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Screening of 'King' mandarin (*Citrus nobilis* Lour) × *Poncirus trifoliata* ((L.) Raf.) hybrids as citrus rootstocks tolerants to iron chlorosis

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ABSTRACT

Iron (Fe) chlorosis is one of the commonest problems in calcareous soils and plant tolerance is determined mainly by rootstocks. This work evaluates new citrus hybrids' tolerance to Fe chlorosis compared with the most widely used rootstocks in Spain: *Citrus macrophylla* (CM, Fe chlorosis-tolerant) and Carrizo citrange (CC, Fe chlorosis-sensitive). Growth parameters, Fe concentration, photosynthetic parameters, ferric chelate reductase (FC-R) activity and proton (H^+) extrusion capacity were assessed in plants irrigated with 20 (control, Ct) or 0 (Fe-deficient, -Fe) μM FeEDDHA. Some—Fe hybrids presented marked Fe chlorosis symptomatology reflected by the root:shoot ratio and Chl *a* and *b* concentrations sharply dropping, and an increase in the Chl *a*/*b* ratio. These effects were very strong in the 050119, 050124-B and 050110—Fe species. The net CO_2 assimilation rate significantly lowered in the 05019, 050131, 050125, 050112 and 05013—Fe species. The 050120, 050125, 050112 and 050124-B—Fe plants presented lower stomatal conductance than the Ct ones. Conversely, the internal CO_2 concentration (Ci) tended to increase in Fe-deprived plants. Fe-deficiency increased FC-R activity and H^+ extrusion in some 0501 species. Both responses were significantly induced in the CM, 050114, 05019, 050131 and 050125—Fe plants. Fe^{2+} accumulation in—Fe plants related inversely with FC-R activity. Most—Fe species with the greatest FC-R activity also accumulated the most Fe^{3+} ions and, therefore, the Fe pool was much larger in the root apoplast. Collectively, the main trait that determined Fe-chlorosis tolerance among these genotypes was the ability to: (1) boost Fe^{3+} reduction in response to Fe-deficiency; (2) acidify root media; (3) benefit the Fe amounts stored in the root apoplast.

1. Introduction

Iron (Fe) deficiency is one of the most important nutritional disorders as it participates in some life-sustaining processes, such as respiration and photosynthesis, where it is involved in electron transfer through Fe^{2+}/Fe^{3+} redox reactions. Despite it being relatively abundant in many cultivated soils, its acquisition by crop plants is often impaired by certain soil properties, like high carbonate content, which alkalinises soil solution and reduces Fe availability for plants (Hell and Stephan, 2003; Msilini et al., 2009). These conditions affect most *citrus* cultivated in the Mediterranean basin, which frequently develop Fe chlorosis symptoms, mainly interveinal yellowness in leaves, stunted vegetative growth, worse yields and poor fruit quality.

A widely applied system to prevent Fe-deficiency in fruit tree crops is to use Fe chlorosis-tolerant genotypes as rootstocks (Jiménez et al., 2008, 2011; Ksouri et al., 2006, 2007; Molassiotis et al., 2006). For this purpose, Fe chlorosis tolerance of citrus rootstocks has been tested in several studies (Castle et al., 2009; Chouliaras et al., 2004; Pestana et al., 2005). However, availability of citrus rootstocks that combine positive plant responses, like tolerance to iron chlorosis, CTV (citrus tristeza virus) and *Phytophthora* spp., is still scarce. Therefore, it is essential to search for new rootstocks that cover all three characteristics. To this end, The Valencian Institute of Agrarian Research (IVIA) carries out an ambitious breeding programme of citrus rootstocks to evaluate the behaviour of new citrus rootstocks against several abiotic disorders,

Abbreviations: CC, Carrizo citrange; CM, Citrus macrophylla; -Fe, iron-deprived; FC-R, ferric chelate reductase; H^+-ATPase, proton-ATPase; IRT, iron transporter; EDDHA, Ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid); EDTA, Ethylenediaminetetraacetic acid; Chl, chlorophyll concentration; Pn, net photosynthesis rate; gs, stomatal conductance; Ci, internal CO_2 concentration; MES, morpholineethanesulfonic acid; BPDS, bathophenanthroline-disulfonic acid disodium salt hydrate; DW, dry weight; FW, fresh weight.

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Table 1
Dry weight (DW; in g) and total Fe concentration (in $\mu g\,g^{-1}$ DW) in shoots and roots from thirteen citrus species irrigated for 8 weeks in nutrient solutions either with (control, Ct) or without (-Fe) FeEDDHA. The values are means of six independent plants ($n = 6$). Within each species, comparisons among treatments were made using Fisher's least significance difference (LSD) test at $^{*}P < 0.05$. CM: *Citrus macrophylla*; CC: Carrizo citrange.

