasonal rise in soil pH was observed when conditioning of irrigation water was less frequent during the rainy season. To sustain the effect of acidification on soil pH, the application of elemental S to soil during the rainy season is required to release acidity for weeks to months depending on the particle size of the S. A conclusion of this survey is that repeated applications of elemental S may be required until the soil pH drops below 6.5.

3.3. Acidification Field Trials

Irrigation water acidification (target pH of 6.0, 5.0, and 4.0) began in spring 2014 and continued to spring 2017 in two Valencia on Swingle rootstock groves. Sulfur applications were made to treatment subplots in January and June of 2015 and 2016, and January 2017. Irrigation water pH reflected treatment targets during the three-year study (Figure 4A). Irrigation water pH was maintained within ~0.5 units of the treatment target pH. Bicarbonate concentrations of the irrigation water was reduced by acidification from greater than 130 mg L^{-1} to less than 110 mg L^{-1} at pH 6 and below 80 mgL^{-1} at pH less than 6.0 (Table 3). The corresponding reduction in irrigation water bicarbonates in response to acidification resulted in a gradual lowering of soil pH from above 7.0 for all treated plots in both the young tree grove and mature tree grove. These results would indicate that irrigation water should be maintained at a pH of 5 or less to reduce bicarbonates below a target of 100 mg L^{-1}.

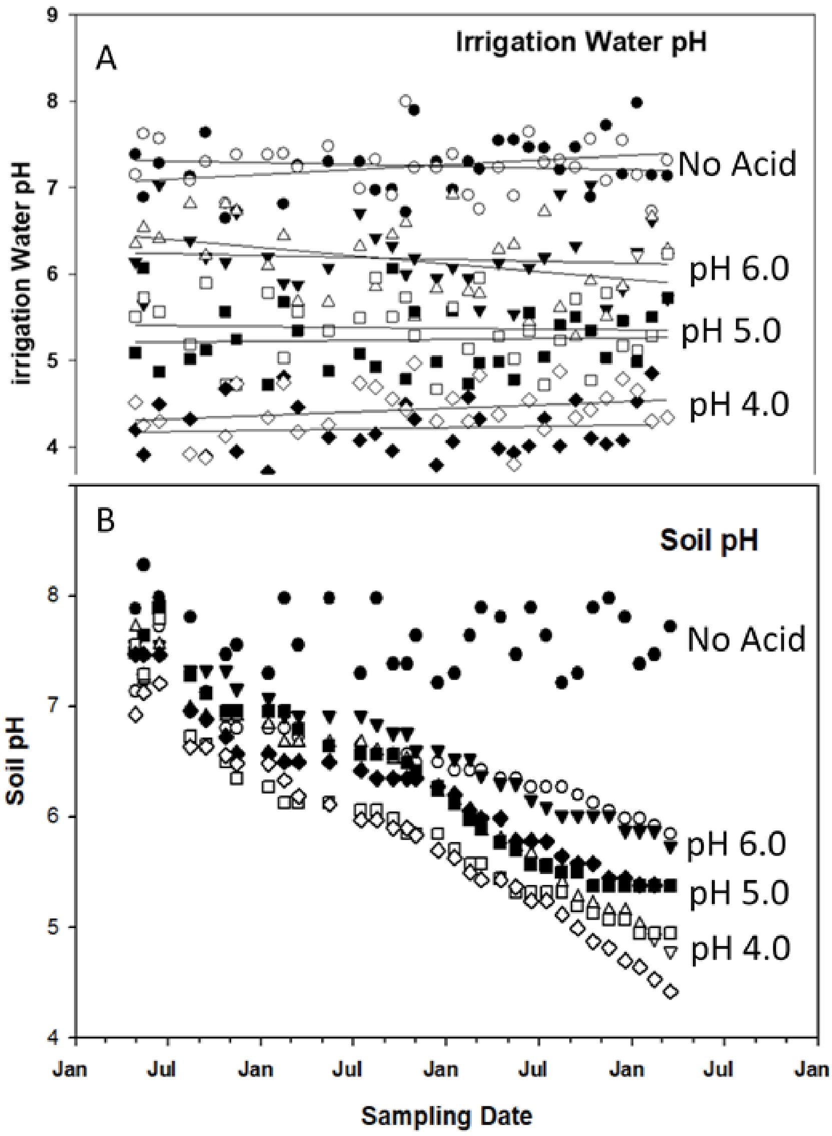

Figure 4. Mean monthly (**A**) irrigation water pH and (**B**) soil pH in response to treatments of irrigation water with sulfuric acid and soil with elemental sulfur from April 2014 to April 2017.

