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Influences of different iron levels on plant growth and photosynthesis of W. Murcott mandarin grafted on two rootstocks under high pH conditions

Meral İNCESU*, Turgut YEŞİLOĞLU, Berken ÇİMEN, Bilge YILMAZ
Department of Horticulture, Faculty of Agriculture, Çukurova University, Adana, Turkey

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Abstract: W. Murcott scion, budded onto two rootstocks, was evaluated under high pH conditions supplied with different Fe levels. Plant dry weight, leaf area, iron chlorosis symptom scale, leaf chlorophyll concentration, net photosynthetic rate, and ferric-chelate reductase (FCR) activity variables were investigated under high pH conditions. Control plants (T1) produced the most leaf area, whereas plants grown without Fe (T2) produced the least. Dry weight was highest in 'Volkameriana' T1 (control) plants and lowest in 'Swingle citrumelo' T3 (10 µM Fe and 7.8 pH) and T2 (0 µM Fe and 7.8 pH) treatments. Significant differences in SPAD and iron chlorosis scale reading were found between rootstocks and treatments. Treatments significantly affected the net photosynthetic rate of the W. Murcott mandarin. Moreover, it was found that tolerant rootstock had higher FCR activity in application T2 than in applications T3 and T4 (100 µM Fe and 7.8 pH). The data of the present study suggested that scion budded onto Volkameriana rootstock showed a higher tolerance to iron deficiency than those budded onto Swingle citrumelo under high pH conditions.

Key words: Iron chlorosis, high pH, W. Murcott, photosynthesis, Swingle citrumelo, Volkameriana

1. Introduction

Iron is the fourth most abundant element on earth, and soil typically contains 1%–5% total iron. Most iron in the soil is found in silicate minerals or iron oxides and hydroxides, forms that are not readily available for plant use (Schulte, 2004). Many Mediterranean soils contain relatively small amounts of iron oxides (Torrent and Barron, 2013) and high calcium carbonate concentrations (Jones et al., 2012). It is estimated that 20%–50% of fruit trees in the Mediterranean basin suffer from iron deficiency (Jaegger et al., 2000). The major cause of iron deficiency in these regions is high concentrations of calcium and bicarbonate in soils (Benyahia et al., 2011). On calcareous soils, high pH and $CaCO_3$ content can induce iron deficiency (Çelik and Katkat, 2007). Generally, the solubility of Fe decreases 1000-fold for every unit increase in pH above 4, and is lowest between pH 7.4 and 8.5 (Bohn, 1967).

Many fruit trees grown on high pH, and calcareous soils exhibit iron deficiency chlorosis (Huang et al., 2012). Citrus tree rootstocks vary in their ability to take up iron from the soil (Pestana et al., 2005; Castle et al., 2009; Pestana et al., 2011; Incesu et al., 2012; Cimen et al., 2014). Previous studies indicated that Volkameriana was very tolerant to low Fe stress, whereas Swingle citrumelo had lower tolerance to Fe stress (Rambola and Tagliavini, 2007; Castle et al., 2009). Leaves from tolerant rootstocks exhibited Fe deficiency symptoms only at lower levels of Fe in solution (<5 mmol Fe dm^{-3}), whereas leaves from sensitive rootstocks were chlorotic at Fe concentrations below 20 mmol Fe dm^{-3} (Pestana et al., 2005). These results indicated that sensitive and tolerant rootstocks have a differential response to the inhibitory effects of bicarbonate ions in the absorption and translocation of Fe.

Physiological and biochemical responses of citrus rootstocks have been determined by evaluating tree responses at equal iron concentrations under high pH conditions (Chouliaras et al., 2004b) by applying different iron doses at moderate pH (pH 6 and 7.4) (Pestana et al., 2001). However, we are not aware of studies evaluating tree responses from low-Fe sensitive and tolerant citrus rootstocks grown under high pH conditions with variable levels of iron fertilization. These soil conditions are common throughout the citrus-growing regions of the Mediterranean. In the past, many growers planted sour orange rootstock under high pH soil conditions. However, this rootstock is very sensitive to the tristeza virus; thus, there is a need to evaluate other rootstocks under low Fe conditions. In this study, we evaluated tree responses of W. Murcott scions on Volkameriana and Swingle citrumelo rootstocks grown under different Fe

* Correspondence: mincesu@cu.edu.tr

levels. The Volkameriana has been shown to tolerate low-Fe stress (Cimen et al., 2014), whereas Swingle citrumelo has lower tolerance to Fe stress (Pestana et al., 2005). Plant dry weight, leaf area, iron chlorosis scale, leaf chlorophyll concentration, net photosynthetic rate, and FCR activity were evaluated at different iron levels (0, 10, and 100 μM) under high pH conditions.