		Dry weight (g)				Total Fe concentration ($\mu g\,g^{-1}$ DW)			
Genotype	Treatment	Shoot		Root		Shoot		Root	
CM	Ct	17.7	**	4.5	*	34.8	**	256.7	***
	-Fe	12.2		3.6		22.1		79.1	
CC	Ct	9.5	***	4.7	***	35.3	ns	220.6	***
	-Fe	5.6		3.1		28.8		100.3	
050119	Ct	8.8	ns	5.0	**	42.0	***	307.0	**
	-Fe	9.2		3.3		23.8		185.8	
050114	Ct	6.5	ns	3.7	***	52.9	**	243.7	**
	-Fe	6.9		2.4		39.5		160.2	
05019	Ct	9.4	***	4.0	***	32.6	*	416.2	***
	-Fe	5.7		3.3		24.5		125.3	
050131	Ct	7.2	*	2.6	ns	86.0	**	360.1	**
	-Fe	5.7		2.9		37.5		198.9	
050120	Ct	7.5	***	2.4	ns	58.5	*	178.3	**
	-Fe	6.2		2.9		35.0		105.1	
050125	Ct	10.7	*	2.7	*	41.0	ns	233.4	***
	-Fe	8.2		3.6		35.4		71.8	
050112	Ct	12.9	ns	5.1	ns	42.0	**	235.7	***
	-Fe	12.6		5.1		28.0		81.4	
05013	Ct	7.6	**	2.4	ns	49.2	ns	307.9	***
	-Fe	6.0		2.4		35.8		104.2	
050110	Ct	13.0	***	4.0	ns	34.5	*	400.0	***
	-Fe	5.8		3.7		24.0		88.9	
050124-B	Ct	5.7	**	2.9	ns	30.6	ns	203.1	**
	-Fe	7.5		2.5		32.2		123.0	
050129	Ct	5.9	**	2.0	ns	43.2	*	300.2	**
	-Fe	3.3		1.8		26.7		154.5	
ANOVA	G		***		***		***		***
	T		***		***		***		***
	G × T		***		***		***		***

like salinity, flooding or iron chlorosis (Forner et al., 2003; Forner-Giner et al., 2009; García-Sanchez et al., 2007; Gimeno et al., 2012; Gonzalez-Mas et al., 2009; Yang et al., 2003). One of the combinations of this programme was hybrids of Mandarino King (tolerant to bicarbonate and *Phytophthora*) and *Poncirus trifoliata* (tolerant to CTV), denominated as 0501 species. As conventional breeding is very slow for woody plants, using physiological screening methods is an excellent tool for the quick characterisation of new citrus genotypes' response to, in this case, Fe-deficient conditions.

Plant growth (length, dry biomass, root/shoot ratio), Fe nutritional status (in leaves and roots), chlorophyll concentrations (directly or estimated by the SPAD index) and photosynthetic activity are common determinations used to evaluate Fe-chlorosis tolerance in plants (Jelali et al., 2010; Martínez-Cuenca et al., 2013a,b; Pestana et al., 2005, 2011). It is known that dicotyledonous and non-grass monocotyledonous species have developed certain adaptive mechanisms, called Strategy I, to increase Fe-uptake capacity under Fe-deficiency conditions, (Kim and Guerinot, 2007; Schmidt, 2005). Strategy I includes, among other responses, enhanced proton (H^+) extrusion by roots that lowers the rhizosphere pH (Santi and Schmidt, 2009), and an enhanced capacity to reduce ferric forms (Fe^{3+}) to ferrous forms (Fe^{2+}) (Kim and Guerinot, 2007) by inducing Ferric Chelate Reductase (FC-R, EC 1.16.1.7) enzyme (Connolly et al., 2003; Jelali et al., 2010; Waters et al., 2002). Both analyses are also run in screening works to determine Fe-deficiency tolerance in plants (Gogorcena et al., 2004; Martínez-Cuenca et al., 2013a; Pestana et al., 2011).

Another aspect to be considered is the form in which Fe is present inside plants. As Fe^{2+} is the functional one for plant development, the balance of ferrous and non-ferrous Fe in roots evidences FC-R enzyme activity in roots. Fe accumulates in the root apoplast, mostly as the Fe^{3+} form, and can operate as an Fe storage pool, and an extra-plasmatic Fe source accumulates and re-translocates from roots to shoots in plants grown under Fe-deprived conditions (Longnecker and Welch, 1990). All of this suggests genotypical differences in Fe-deficiency resistance between species.

Since the relative importance of Fe-acquisition system components seems to differ considerably between plant species and genotypes, some studies have focused on differences in root responses to Fe-deficiency among plant species (DellíOrto et al., 2000a; Donnini et al., 2009; Gharsalli and Hajji, 2002), including citrus (Chouliaras et al., 2004; Pestana et al., 2005, 2011). The main objective of this work was to evaluate the behaviour of 11 new citrus rootstocks under Fe- deficient conditions according to several determinations: (1) physiological plant responses (biomass, chlorophyll concentration and photosynthesis rate); (2) absorption and distribution of Fe content in plants; (3) acidification response of roots; (4) activity of the key enzyme involved in the ferrous reduction process; (5) quantification of Fe content in the ferrous form present in the root system. For this purpose, assays were carried out in 11 Fe-deficient and Fe-sufficient seedlings of 0501 hybrids, and were compared with two of the main rootstocks widely used as citrus rootstocks in Spain, known for their different tolerance Fe chlorosis (Castle et al., 2009): *Citrus macrophylla* (CM, Fe chlorosis-tolerant genotype) and Carrizo citrange (CC, Fe chlorosis-sensitive genotype).

2. Materials and methods

2.1. Plant material and growth conditions

Seeds from 11 citrus hybrids, a combination of 'King' mandarin (*Citrus nobilis* Lour) × *Poncirus trifoliata* ((L.) Raf.) mother plants (named 0501 hybrids), *Citrus macrophylla* W. and Carrizo citrange [hybrid of *Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.], were germinated in a glasshouse in a sterile substrate that comprised peat, coconut fibre, sand and perlite