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Root-reducing capacity, rhizosphere acidification, peroxidase and catalase activities and nutrient levels of *Citrus taiwanica* and *C. volkameriana* seedlings, under Fe deprivation conditions

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Abstract – The Fe deficiency created by either deprivation of Fe, low Fe supply or addition of $\text{CaCO}_3/\text{NaHCO}_3$ to Hoagland nutrient solution resulted in significant changes of the physiological and biochemical parameters of the rootstocks *C. taiwanica* and *C. volkameriana*. For instance, the lack of Fe and low Fe supply of 3 μM Fe-EDDHA induced an increase in the Fe root-reducing capacity and rhizosphere acidification. Seedlings treated with adequate Fe (20 μM Fe-EDDHA) showed a decline in the Fe-reducing capacity and in rhizosphere acidification. Fe deficiency was associated with a significant decline in peroxidase and catalase activities. Fe deprivation conditions increased the concentration of N, P, Ca, Mg and Mn and reduced the concentration of Fe and Zn in young leaves of both rootstocks. *C. volkameriana* was found to be more sensitive to Fe chlorosis than *C. taiwanica*.

catalase / citrus / Fe deficiency / peroxidase / rhizosphere acidification / root-reducing capacity

Résumé – Impact de la privation de Fe sur la capacité racinaire de réduction, l'acidification rhizosphérique, l'activité de peroxidase et catalase ainsi que sur le niveau nutritif chez le *Citrus taiwanica* et le *C. volkameriana*. Le déficit en fer provoqué par la privation, l'apport à faible dose ou l'addition de $\text{CaCO}_3/\text{NaHCO}_3$ provoque des modifications physiologiques et biochimiques chez les souches du *C. taiwanica* et du *C. volkameriana*. Par exemple, le déficit en fer induit une augmentation de la capacité racinaire de réduction du Fe, ainsi qu'une acidification de la rhizosphère. Des plants de semis présentent une diminution de la capacité de réduction du fer lorsqu'ils sont traités avec une quantité suffisante de Fe (20 μM MM Fe-EDDHA). Le déficit en fer est associé à l'activité de la catalase et de la peroxidase. Il est également observé que la privation de Fe augmente la concentration de N, P, Ca, Mg, Mn et diminue celle de Fe et de Zn chez les feuilles jeunes des deux souches. *C. volkameriana* est plus sensible à la chlorose en fer que *C. taiwanica*.

catalase / citron / déficit de Fe / peroxidase / acidification de rhizosphère / capacité racinaire de réduction

1. INTRODUCTION

Although Fe is abundant in most well-aerated soils, it is not readily available for plant uptake [33], primarily because it is relatively insoluble. Under conditions of limited Fe supply, the roots of plants undergo physiological, biochemical and morphological alterations in order to increase the rate of Fe absorption [6]. The first strategy exhibited by most plants, e.g. dicotyledons and monocotyledons except for grasses, consists of four types of root responses: (a) enhancement of H^+ -ion release [33, 34] that promotes solubilization and reduction of Fe^{3+} to Fe^{2+} ; (b) formation of rhizodermal transfer cells [18] that are related to the rhizosphere acidification [32]; (c) enhancement of the release of reducing compounds [21], and (d) enhancement of Fe^{3+} reduction to Fe^{2+} due to the increase in activity of the enzyme chelate reductase [21].

Bicarbonate presence is one of the major factors in the appearance of Fe chlorosis in plants grown on calcareous soils or irrigated with nutrient solution [8, 24]. High bicarbonate concentration in roots might decrease the physiological availability of Fe in leaves due to an increase in the pH of the leaf apoplast [19, 21, 25, 46]. Thus, the addition of bicarbonate to nutrient solution is widely used as a technique to screen cultivars tolerant to Fe chlorosis [9].

One of the analytical techniques used to study Fe chlorosis is the measurement of the total Fe concentration in leaves. However, this approach presents major problems because Fe-chlorotic leaves often have high Fe concentrations, and this condition is termed the "chlorosis paradox" [26]. In contrast to leaf analysis, the activity of two heme-proteins, peroxidase (PRX) (EC 1.11.1.7) and catalase (CAT) (EC 1.11.1.6), have been proposed as an indicator of the Fe nutritional status of

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plants [1, 2, 40]. PRX activity has also been proposed as a criterion to distinguish Fe from Mn chlorosis in Citrus [5], tomato and cucumber [39]. Furthermore, Fe chlorosis may affect the nutritional status of plants [41].

