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BRIEF COMMUNICATION

Iron chlorosis in grafted sweet orange (*Citrus sinensis* L.) plants: physiological and biochemical responses

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Abstract

Fe deficiency was imposed in *Citrus sinensis* L. cultivars Valencia and New Hall grafted on *C. aurantium* and *Swingle citrumelo* rootstocks by the absence of Fe (-Fe) or by the presence of bicarbonate in the Hoagland nutrient solution. In Fe-deprived leaves total and active Fe concentration, and peroxidase and catalase activities were decreased while the ratios carotenoids/chlorophylls, P/Fe, and K/Ca were increased. Fe(III) chelate reductase activity was induced in (-Fe)-treated roots whereas it was depressed in bicarbonate-treated roots.

Additional key words: carotenoids, catalase, chlorophylls, Fe(III) chelate reductase, peroxidase.

Bicarbonate is one of the most important factors causing Fe chlorosis in plants grown on calcareous soils (Mengel *et al.* 1984). Enhanced Fe(III) reduction under Fe deficiency by the root Fe(III) chelate reductase (FCR) is the most typical feature of dicotyledonous and monocotyledonous species, with the exception of the graminaceous plants (Romera *et al.* 1991). Thus, determination of the FCR activity has been widely used as a screening technique for selecting Fe chlorosis tolerant genotypes. Fe-chlorotic leaves often have high Fe contents (Fe chlorosis paradox) (Morales *et al.* 1998) and thus the total leaf Fe contents appear to be not potentially useful in Fe nutritional studies. The activities of the Fe-containg metalloenzymes, superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (PRX, EC 1.11.1.7), and catalase (CAT, EC 1.11.1.6) have been proposed as an indicator of environmental stress, including Fe deficiency (Nenova and Stoyanov 2000, Almansa *et al.* 2002). Furthermore, Fe deficiency may induce changes in regulation of cation uptake (Welch *et al.* 1993). Since Fe chlorosis is a widespread problem in citrus, the aim of this work was to find out how Fe deficiency is linked with physiological and biochemical factors in grafted plants of citrus.

One-year-old sweet orange (*Citrus sinensis* L.) cvs. Valencia (V) and New Hall (NH) plants grafted on two-year-old *C. aurantium* (A) and Swingle citrumelo (*C. paradisi* × *P. trifoliata*; C) rootstocks were planted in plastic bags containing 3000 cm^3 of sand/perlite mixture (1/1) and grown in a heated greenhouse (20 - 24 °C) from September to March in the Aristotle University farm of Thessaloniki, Greece. The experimental plants were irrigated every two days with 300 cm^3 of Hoagland's nutrient solution (Hoagland and Arnon 1950). Six plants (replications) were included in each one of the six nutrient solutions treatments, as follows: *1*) 20 μM Fe-EDDHA (pH 6.0) (+Fe) as control, *2*) 0 μM Fe-EDDHA (pH 6.0) (-Fe), *3*) 10 μM Fe-EDDHA + 0.5 g dm^{-3} $CaCO_3$ + 5 mM $NaHCO_3$ (pH 7.2) (bic_1), *4*) 10 μM Fe-EDDHA + 0.5 g dm^{-3} $CaCO_3$ + 10 mM $NaHCO_3$, (pH 7.5) (bic_2), *5*) 10 μM Fe-EDDHA + 0.5 g dm^{-3} $CaCO_3$ + 20 mM $NaHCO_3$ (pH 7.8) (bic_3), and *6*) 10 μM Fe-EDDHA + 0.5 g dm^{-3} $CaCO_3$ + 40 mM $NaHCO_3$ (pH 8.2) (bic_4). $NaHCO_3$ and $CaCO_3$ were

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Abbreviations: Car - carotenoids, CAT - catalase; Chl - chlorophyll; EDDHA - ethylenediamine-di-(*o*-hydroxyphenyl)-acetic acid; FCR - Fe(III) chelate reductase; PRX - peroxidase; SOD - superoxide dismutase.
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added to accelerate the Fe deficiency process. The experimental layout was a 2 × 2 × 6 factorial with two cultivars, two rootstocks, and six nutrient solutions treatments. The degree of Fe chlorosis in young leaves was estimated according to Alcantara *et al*. (1988). Chlorophyll (Chl) content was estimated at the end of the experiment by extraction from leaf disks (0.77 cm^2) with 96 % ethanol in a water bath at 78 °C, until complete discoloration of the disks. After Chl extraction the absorbance at 649 and 665 nm was measured, and Chl *a* and *b* contents were estimated according to the equations of Wintermans and Mots (1965). Carotenoids (Car) were extracted from six leaf disks by pestle and mortar, using cold ethanol, in a dark room. The absorbance of the extracts was measured at 425 and 437 nm. The content of Car was estimated according to the equations of Britton (1991).