2. Materials and methods

2.1. Plant material, treatments, and growth conditions

W. Murcott (*Citrus reticulata* Blanco) scions budded on Volkameriana (*Citrus volkameriana* V. Ten. & Pasq) and Swingle citrumelo (Duncan grapefruit × *Poncirus trifoliate*) were used as plant materials. Bud unions and seeds were obtained from the Tuzcu citrus collection of the Faculty of Agriculture, Çukurova University.

Seeds were grown in plastic trays with sterilized 1:1 peat:soil, and were germinated in the dark at 22 °C. After germination, the seedlings were grown in pots in a greenhouse for 8 months. Uniform rootstock seedlings were then grafted and grown in a Hoagland nutrient solution modified for citrus for 8 months. Prior to the beginning of the experiment the plants were transferred to 3-L pots that were filled with quartz sand and placed in a plant growth chamber.

All plants were irrigated with a solution of the following composition: 1.25 mM KNO_3, 0.625 mM KH_2PO_4, 2.00 mM $MgSO_4$, 2.00 mM $Ca(NO_3)_2$, EDTA-Fe (125 μM), 25.0 μM H_3BO_3, 2.00 μM $MnSO_4$, 2.00 μM $ZnSO_4$, 0.50 μM $CuSO_4$, and 0.065 μM $(NH_4)_6Mo_7O_{24}$ for 2 months in half concentration in order to ensure plant adaptation to growth chamber conditions. Final solution pH and EC were adjusted to 5.8 and 1.05 mS, respectively. Plants were divided into one of four nutrient solutions treatments as follows: 1) 10^{-4} M Fe EDTA pH: 6 (T1), 2) 0 mM Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8 (T2), 3) 10^{-5} M Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8 (T3), and 4) 10^{-4} M Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8 (T4). These treatments were conducted for 5 months.

Relative humidity, day/night temperature, and photosynthetic photon flux density (PPFD) (approximately 20 cm above the plants) in the growth chamber were 65%, 26 °C/20 °C, and 450 ± 25 μmol m^{-2} s^{-1}, respectively. Cool white fluorescent lamps provided a 16-h light/8-h dark photoperiod.

2.2. Plant growth measurements and symptom score

At the end of the experiment the plants were harvested, dried in a forced air dryer (60 °C), and weighed. The seedlings were rated for chlorosis according to the method described by Byrne et al. (1995). The new fully expanded leaves were evaluated as follows: 1 = healthy green leaves; 2 = yellowish-green interveinal areas, green veins; 3 = greenish-yellow interveinal areas, green veins; 4 = yellow interveinal areas, green veins; 5 = yellow-white interveinal areas, pale green veins, and some defolation.

2.3. Leaf chlorophyll concentration

Leaf chlorophyll concentration was estimated using a portable SPAD-502 meter (Minolta, Japan). Readings were taken from the two youngest fully expanded leaves of each replicate at the end of the experiment. SPAD readings were used to estimate leaf chlorophyll concentration, because there is a strong relationship between SPAD readings and chlorophyll levels in citrus leaves (Cimen et al., 2014).

2.4. Gas exchange measurements

Five measurements were taken on fully expanded leaves of W. Murcott for each rootstock in all treatments. Net photosynthetic rate (P_N) [μmol(CO_2) m^{-2} s^{-1}] was measured at the end of the experiment using a portable photosynthesis system (model LCA-4, ADC Bioscientific Ltd., Hoddesdon, UK). Measurements were taken from attached mature leaves of about 6 months of age. Leaf temperature ranged between 26 and 28 °C and the relative humidity was 65% during the experimental period. Light levels had a range of 270–310 μmol m^{-2} s^{-1} during the measurement period.

2.5. Leaf area index

Leaf area index (LAI) was measured at the end of the experiment in all treated plants using a LI-COR LI-3000 leaf area meter (LI-COR, Lincoln, NE, USA).