The application of acidified water reduced soil pH more gradually than expected (Figure 4B), resulting in lower but non-significant differences in soil pH between treatments compared with the control from April 2014 to April 2016 (Table 3). Soil samples collected in April 2014 to April 2016 indicate that soil pH in the plots receiving S applications decreased by approximately 0.6 pH units below the soil pH of plots receiving only acidified irrigation water at both sites (Table 3). Soil pH was significantly lower in the acidification treatments in the last year of the trials (April 2016 to April 2017) compared with the initial years of this study. Soil pH decreases from application of S in conjunction with acidified irrigation were less than 0.1 pH units after April 2016. Mean irrigation water bicarbonate significantly decreased with lower pH (Table 3). Irrigation water acidification can be used as needed once soil samples indicates a pH greater than 6.

Table 3. Mean monthly irrigation water pH and bicarbonate, and soil pH for the three years after acidification treatments began.

Target Irrigation Water pH and Soil S Application Treatments	Mature Trees				Young Trees			
	Irrigation Water		Soil		Irrigation Water		Soil	
	pH	Bicarbonates (mg L^{-1})	pH (4/14 to 4/16)	pH (4/16 to 4/17)	pH	Bicarbonates (mg L^{-1})	pH (4/14 to 4/16)	pH (4/16 to 4/17)
Control without S	7.45	126.55 AB †	7.53	7.43	7.52 A	144.02 A	7.59	7.65 A
Control with S	7.52	136.55 A	6.89	6.50	7.47 A	157.00 A	6.52	6.11 AB
6.0 without S	6.19	98.82 B	6.90	6.25	6.17 AB	113.60 AB	6.59	6.09 AB
6.0 with S	6.14	88.71 BC	6.41	6.22	6.15 AB	101.63 B	6.16	6.05 AB
5.0 without S	5.23	64.35 CD	6.55	5.68	5.24 B	74.32 C	6.28	5.39 B
5.0 with S	5.39	68.70 C	6.07	5.34	5.38 B	80.00 BC	5.86	5.04 BC
4.0 without S	4.17	55.63 D	6.56	4.82	4.22 BC	65.23 C	5.72	4.49 C
4.0 with S	4.39	61.53 D	6.00	4.97	4.43 C	69.92 C	5.75	4.67 C
Significance (*p*)	0.098	<0.0001	0.886	0.0462	0.0134	<0.0001	0.2851	0.0298

† Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

At the end of the three-year study, soil pH was near the treatment target pH with the exception of the treatment with a target pH of 4.0 (Figure 4B). A high Ca content of the soil increases the buffering capacity and reduced the effect of acidified irrigation water on soil pH (Ghasemi et al., 2010, Hanlon et al., 1997). The application of time-released S products reduced soil pH in the control plots by approximately 0.85 pH units in the initial two years but less than 0.3 units during the last year. A reduction of soil pH with S applications alone was also detected in plots receiving acidified water but was also greater in the first two years than the final year of the study. These results would indicate that application of S in a controlled-release method alone could bring down soil pH if acidification of irrigation water is not feasible as a result of equipment or personnel limitations. Soil pH increased during the summer rainy season when less irrigation water is applied (Data not shown). The use of controlled release S material during this period of the year would be beneficial. No significant differences were found in soil nutrient concentrations between treatments (Table 4).

Table 4. Mean monthly soil analysis for 0–15 cm depth for young (five-year-old) and mature (10-year-old) Valencia trees on Swingle citrumelo rootstock.

Target Irrigation Water pH and Soil S Application Treatments	Mature Trees					Young Trees				
	Soil Nutrient Concentrations									
	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})
Control without S	269.75	10.11	3.15	2.32	0.42	260.39	9.48	2.94	2.16	0.38
Control with S	317.76	8.07	4.01	2.80	0.40	301.24	7.36	3.60	2.70	0.37
6.0 without S	316.91	8.39	2.99	2.27	0.34	302.02	7.91	2.77	2.11	0.33
6.0 with S	304.83	9.14	3.81	2.81	0.37	291.32	8.60	3.59	2.62	0.35
5.0 without S	326.43	8.10	3.23	2.18	0.48	317.55	7.81	3.03	2.12	0.46
5.0 with S	345.68	8.57	3.57	2.59	0.47	322.24	7.95	3.37	2.51	0.45
4.0 without S	371.94	9.79	3.68	2.28	0.38	357.89	9.46	3.52	2.18	0.35
4.0 with S	399.63	10.35	3.30	3.10	0.38	380.37	10.00	3.09	2.93	0.37

Tree canopy volume was not increased by irrigation water acidification or the application of S during the first two years of this study. Therefore, average mature leaf concentrations can be considered a good indicator of relative nutrient uptake. Morgan et al. [28] found greater tree growth and yield for HLB affected trees with foliar applications of Mn and Zn at rates about three times greater than those recommended for trees not affected by HLB.