Citrus is considered to be a Fe chlorosis-susceptible genus and Fe chlorosis is one of the most frequent nutritional problems in its production on calcareous soils [7]. A common practice for recovering Fe chlorosis is soil application of Fe-chelates as ethylenediamine-di-(*o*-hydroxyphenyl)-acetic acid (Fe-EDDHA). However, the application of Fe chelates does not represent a suitable way to prevent or cure Fe chlorosis because of their cost and of the environmental risks associated with their use. Therefore, the use of tolerant rootstocks represents an alternative approach to solving this problem. Horticultural expertise also shows that there are differences in susceptibility between various rootstocks used for Citrus.

This study was conducted in order to evaluate Citrus rootstocks' tolerance to Fe chlorosis based on the Fe-reducing capacity, rhizosphere acidification, and activity of two heme-proteins, peroxidase (PRX) and catalase (CAT).

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Uniform healthy seedlings of the rootstocks *C. taiwanica* and *C. volkameriana* were used. Four seedlings of about 15 cm in height per rootstock were stabilized on an opaque lid in such a way that the roots were immersed in a 15-L modified (with respect to Fe) Hoagland nutrient solution [13] in the plastic bucket placed underneath. The nutrient solution was constantly aerated with the aid of an air pump and was changed every week over a 12-week period. The system was placed in a greenhouse at the farm of the Aristotle University of Thessaloniki, Greece. The greenhouse had natural irradiation with a transmission factor of around 70% for photosynthetically active radiation (PAR) at plant height (photosynthetic photon flux of $1010 \text{ mmol m}^{-2} \text{ s}^{-1}$) at 12:00 h. The mean temperature during the study varied between 20 and 26 °C.

Five replications (plastic buckets with 4 plants) were established for each one of the four nutrient treatments, as follows: 20 μM Fe-EDDHA (pH 6.0) as control, 0 μM Fe-EDDHA (pH 6.0), 3 μM Fe-EDDHA (pH 6.0) and 10 μM Fe-EDDHA plus 0.5 g L^{-1} CaCO_3 plus 10 mM NaHCO_3 (pH 7.5).

The Fe chlorosis score was visually estimated according to a scale from 0 (green) to 5 (severe chlorosis with necrosis) [31]. After a period of 12 weeks young and old leaves, shoot tips, stems and roots were collected.

2.2. Quantification of root-reducing capacity

Root-reducing capacity was estimated quantitatively every three weeks by measuring the capacity of the root to reduce Fe^{3+} to Fe^{2+} according to [17, 43]. Root tips of 5–8 mm length and 20 mg fresh weight were washed for 5 min with 0.2 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and placed in tubes containing 15 ml of aerated solution. The previous solution consisted of 0.2 mM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mM Fe(III)-EDTA (ferric ethylene diamine-

tetraacetate) and 0.3 mM BPDS (Na_2 -bathophenanthroline-disulfonic 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 at 23 °C in the dark 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 $22.14 \text{ mM}^{-1} \text{ cm}^{-1}$ [43]. The control consisted of complete solution without roots.

2.3. Rhizosphere acidification

The ability of the roots to acidify the rhizosphere was estimated according to [2]. At the end of the 11th week, a 10-ml sample of the treatment solution of each container was taken for pH determination using a combination pH electrode. An assay solution not containing roots, exposed to the same ambient conditions, was used as a control.

2.4. Plant enzyme extracts and assays

Plant parts (young and old leaves, shoot tips, and bark and young roots) were extracted by pestle and mortar with an ice-cold 10 mM Na-phosphate buffer (pH 6.5) containing 2.5% (w/v) insoluble polyvinylpyrrolidone (PVPP). The ratio of fresh weight to extraction buffer was 1:6 (w/v) for young leaves, shoot tips and young roots and 1:8 (w/v) for old leaves and bark. After homogenization the mixture was centrifuged for 30 min at 15 000 g (2–4 °C) and the initial supernatant was collected and used for determination of soluble peroxidase and catalase activities. The pellet was resuspended with a 0.1 M Na-phosphate buffer (pH 6.5) containing 1 M NaCl, incubated for 24 h at 2–4 °C and centrifuged for 30 min at 15 000 g (2–4 °C). The supernatant was collected for estimation of the ionically-bound-to-cell-wall PRX activity.