2.6. Ferric-chelate reductase activity

Ferric-chelate reductase activity in the roots was determined according to Chouliaras et al. (2004a). Root tips of 5–8 mm length and a fresh weight of 20 mg were washed for 5 min with 0.2 mM $CaSO_4 2H_2O$ and placed in tubes containing 15 mL of aerated solution. The previous solution consisted of 0.2 mM $CaSO_4 2H_2O$, 0.1 mM Fe(III)-EDTA (ferric ethylene diamine tetra acetate), and 0.3 mM BPDS (Na2-bathophenanthrolinedisulfonic acid). The solution was buffered at pH 5.5 with 5 mM MES (2-(N-morpholino) ethanesulfonic acid). The tubes were incubated in a water bath in the dark at 23 °C for 120 min. Root-reducing capacity was expressed as the concentration of the resulting formation of Fe(II)-BPDS in the solution, and was estimated at 535 nm using a molecular extinction coefficient of 22.14 mM^{-1} cm^{-1}. The control consisted of a complete solution without roots (Chouliaras et al., 2004a).

2.7. Statistical analysis

The experiment was arranged as 4 × 2 × 7, four treatments, two rootstocks, and seven replicates, respectively, in a complete randomized design. Data were subjected to a two-way analysis of variance (ANOVA). Significant differences between means were calculated by using Tukey's multiple range test at a significance level of $P \leq 0.05$. The correlation coefficients between all measured parameters were also calculated. All statistical analyses

were performed with SAS v9.00 statistics software, and SigmaPlot 11.00 (Systat Software, San Jose, CA, USA) was used for data presentation.

3. Results

3.1. Plant growth

Rootstocks and different iron levels significantly affected plant dry weight and leaf area (Table 1). Volkameriana rootstock produced significantly more dry weight than Swingle citrumelo plants, and treatment T1 produced the largest plants (Table 2). In interactions, the dry weight of Volkameriana plants was higher than that of Swingle citrumelo in all treatments. Dry weight was greatest in Volkameriana T1 plants and lowest in Swingle citrumelo T2 and T3 plants (Table 2). Rootstock and iron treatment significantly affected leaf area (Table 1). Volkameriana plants had a larger leaf area compared to Swingle citrumelo. The largest leaf area among treatments was found in T1 plants, whereas T2 had the lowest (Table 2).

3.2. SPAD and symptom scores

At the end of the experiment, SPAD readings ranged between 18.60 and 56.50 (Table 2). A two-way ANOVA indicated a significant main effect of rootstock and Fe treatment and also their interaction ($P \leq 0.05$) on the SPAD and Fe chlorosis scale (Table 1). Plants grown in treatments T2 and T3 had the lowest SPAD value in the experiment. Swingle citrumelo T1 plants had the highest SPAD readings, followed by Volkameriana T1 and T4 plants (Table 2). The lowest SPAD readings were determined in the leaves of scion budded onto Swingle citrumelo T2, T3, and T4. Moreover, significant differences were determined in terms of symptom score observations. At the end of the experiment, extremely chlorotic leaves were observed on Swingle citrumelo T2, T3, and T4 plants. T1 plants had healthy green leaves in both rootstocks (Table 2).

3.3. Photosynthetic rate

Photosynthetic measurements showed that rootstock main effect on net photosynthetic rate (P_N) was significant on scion photosynthesis. Treatments significantly affected the net photosynthetic rate of W. Murcott mandarin. Furthermore, a significant rootstock × treatment interaction effect was observed according to the two-way ANOVA analysis (Table 1). The highest net photosynthetic rate was recorded on the leaves of W. Murcott under treatment T1. Increases of Fe concentration in the nutrient solution increased net photosynthetic rate. The leaves of W. Murcotton, both Volkameriana and Swingle citrumelo, had the highest net photosynthetic rate under T1. Treatments T2 and T3 resulted in the lowest net photosynthetic rate on the leaves of scion in both rootstocks (Table 2).

3.4. FCR activity

A two-way ANOVA indicated a significant main effect of rootstock, Fe treatment, and their interaction ($P \leq 0.05$) on the root ferric chelate reductase (FCR) activity (Table 1). Treatment T2 resulted in the highest FCR activity in the roots of Volkameriana, whereas in T1 it was highest in the roots of Swingle citrumelo (Table 2). In the Swingle citrumelo rootstock, FCR activity significantly increased at higher Fe levels in the nutrient solution (Table 2). In the present study, FCR activity of Fe-tolerant rootstock Volkameriana increased in T2 compared to T1. On the other hand, FCR activity of Fe-susceptible rootstock Swingle citrumelo was lower in T2 than in T1 (Table 2).

3.5. Correlation coefficients analysis

Significant correlations were determined between the investigated parameters. The correlations between chlorophyll content and P_N (0.77) and between leaf area and chlorophyll content (0.64) were very significant (Table 3).

Table 1. Results of two-way analysis of variance (ANOVA) of rootstock (R) and Fe treatment (T) effects and their interaction (R × T) for the dependent variables considered.