Leaf Ca, Mg, Mn, and Zn concentrations were higher in response to reduced water and/or soil pH compared to the nontreated controls in both the young trees and mature trees (Figure 5A–D). Leaf Ca, Mg, Mn, and Zn were consistently in the optimum or high range for citrus trees [11] at soil pH 6.5 or lower. When averaged over the three-year treatment period, leaf Ca, Mg, Mn, and Zn concentrations were significantly different between treatments at the mature tree site (Table 5) and the young tree site (Table 6). Leaf B at both young and mature tree sites was significantly different between treatments if only the last year of data is considered (Tables 5 and 6). Moderation of soil pH improved leaf concentrations of Ca, Mg, Mn and Zn in plots with a soil pH below 6.5 suggesting an increased root uptake of these nutrients (Tables 5 and 6).

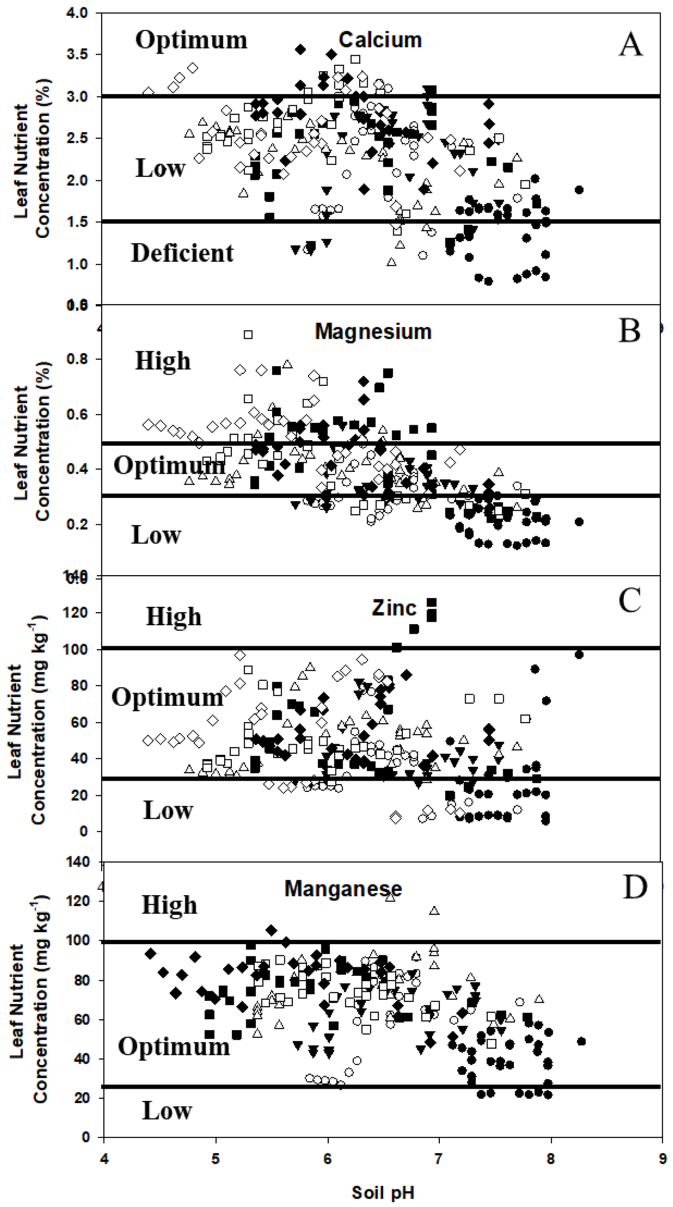

Figure 5. Leaf nutrient concentrations of (**A**) Ca, (**B**) Mg, (**C**) Zn, and (**D**) Mn for five-year-old Valencia trees on Swingle citrumelo rootstock in response to treatments of irrigation water with sulfuric acid and soil with elemental sulfur to reduce soil pH from April 2014 to April 2017 (see Figure 4 for identification of symbols). Low, optimum, and high leaf nutrient concentrations (28).