Peroxidase activity was estimated at room temperature as described by [28], with a UV-1601 Shimadzu spectrophotometer at 590 nm. One unit of peroxidase activity (U) was defined as the increase in one unit of absorbance per minute under the assay conditions. Enzymatic activity was referred to fresh weight (U/g FW).

Catalase (CAT) activity was measured according to the floating disc method [42]. Thirty μl of crude enzyme extract were applied onto a disc of 1 cm in diameter, cut from GF 52 filter paper (Schleicher and Schuell) and placed at the bottom of a Lamott test tube (CODE 0829). Twenty ml of 30 mM H_2O_2 were added and the elapsed time for the disc to float was recorded. The activity of catalase was quantified based on the activity of bovine liver catalase (Sigma). One unit of catalase activity (U) was defined as the decomposition of 1 μmol H_2O_2 /min at pH 7.0 and at 25 °C.

2.5. Mineral composition

Young and old leaves, roots, young shoots and stems were washed with a 0.01% soap plus 0.1 N HCl solution, followed by 2 rinses of distilled water. After drying at 68 °C for three days, the plant material was ground to pass through a 30 mesh screen and 0.5 g ashed at 500 °C for 5 h. The ash was diluted in 3 ml 6N HCl and brought to a volume of 50 ml with distilled

water. K, Na, Ca Mg, Fe, Mn and Zn were measured by atomic absorption spectroscopy (Perkin Elmer 2340) and expressed as $\mu\text{g/g}$ dry weight (DW) (Fe, Mn and Zn) or % DW (K, Na, Ca and Mg). The concentration of P was measured spectrophotometrically according to the phosphovanado-molybdate method, while N was determined by the Kjeldahl method.

2.6. Experimental design and statistic

The experimental layout was a 2×4 factorial. Analysis of variance (ANOVA) of all variables was performed using the statistical program SPSS. For the comparison of the means the Duncan's multiple range test was used and the standard error of the means was also calculated for the figures.

3. RESULTS

Visual symptoms of Fe deficiency (leaf chlorosis) appeared in young leaves at different times, depending upon the Fe deficiency treatments and rootstock. At the end of the 12th week the highest values of chlorotic scores were recorded in bicarbonate treatments in both rootstocks (Tab. I).

A time-course study of the two rootstocks showed that adequate Fe supply ($20 \mu\text{M}$ Fe-EDDHA) caused a continuous decline in root Fe-reducing capacity, while in all other treatments an increase was recorded (Fig. 1 and Fig. 2). Low Fe application ($3 \mu\text{M}$ Fe-EDDHA) resulted in the greatest increase in root Fe-reducing capacity for both the rootstocks.

The rootstock *C. taiwanica* showed greater absolute values of proton release than the rootstock *C. volkameriana* in the same Fe treatment (Tab. I). Low Fe application ($3 \mu\text{M}$ Fe-EDDHA) in the rootstock *C. taiwanica* exhibited a decline in the pH of the tested solution by 1.8 units, while in the same treatment the decline in the pH for the rootstock *C. volkameriana* was 1.25 units (Tab. I). Adequate Fe application ($20 \mu\text{M}$ Fe-EDDHA) caused pH decline in the rhizosphere by 0.65 and 0.5, respectively, for the rootstocks *C. taiwanica* and *C. volkameriana*. Acidification of the rhizosphere is positively correlated to the root Fe-reducing capacity throughout the experiment, in both rootstocks (data not shown).

Table I. Effect of the Fe treatments on chlorosis scores (0=green, 5=severe chlorosis with necrosis) of young leaves and rhizosphere acidification of the rootstocks *C. taiwanica* (T) and *C. volkameriana* (V), at the end of 12 weeks. Values with a different letter in the same column differ significantly at $P = 0.05$ (Duncan's multiple range test).

Rootstock	T	V	T	V
Treatments	Chlorosis score ^a		Rhizosphere acidification	
20 ^b (control)	0d	0.4d	-0.65c	-0.50d
3	0.8c	1.5c	-1.80a	-1.25a
0	1.5b	2.4b	-1.20b	-1.00ab
Bicarbonate ^c	2.8a	3.9a	-1.40ab	-0.80c

^a Average values of 5 replications (15 samples).