Dependent variable	Independent variable		
	R	T	R × T
DW	220.61*	8.64*	7.32*
Leaf area	8.10*	12.78*	4.36*
SPAD	50.98*	100.46*	17.86*
Iron symptom score	50.43*	67.81*	7.89*
P_N	8.97*	24.81*	4.19*
FCR activity	36.77*	19.63*	65.04*

[1]Numbers represent *F* values. * $P \leq 0.05$.

Table 2. Results of rootstock and Fe treatment effects and their interaction on the dependent variables considered.

	DW (g)	Leaf area (cm^2)	SPAD readings	Iron symptom score [1]	P_N [$\mu mol(CO_2)\ m^{-2}\ s^{-1}$]	FCR ($mM^{-1}\ cm^{-1}$)
Rootstock (R)						
Vo	35.27 a	17.55 a	40.31 a	1.91 b	2.11 a	0.82 a
Sw	28.41 b	16.06 b	31.81 b	2.75 a	1.83 b	0.69 b
Treatment (T)						
T1 control	33.60 a	19.25 a	53.39 a	1.00 c	3.73 a	0.77 b
T2 0	30.64 b	14.99 c	26.84 c	2.88 b	1.04 c	0.89 a
T3 10	31.37 b	15.82 bc	29.83 c	3.20 a	1.27c	0.66 c
T4 100	31.75 b	17.16 b	34.18 b	2.25 b	1.86 b	0.70 c
R × T						
Vo × T1 [2]	37.38 a	19.56 a	50.28 b	1.00 e	3.97 a	0.71 c
Vo × T2	33.27 c	15.98 a	35.43 d	2.50 c	0.92 c	1.21 a
Vo × T3	34.55 bc	16.53 a	35.08 d	2.40 c	1.30 c	0.72 c
Vo × T4	35.88 ab	18.11 a	40.46 c	1.75 d	2.25 b	0.66 cd
Sw × T1	30.24 d	18.94 a	56.50 a	1.00 e	3.49 a	0.84 b
Sw × T2	26.87 e	13.99 a	18.60 f	4.00 a	1.17 c	0.57 e
Sw × T3	26.73 e	15.12 a	24.22 e	3.25 b	1.24 c	0.60 de
Sw × T4	29.82 d	16.20 a	27.90 e	2.75 c	1.46 bc	0.74 c

Means followed by different letters in the same column are significantly different (Tukey's multiple range test at $\alpha = 0.05$).
[1] 1 = healthy green leaves; 2 = yellowish-green interveinal areas, green veins; 3 = greenish-yellow interveinal areas, green veins; 4 = yellow interveinal areas, green veins; 5 = yellow-white interveinal areas, pale green veins, and some defolation.
DW: dry weight, P_N: net photosynthetic rate, FCR: Ferric chelate reductase activity
[2] T1) 10^{-4} M Fe EDTA pH: 6
T2) 0 mM Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8
T3) 10^{-5} M Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8
T4) 10^{-4} M Fe EDTA + 2 g/L $CaCO_3$ + 3 mM $NaHCO_3$ pH: 7.8

Table 3. Correlation coefficients analysis between investigated parameters.

Variable	Symptom score	Leaf area	DW	SPAD	P_N	FCR
Symptom score	1.00	-0.64^{***}	-0.45^{**}	-0.89^{**}	0.33^{*}	-0.19^{ns}
Leaf area		1.00	0.40^{*}	0.64^{***}	0.57^{***}	0.24^{ns}
DM			1.00	0.46^{**}	0.46^{**}	0.42^{**}
SPAD				1.00	0.77^{***}	0.17^{ns}
P_N					1.00	0.51^{**}
FCR						1.00

[1] ***: $P \leq 0.001$, **: $P \leq 0.01$, *: $P \leq 0.05$, ns: not significant.

4. Discussion

Plant dry weight and leaf area were evaluated to compare the effects of iron treatments in high pH conditions. Plant dry weight was negatively affected by high pH treatments. In the present study, the highest plant dry weight was observed in the control plants. T2, T3, and T4 were lower than the control plants in terms of plant dry weight. Previous studies have also found that high pH (Yang et al., 2008) and bicarbonate ions (Mengel, 1994; Pestana et al., 2005) decrease plant growth and development. In this study, high pH and low iron had a significant effect on reducing dry weight. Similarly, total fresh weight of olive and peach rootstock was lower with higher bicarbonate and higher iron stress (De la Guardia and Alcantara, 2002). Furthermore, Shi et al. (1993) found large growth reduction in three peach rootstocks growing with bicarbonate. Decreased plant biomass and leaf area have been reported for various plant species grown under Fe-deficient conditions (Nenova, 2006).