Table 5. Mean monthly leaf tissue analysis for mature (10-year-old) Valencia trees on Swingle citrumelo rootstock in the first two years (April 2014 to April 2016) and the third year (April 2016 to April 2017) after acidification treatments began.

Target Irrigation Water pH and Soil Sulfur Application Treatments	April 2014 to April 2016					April 2016 to April 2017				
	Leaf Nutrient Concentrations									
	Ca (%)	Mg (%)	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})	Ca (%)	Mg (%)	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})
Control without S	1.49 C †	0.22	41.75 D	26.23 D	94.01	1.45 C	0.19 D	41.96 C	22.33 C	77.03 A
Control with S	2.42 B	0.35	69.35 BC	39.03 D	78.13	2.57 AB	0.37 BC	64.78 B	48.64 B	74.01 AB
6.0 without S	2.38 B	0.34	66.03 C	41.16 C	74.12	2.31 B	0.34 C	66.90 B	47.10 B	63.16 B
6.0 with S	2.06 BC	0.42	79.99 AB	52.30 B	73.33	2.36 B	0.47 B	77.50 AB	55.83 A	68.84 B
5.0 without S	2.37 B	0.45	76.29 B	80.87 A	78.55	2.44 AB	0.52 AB	76.68 AB	50.38 B	76.61 A
5.0 with S	2.67 AB	0.45	80.72 A	52.02 B	78.32	2.67 A	0.52 AB	80.32 A	54.78 AB	73.22 AB
4.0 without S	2.74 A	0.46	73.48 B	57.43 AB	84.62	3.01 A	0.51 AB	79.23 A	54.04 AB	82.32 A
4.0 with S	2.53 AB	0.51	80.35 A	52.79 B	79.00	2.45 AB	0.58 A	85.59 A	55.28 A	70.37 AB
Significance (p)	0.0001	0.0118	0.0006	0.0144	0.2847	0.0491	0.0387	0.0188	0.0287	0.0388

† Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

Table 6. Mean monthly leaf tissue analysis for young (five-year-old) Valencia trees on Swingle citrumelo rootstock in the first two years (April 2014 to April 2016) and the third year (April 2016 to April 2017) after acidification treatments began.

Target Irrigation Water pH and Soil Sulfur Application Treatments	April 2014 to April 2017					April 2016 to April 2017				
	Leaf Nutrient Concentrations									
	Ca (%)	Mg (%)	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})	Ca (%)	Mg (%)	Mn (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})
Control without S	1.41 C [†]	0.21 C	39.22 C	25.35 C	87.01	1.19 C	0.18 C	34.16 D	26.15 C	48.28 C
Control with S	2.30 A	0.34 B	63.86 B	37.26 BC	72.79	2.15 B	0.38 BC	47.64 C	42.65 BC	52.71 BC
6.0 without S	2.22 AB	0.33 B	63.79 B	39.21 BC	69.10	1.83 BC	0.34 BC	57.73 C	45.09 BC	47.77 C
6.0 with S	2.14 B	0.41 AB	76.81 A	49.76B	69.19	2.47 AB	0.45 B	67.11 BC	48.03 B	51.50 BC
5.0 without S	2.32 A	0.45 A	74.55 A	58.29 A	73.43	2.21 B	0.46 B	72.13 B	51.51 B	53.66 B
5.0 with S	2.54 A	0.47 A	79.60 A	49.60 B	74.39	2.54 AB	0.50 AB	74.07 B	51.95 B	53.65 B
4.0 without S	2.78 A	0.45 A	73.53 AB	56.10 A	82.62	2.96 A	0.50 AB	80.48 AB	56.32 AB	72.28 A
4.0 with S	2.57A	0.52 A	80.59 A	52.22 AB	78.34	2.65 A	0.59 A	81.66 A	62.50 AB	70.27 A
Significance (p)	<0.0001	0.0400	0.0006	0.0285	0.2362	0.0432	0.0451	0.0365	0.0165	0.0251