^b μM Fe-EDDHA in all tables.

^c Treatment with 10 mM bicarbonate in all tables.

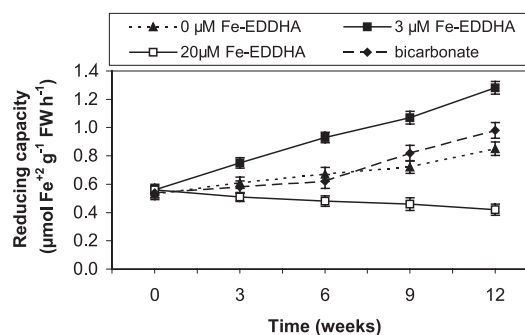


Figure 1. Changes in the reducing capacity ($\mu\text{mol Fe}^{2+} \text{g}^{-1} \text{FW h}^{-1}$) of *C. taiwanica* during a period of 12 weeks, as a function of different Fe treatments. Vertical bars represent mean values for three replications.

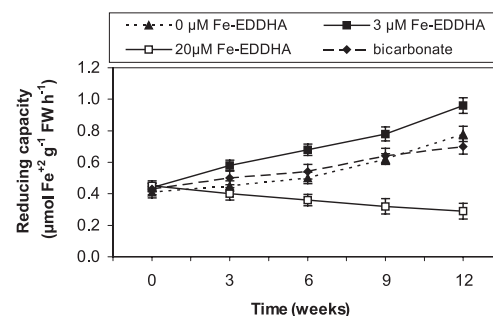


Figure 2. Changes in the reducing capacity ($\mu\text{mol Fe}^{2+} \text{g}^{-1} \text{FW h}^{-1}$) of *C. volkameriana* during a period of 12 weeks, as a function of different Fe treatments. Vertical bars represent mean values for three replications.

Significant differences between Fe treatments appeared in both Citrus rootstocks concerning soluble and ionically-bound peroxidase (PRX) activities (Tab. II and Tab. III). In all the plant parts, except the bark, the absolute values of soluble and ionically-bound-to-the-wall peroxidase activities were higher in *C. taiwanica* than in *C. volkameriana*. The highest absolute values of peroxidase activity were observed in young roots, while the lowest peroxidase activity appeared in shoot tips for the soluble peroxidase and in young leaves for the wall-bound peroxidase. Among Fe treatments the highest peroxidase activity in almost all plant parts of both rootstocks appeared in the treatment of adequate Fe application ($20 \mu\text{M}$ Fe-EDDHA). Unexpectedly, high wall-bound peroxidase activity appeared in the young roots of *C. taiwanica* and in the bark of *C. volkameriana* in the treatment of application of carbonate salts. The low ($3 \mu\text{M}$ Fe-EDDHA) and the -Fe treatments, as well as the presence of bicarbonate salts, negatively influenced catalase activity. The greatest absolute values of catalase activity appeared in the bark, followed by that in old leaves. The youngest plant parts (young leaves and roots and shoot tips) showed low catalase activity (Tab. IV).

Fe deficiency treatments increased the concentration of the elements N, P, K, Ca, Mg and Mn, but not Fe and Zn, the concentration of which declined in *C. taiwanica* (Tab. V) and *C. volkameriana* (data not shown). All fundamental elements studied, except Fe and Zn, showed greater concentration in

Table II. The effect of various Fe treatments on soluble peroxidase activity (U/g FW) of young and old leaves, bark, shoot tips and young roots of the rootstocks *C. taiwanica* (T) and *C. volkameriana* (V), at the end of 12 weeks. Values with a different letter in the same column differ significantly at $P = 0.05$ (Duncan's multiple range test).

Rootstocks Treatments	Young leaves		Old leaves		Bark		Shoot tips		Young roots	
	T	V	T	V	T	V	T	V	T	V
20	5.8a	5.2a	8.6a	7.6a	8.1a	10.4a	4.7a	5.9a	14.2a	12.0a
3	4.3b	3.5b	6.6b	5.4b	6.7b	9.6ab	4.0ab	3.6b	11.6ab	10.8ab
0	3.7bc	2.8c	5.1c	4.9bc	5.8c	8.5c	3.5b	3.0bc	10.2b	9.6bc
Bicarbonate	3.2c	2.4c	3.6d	3.4d	4.9d	7.4d	2.8c	2.4c	9.3b	8.2c

Table III. The effect of various Fe treatments on the ionically-bound-to-cell-wall peroxidase activity (U/g FW) of young and old leaves, bark, shoot tips and young roots of the rootstocks *C. taiwanica* (T) and *C. volkameriana* (V), at the end of 12 weeks. Values with a different letter in the same column differ significantly at $P = 0.05$ (Duncan's multiple range test).