For enzyme determination, a 200-mg sample of young leaves was homogenised in a cold mortar in a sixth-fold volume of 10 mM Na-phosphate buffer (pH 6.5) containing 2.5 % (m/v) insoluble polyvinylpolypyrrolidone (PVPP) and 1 M NaCl. After homogenization the mixture was centrifuged (15 000 *g*, 30 min, 4 °C) and the supernatant was used as crude enzyme extract for peroxidase (PRX) and catalase (CAT) activities. PRX activity was assayed according to Ngo and Lenhoff (1980) in a *UV-1601 Shimadzu* (Japan) spectrophotometer at 590 nm. One unit of POD activity (U) was defined as the increase of one unit of absorbance per minute, under the assay conditions. CAT activity was measured according to the floating disc method (Wang 1995). One unit of CAT activity (U) was defined as the decomposition of 1 μmol H_2O_2 min^{-1} at pH 7.0 and 25 °C. The root Fe (III) chelate reductase activity was estimated according to Welch *et al.* (1993) and Albano and Miller (1996). Root tips of 5 - 8 mm length and 20 mg fresh mass were washed for 5 min with 0.2 mM $CaSO_4 . 2\ H_2O$ and were placed in tubes containing 15 cm^3 of aerated solution consisted of 0.2 mM $CaSO_4 . 2\ H_2O$, 0.1 mM Fe(III)-EDTA (ferric ethylene diamine-tetraacetate), and 0.3 mM Na_2-bathophenanthrolinedisulfonic acid (BPDS). The solution was buffered at pH 5.5 with 5 mM

Table 1. The effect of Fe treatments (for explanation of treatments see the text) on total and active Fe, PRX, and CAT activities in citrus leaves, and root FCR activity of various scion and rootstock combinations (V × A, V × C, NH × A, NH × C). Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, $P \leq 0.05$).

Parameters	Treatment	V × A	V × C	NH × A	NH × C
Total Fe [mg kg^{-1}(d.m.)]	+Fe (control)	93e	81c	102e	88d
	–Fe	69c	67b	88d	52d
	bic_1	81d	69b	75c	71c
	bic_2	67c	66b	53b	42a
	bic_3	52b	43a	45ab	49ab
	bic_4	43a	40a	37a	40a
Active Fe [mg kg^{-1}(d.m.)]	+Fe (control)	58d	56d	60e	58e
	–Fe	40b	38bc	40c	39c
	bic_1	49c	43c	52d	48d
	bic_2	39b	41c	39c	41c
	bic_3	34ab	32b	26ab	29b
	bic_4	29a	24a	20a	17a
PRX [U g^{-1}(f.m.)]	+Fe (control)	5.8d	5.6c	6.2d	5.9e
	–Fe	4.2c	4.1b	4.3bc	3.9bc
	bic_1	3.9bc	4.3b	4.5c	4.5d
	bic_2	3.6b	4.0b	3.9ab	4.0c
	bic_3	3.5ab	3.4a	3.7a	3.5ab
	bic_4	2.9a	3.2a	3.5a	3.1a
CAT [U g^{-1}(f.m.)]	+Fe (control)	17.0e	15.6e	16.2d	15.9d
	–Fe	14.0c	13.8c	14.3c	14.0c
	bic_1	14.5d	14.4d	14.5c	14.3c
	bic_2	14.0c	13.6bc	14.2c	13.3b
	bic_3	13.4b	13.2b	12.9b	13.0b
	bic_4	12.9a	12.4a	11.8a	12.2a
FCR [μmol Fe^{+2} g^{-1}(f.m.) h^{-1}]	+Fe (control)	0.82e	0.79e	0.84e	0.74b
	–Fe	0.98f	0.82e	1.02f	0.81e
	bic_1	0.74d	0.69d	0.71d	0.65c
	bic_2	0.58c	0.52c	0.60c	0.52b
	bic_3	0.46b	0.38b	0.44b	0.36a
	bic_4	0.34a	0.28a	0.32a	0.32a

2-N-morpholino ethanesulfonic acid (MES). The tubes were incubated, in the dark, in a water bath at 23 °C, for 120 min. Root Fe (III) chelate reductase was estimated at 535 nm, using a molecular coefficient of absorbance 22.14 $mmol^{-1}$ cm^{-1} (Welch *et al.* 1993). The control consisted of complete solution without roots.