[†] Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

During the first two years of the study, leaf Ca concentration increased for the lowest irrigation water pH treatment compared with the nontreated control by 65.1% and 72.3% for mature and young trees, respectively (Tables 5 and 6). Likewise, during the first two years of the study, leaf Mg, Mn, and Zn increased by an average of 94.5%, 82.4%, and 113.8%, respectively, for the mature trees; and 109.5%, 90.7%, and 100.6%, respectively, for the young trees. During the last year of the study, leaf Mg, Mn, and Zn increased by an average of 157.9%, 85.2%, and 136.9% for the mature trees, respectively; and 161.1%, 111.4%, and 101.0% for the young trees, respectively. These results indicate an increased nutrient uptake from irrigation water ranging in pH from 6.2 to 4.5 compared to soil with a pH range from 6.9 to 5.7 because of the lowering of pH in the soil solution. No significant differences were found in soil nutrient concentrations between treatments, indicating the increase in leaf concentration was from improved nutrient uptake as a result of increased availability and not increased soil nutrient concentrations.

Results of quarterly PCR analysis indicated that all trees in the treatment blocks of both the young and mature groves were positive for HLB from the beginning of the study. Canopy volume and fruit drop were not significantly different between irrigation acidification or S applications (data not shown).

Irrigation water treatments with a target pH of 6.0 or lower were significantly greater in estimated root length than the control (Table 7). Root length was not significantly different between soil pH moderation treatments in the first two years. However, mean root length was significantly different at all three depths (0–15 cm, 15–30 cm, and 30–45 cm) at pH ≤ 6.0 in both young tree and mature tree groves in the third year. Root length decreased with soil depth for both the young and mature trees. The positive relationship between root density and reduction in soil pH from greater than 7.0 to less than 5.0 confirms the root density–soil pH relationship in the survey comparing groves with high versus low bicarbonates.

Table 7. Mean monthly observed root length measurements for young (five-year-old) and mature (10-year-old) Valencia trees on Swingle citrumelo rootstock from April 2016 to April 2017.

Target Irrigation Water pH and Soil Sulfur Application Treatments	Mature Trees			Young Trees		
	Mean Observed Root Length (cm)					
	0–15 cm Depth	15–30 cm Depth	30–45 cm Depth	0–15 Cm Depth	15–30 cm Depth	30–45 cm Depth
Control without S	7.80 C [†]	5.97 C	2.60 C	8.34 C	4.57 C	3.74 D
Control with S	8.74 BC	6.07 B	2.79 C	8.71 C	7.84 B	4.36 C
6.0 without S	9.87 B	9.34 A	3.41 B	14.60 B	19.78 A	9.04 B
6.0 with S	12.09 AB	8.64 AB	3.31 BC	17.34 AB	17.89 AB	8.89 B
5.0 without S	18.08 A	8.10 AB	4.88 A	13.48 B	17.94 AB	9.84 A
5.0 with S	15.80 AB	8.98 A	4.07 A	18.97 A	18.95 AB	8.89 B
4.0 without S	17.89 A	9.78 A	3.89 AB	18.07 A	19.09 A	8.23 BC
4.0 with S	18.79 A	8.98 A	4.23 A	19.87 A	19.89 A	8.97 A
Significance (p)	0.047	0.027	0.034	0.043	0.036	0.021

[†] Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

No significant differences were found in fruit number per tree in young and mature trees for any treatment or year (Tables 8 and 9). However, a significantly greater weight per fruit and number of 40 kg boxes per tree for young and mature trees were observed between irrigation water acidification treatments in the second and third years. However, soluble solids in the young tree block were reduced with lower pH irrigation water, whereas solids in mature trees increased with lower pH. This fruit response was reflected in significant yield increases in the last two years of acidification treatment. Fruit soluble solids also increased with irrigation water acidification and S applications except for a decrease in soluble solids in the young tree block in the first year. This may have been due to the larger fruit size of the young trees [24].

Table 8. Yields from young (five-year-old) Valencia trees on Swingle citrumelo rootstock for harvest years 2014/2015, 2015/2016, and 2016/2017.