In the present study, increases in plant leaf areas occurred with the rise in iron doses despite high pH. When dry weight and leaf area parameters are taken into account, it can be stated that in the presence of iron, Swingle citrumelo benefited from Fe. Moreover, in previous studies plant leaf area has been found to decrease depending on high pH (Colla et al., 2010). It is known that a low Fe supply negatively affects the chlorophyll content and other components of chloroplasts, which reduces growth capacity (De La Guardia and Alcantara, 2002). In the present study, it was observed that as iron doses rise, SPAD values increase, which is supported by other research findings (Pestana et al., 2001; De La Guardia and Alcantara, 2002; Pestana et al., 2011). This is because iron functions as a component of proteins in significant cellular events such as respiration and cell division; moreover, it has a role in the reduction steps of important biological events, such as transpiration and photosynthesis, and also in chlorophyll biosynthesis (Einsenstein and Blemings, 1998; Zocchi et al., 2007). It has been stated that this decrease in chlorophyll is due to the S-aminolevulinic acid functioning in chlorophyll biosynthesis and the effectiveness of Fe during the formation of protochlorophyll (Molassiotis et al., 2006). Chouliaras et al. (2004b) stated that the increase in pH in the same iron dose is accompanied by a decrease in the amount of chlorophyll in Valencia orange onto Swingle citrumelo and sour orange. However, this study illustrates that Swingle citrumelo rootstock can benefit from it in the presence of iron despite high pH; furthermore, Volkameriana rootstock utilizes considerably more iron under high pH conditions compared to Swingle citrumelo rootstock. Various studies claim that iron deficiency affects the content of chlorophyll and hence photosynthesis in plants (Davis et al., 1986; Hurley et al., 1986; Morales et al., 1994; Bertamini et al., 2001). Indeed, in this study the rate of photosynthesis is higher in the control plants compared to other applications. Chouliaras et al. (2004a) claimed that iron deficiency resulted in a significant decrease of Pn in citrus plants, and several authors indicated that the lime-induced iron deficiency reduced the net photosynthetic rate in the leaves (Morales et al., 1994; Abadía et al., 1999; Larbi et al., 2006; Nenova, 2009). Iron deficiency diminished the activity of the stroma enzymes. Additionally, lack of Fe reduced the formation of thylakoid membranes in chloroplast (Chouliaras et al., 2004a). Physiological parameters, such as chlorophyll and CO_2 gas exchange measurements, can substantiate the tolerance of plants to Fe deficiency or high pH conditions (Cimen et al., 2014). In this study, Volkameriana, known as the tolerant rootstock to iron deficiency, was found to have higher chlorophyll and Pn values than Swingle citrumelo.

FCR activity is reported to have fallen with high pH (Chouliaras et al., 2004b). In this study, the responses of tolerant and sensitive rootstocks were differentiated under T2 conditions. While tolerant rootstock provided the highest FCR activity, the lowest FCR activity was observed in sensitive rootstock in T2 application. Previous studies have reported that in a 7.4 pH environment, plants including low doses of iron secrete more FCR than those containing a higher dose of iron (Pestana et al., 2001); moreover, several studies have shown that FCR activity increases during 0 mM Fe application, whereas in applications containing bicarbonate a decrease in FCR activity occurred (Chouliaras et al., 2004b). The obtained results reveal that sensitive rootstock attempts to absorb Fe in the case of existence of a high dose of iron in the environment under high pH conditions (100 mM). It was found that tolerant and sensitive rootstocks have a differential response when there is little or no iron. This situation shows that in cultivated soils, rootstocks would have almost the same amount of FCR activity in the presence of enough iron and bicarbonate ions.

We described how different Fe concentrations in nutrient solution affected W. Murcott mandarin grafted on Volkameriana and Swingle citrumelo tolerant and susceptible to Fe deficiency, respectively. Plant growth and photosynthesis of W. Murcott mandarin on both rootstocks increased with the presence of Fe in the growth environment. In addition, a significant difference between tolerant and susceptible rootstocks was determined in terms of FCR activity under high pH conditions. This study determined that Swingle citrumelo rootstock, which is known to be susceptible to iron chlorosis, provides

good plant development if it is given sufficient amounts of iron under high pH conditions. Volkameriana performed well in this experiment, and Swingle citrumelo showed more susceptibility to high pH than Volkameriana.

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