Rootstocks Treatments	Young leaves		Old leaves		Bark		Shoot tips		Young roots	
	T	V	T	V	T	V	T	V	T	V
20	2.4a	2.2a	5.5a	5.0a	5.7a	8.5a	3.5a	3.4a	11.5b	9.0a
3	1.6b	1.5b	4.3b	3.4b	3.8bc	6.4bc	2.1b	2.7ab	9.8c	8.3ab
0	1.3bc	1.2bc	3.8b	3.2b	3.0c	5.4c	1.4c	2.0bc	9.1cd	7.2bc
Bicarbonate	1.1c	0.9c	2.4c	2.3c	4.2b	7.2b	2.4b	1.3c	13.3a	6.6c

Table IV. The effect of various Fe treatments on catalase activity (U/g FW) of young and old leaves, bark, shoot tips and young roots of the rootstocks *C. taiwanica* (T) and *C. volkameriana* (V), at the end of 12 weeks. Values with a different letter in the same column differ significantly at $P = 0.05$ (Duncan's multiple range test).

Rootstocks Treatments	Young leaves		Old leaves		Bark		Shoot tips		Young roots	
	T	V	T	V	T	V	T	V	T	V
20	20.2a	18.9a	39.8a	34.4a	63.4a	57.2a	28.2a	25.6a	26.2a	25.8a
3	15.6b	14.4b	19.2b	17.5b	56.3ab	51.3ab	18.3b	16.9b	18.5b	16.9b
0	14.5bc	13.9bc	15.6c	14.9bc	48.2bc	46.9bc	14.4b	14.1bc	14.2bc	14.1b
Bicarbonate	12.8c	11.8c	14.3cd	14.1c	39.8c	33.6c	13.7b	13.0c	13.6c	12.9c

Table V. Mineral composition of young leaves of the rootstock *C. taiwanica*, as affected by various Fe treatments. Values with a different letter in the same column differ significantly at $P = 0.05$ (Duncan's multiple range test).

Treatments	N	P	Ca	Mg	Fe	Zn	Mn
	(% DW)				$(\mu\text{g g}^{-1} \text{DW}^{-1})$		
20	2.65c	0.23b	2.18c	0.30b	96a	18a	12c
3	3.12b	0.26ab	2.63bc	0.32ab	73b	14ab	15abc
0	3.09bc	0.27a	2.75b	0.33ab	61c	13c	19a
Bicarbonate	3.03a	0.27a	2.98a	0.35a	63c	14ab	16ab

plants treated with low Fe than those treated with sufficient Fe (Tab. V).

4. DISCUSSION AND CONCLUSIONS

The two rootstocks showed variability as to their susceptibility to Fe chlorosis. The Fe chlorosis symptoms were more severe in *C. volkameriana* than in *C. taiwanica* seedlings, under the conditions of this study. In contrast to the results reported by [11] and based upon chlorosis scores, seedlings of *C. volkameriana* may be considered as non-tolerant to Fe chlorosis and *C. taiwanica* as tolerant.

Rootstock tolerance to Fe deficiency has been associated with the roots' ability to reduce external Fe^{+3} [30]. Although

this mechanism as a response to Fe deficiency has been widely described for herbaceous plants [6], there are few reports on woody species [4]. Our findings also illustrated the existence of a similar mechanism in the fruit tree species used in this study. In limited Fe supply conditions the rootstocks *C. taiwanica* and *C. volkameriana* obviously expressed the adaptive chelate reductase system. In general, insufficient Fe supply increased the root-reducing capacity of both rootstocks. An interesting point was that the greatest root Fe-reducing capacity was observed under the low Fe supply (3 μM Fe-EDDHA) rather than under the no Fe supply. Although in most published studies, root-reducing capacity was more likely to be induced by absolute Fe absence in the nutrient solution than by very low Fe concentrations, our observation is in agreement with previous reports for Citrus and [20, 38] and peach [3]. Furthermore, the root-reducing capacity system was functioning over a long period (12 weeks), as was also observed by [31, 32].