Target Irrigation Water pH and Soil Sulfur Application Treatments	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)
	2014/2015				2015/2016				2016/2017			
Control without S	151.2	7.04 C †	26.53	60.3 A	130.3	6.88 C	22.45 C	62.7 A	208.5	7.29 C	37.86 B	58.1 B
Control with S	161.7	7.07 C	28.57	62.7 A	150.8	7.23 B	27.35 BC	63.45 AB	181.3	7.18 C	31.43 C	62.7 B
pH 6.0 without S	191.3	7.42 B	35.51	60.8 A	212.5	7.87 AB	41.64 AB	59.8 B	222.4	7.89 AB	43.7 B	59.3 B
pH 6.0 with S	162.0	7.32 BC	26.53	59.3 A	190.6	7.69 AB	36.74 B	61.0 B	184.3	7.72 BC	35.5 BC	60.8 AB
pH 5.0 without S	199.8	7.42 B	37.15	54.6 B	209.3	7.72 AB	40.41 B	59.04 BC	218.9	7.84 AB	42.45 A	56.1 C
pH 5.0 with S	202.2	7.52 AB	37.96	58.8 A	223.1	7.99 A	44.49A	60.75 B	209.8	8.08 A	41.23 B	57.1 BC
pH 4.0 without S	178.7	7.42 B	33.06	54.9 B	196.9	7.87 AB	38.78 AB	57.3 C	196.7	7.77 BC	37.96 BC	88.4 A
pH 4.0 with S	179.4	7.65 A	34.29	55.1 AB	218.8	7.97 A	43.68 A	57.8 C	218.7	7.51 BC	41.23 B	54.9 C
Significance (*p*)	0.4910	0.003	0.217	<0.001	0.441	0.001	0.012	0.001	0.4803	0.0003	0.011	0.0005

† Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

Table 9. Yields from the mature (10-year-old) Valencia trees on Swingle citrumelo rootstock for harvest years 2014/2015, 2015/2016, and 2016/2017.

Target Irrigation Water pH and Soil Sulfur Application Treatments	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)	Total Fruit Number	Fruit Weight (40 Fruit, kg)	Fruit Yield (kg Per Tree)	Soluble Solids (g/kg)
	2014/2015				2015/2016				2016/2017			
Control without S	289.0	8.62 C †	64.09	58.1 BC	258.0	8.82 C	56.74C	58.8 BC	295.1	8.83 C	65.32 C	58.55 B
Control with S	348.8	9.49 B	82.86	56.1 BC	366.3	9.51 B	86.95 BC	55.86 C	362.9	9.83 A	88.99 BC	56.84 BC
pH 6.0 without S	479.3	9.50 B	113.89	51.2 C	484.4	9.51 B	115.11 A	53.41 C	428.4	9.43 B	100.86 A	55.12 C
pH 6.0 with S	359.9	9.94 A	89.40	57.09 BC	381.2	9.97 A	91.03 AB	57.82 BC	390.8	9.96 A	97.29 AB	58.07 B
pH 5.0 without S	340.9	9.38 BC	80.01	56.1 BC	379.7	9.43 BC	89.40 AB	55.37 C	424.2	9.47 B	100.42 A	57.82 B
pH 5.0 with S	302.0	9.74 A	73.48	59.3 AB	364.1	9.73 AB	88.58 AB	60.76 B	406.1	9.78 AB	99.19 A	59.78 AB
pH 4.0 without S	323.2	9.79 A	79.19	60.5 A	389.4	9.72 AB	94.70 A	61.50 A	429.2	9.65 B	103.68 A	63.21 A
pH 4.0 with S	335.4	9.51 B	79.74	61.5 A	374.4	9.51 B	89.03 AB	62.23 A	439.9	9.60 B	105.58 A	63.21 A
Significance (*p*)	0.212	<0.0001	0.3375	<0.0001	0.244	<0.0001	0.037	0.0001	0.2447	0.0001	0.043	0.0077

† Means followed by the same letter within the same column are not significantly different according to Tukey's multiple comparison test at $p \leq 0.05$.

4. Conclusions

Fibrous mass density in the first surveyed groves indicated a greater root density associated with irrigation water from shallow wells (i.e., low bicarbonates) compared with deep wells from the aquifer (i.e., high bicarbonates). Leaf analysis of the survey groves in November 2016 after three seasons of acidification (2013–2016) indicated that the nutrient status of groves with acidification was in the sufficiency range and similar to the groves irrigated with low bicarbonate water without acidification. Flatwood soils increased in soil pH during the rainy season when irrigation amounts decreased. Products are available on the market that incorporate S with or without plant nutrients in a clay matrix that releases S for six to nine months as the particles dissolve in irrigation water, similar to a slow release fertilizer. These products have been found to be effective for reducing soil pH and increasing nutrient availability. The replicated study assessing the effect of irrigation water acidification or the combination of irrigation water acidification and sulfur applications indicated that soil pH is reduced as bicarbonates are reduced. The lower soil pH did not improve tree growth; however, it did improve tree nutrient status, root density, and yield at a soil pH lower than 6.0.

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