For determination of inorganic elements, young leaves dried at 68 °C for 3 d were ground in a mill to pass a thirty mesh screen and 0.5 g of the ground tissue was dry ashed at 540 °C for 5 h. The ash was diluted to 3 cm^3 of 6 M HCl and the filtrate was analysed for K, Ca, and Fe by atomic absorption spectroscopy (*Perkin Elmer 2340*, USA). The active Fe concentration was measured according to Guzman *et al*. (1986). P concentrations were estimated spectrophotometrically by the phospho-vanado-molybdate method. The statistical analysis was performed by the program *SPSS-10* (SPSS, Chigaco, USA).

At the end of the experiment (6.5 months after the initiation) typical symptoms of Fe chlorosis were noticed in the $NaHCO_3$-treated leaves, while slight Fe chlorosis symptoms were observed in the -Fe treatment in both the grafted cultivars (data not shown). The contents of total and active Fe in Fe-deprived leaves were decreased (Table 1). By contrast to total Fe, the active Fe was always negatively correlated to the degree of Fe chlorosis (r = -0.922**, -0.934**, -0.896*, and -0.866* for the scion and rootstock combinations V × A, V × C, NH × A and NH × C, respectively). Furthermore, in Fe-deprived leaves, PRX and CAT activities were depressed. The degree of Fe chlorosis was correlated with the activity of PRX (r = -0.822*, -0.922**, -0.913**, and -0.811* for the combinations V × A, V × C, NH × A, and NH × C, respectively) and CAT (r = -0.932**, -0.903**, -0.925** and -0.943**). Hence, the enzyme activities proved to be a reliable indicator of Fe chlorosis for both orange cultivars grafted on two rootstocks. In (-Fe)-treated roots FCR activity was increased while it was decreased in bicarbonate-treated roots (Table 1). The addition of bicarbonate in the Hoagland nutrient solution increases the pH of the solution due to its pH buffering capacity. Therefore, it may trigger a decrease in FCR activity since its optimal pH is around 5.0 (Moog and Bruggemann 1994). Under Fe deprivation, an increase of the Car/Chl ratio was noticed (Table 2). A similar trend in the changes of Car and Chl contents was observed in the Fe-deprived sunflower leaves (Ranieri *et al.* 2001). According to Fernandez-Lopez *et al.* (1993) the Car/Chl ratio was increased in Fe chlorotic lemon leaves and Fe application had greater influence on Chl rather than on Car. Furthermore, in the Fe-deprivation treatments the P/Fe ratio was increased (Table 2). However, in the 40 mM $NaHCO_3$-treated leaves, although the degree of Fe chlorosis was higher than in the rest $NaHCO_3$ treatments, the P/Fe ratio was not high enough due to the reduction in P concentration in this treatment (data not shown). Contrary to Fernandez-Lopez *et al.* (1991), Fe chlorotic leaves showed an increase in K/Ca ratio (Table 2).

In conclusion, PRX, CAT, and FCR activities, active Fe concentrations, and the ratios Car/Chl, P/Fe, and K/Ca could be efficiently used in screening techniques and for early evaluation of Fe-nutritional status of citrus plants.

Table 2. The effect of Fe treatments (for explanation of treatments see the text) on Car/Chl, K/Ca, and P/Fe ratios in young citrus leaves of various scion and rootstock combinations (V × A, V × C, NH × A, NH × C). Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, $P \leq 0.05$).

	Car/Chl				K/Ca				P/Fe			
	V × A	V × C	NH × A	NH × C	V × A	V × C	NH × A	NH × C	V × A	V × C	NH × A	NH × C
+Fe	0.08a	0.09a	0.08a	0.09a	0.35a	0.36a	0.35a	0.32a	31.5a	35.8a	31.6a	32.1a
–Fe	0.09b	0.11b	0.09b	0.13c	0.52b	0.57e	0.44c	0.48cd	47.5d	48.7d	47.1c	42.1bc
bic_1	0.10b	0.11b	0.09ab	0.11b	0.51b	0.47c	0.40b	0.39b	40.0b	44.8c	41.7b	41.5b
bic_2	0.12c	0.12b	0.09ab	0.14c	0.50b	0.43b	0.42bc	0.43bc	49.3b	45.6cd	51.2d	44.9c
bic_3	0.13c	0.17c	0.12c	0.16d	0.97d	0.45bc	0.50d	0.46c	67.3e	71.8e	55.6e	65.7d
bic_4	0.15d	0.19d	0.15d	0.18e	0.64c	0.51d	0.55de	0.50cd	44.2c	41.0b	40.5b	42.9bc

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