The ability of the two rootstocks to decrease the rhizospheric pH, which is a major characteristic of plants following the Strategy I, was also different. In our study, Fe deficiency treatments were correlated with a significant increase in proton release, compared with the adequate Fe application in both rootstocks. Although the proton release rate by roots in calcareous soils has no major influence on soil Fe-salts dissolution [12], in hydroponical culture it influences Fe availability [15]. The acidification of the external medium of a root cell is brought about by the plasmalemma located ATPase, which is responsible for the pumping of protons into the outer medium of the cell and for the polarization of the membrane [36]. It might be possible that a different activity of this enzyme explains the differential responses of the two rootstocks to lower rhizospheric pH. It was recently suggested that the redox status of Fe was influenced by apoplastic pH [16]. Under alkaline nutrient conditions, as in the case of NO_3^- , the apoplastic pH increase and Fe^{+3} reduction is depressed. The presence of HCO_3^- in the liquid nutritional medium or in soil solution does not influence xylem sap pH, but the neutralization process of HCO_3^- traps all the protons released by the proton pumps of the membranes. As a result, Fe^{+3} is trapped in the apoplast of the root epidermis.

Omission of Fe or addition of bicarbonate salts to the nutrient solution resulted in a significant decline in soluble and ionically-bound-to-cell-wall peroxidase and catalase activities, in all plant parts of both rootstocks. The high correlation found between Fe and enzyme activities might indicate that the activity of the hemoprotein was controlled by the availability of Fe in the tissue [14]. Fe concentration was highly correlated with peroxidase and catalase activities, when plants were grown in water or sand culture [23]. Fe deficiency was associated with low peroxidase and catalase activities in sunflower [29] and maize [27] plants, both involved in H_2O_2 metabolism. Severe Fe deficiency for a long time leads to cell senescence, as the plant does not metabolize H_2O_2 effectively [29]. Changes in peroxidase activity could be due to a general change in all isoenzymes [27], resulting in the appearance of new isoenzymes or disappearance of already existing isoenzymes [10]. Furthermore, changes of peroxidase isoenzymes were recorded in peach rootstocks under Fe deficiency conditions (Molassiotis, personal communication). peroxidase (soluble and ionically-bound to the wall) and catalase activities were higher in the case

of tolerant *C. taiwanica* rather than in the non-tolerant *C. volkameriana*. The ability of plants to synthesize more peroxidase and catalase could be a feature associated with tolerance to Fe deficiency.

The Fe concentration of chlorotic leaves was significantly lower compared with that of the non-chlorotic plants. Even if the Fe “chlorosis paradox” occurs in field experiments [19, 26], the previous observation concludes that this phenomenon did not occur in young Citrus plants, grown hydroponically. Furthermore, the nutrient concentration in young leaves was affected by Fe chlorosis. The increase in P concentration in the Fe deficiency treatments cannot be explained by P availability. According to [25] the high P concentration in chlorotic leaves is the result and not the cause of Fe chlorosis. The higher concentration of Mn in young leaves in the Fe treatment in comparison with the other treatments indicates an antagonistic relationship between Fe and Mn. A similar relationship has been found in numerous plant species [2, 22, 35, 44]. Although the bicarbonate leads to Fe chlorosis of plants grown on soil, sand or nutrient solution [37], this work showed that bicarbonate causes a significant decline in Zn concentration in young leaves. Bicarbonate has been regarded as a major factor of Zn deficiency in rice [45], especially in lowland soil. The changes in leaf Mn and Zn in response to Fe chlorosis were mainly attributed to the presence of bicarbonate, which modifies the redox status of rhizosphere and so affects the availability of these two metals.

In conclusion, the differentiation in Fe chlorosis between *C. taiwanica* and *C. volkameriana* seedlings likely reflects differences in the root Fe-reducing capacity, rhizosphere acidification, peroxidase and catalase activities and nutrient levels, but also genotypic differences. These physiological parameters could be efficiently used in screening techniques for selecting Citrus rootstocks tolerant to Fe chlorosis and also to forecast Fe chlorosis in advance of the appearance of chlorotic symptoms. The search for new Citrus rootstocks and new cultivar/rootstock combinations using the previously mentioned physiological parameters is one of the most important lines of investigation needed to prevent this nutritional problem.

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