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BRIEF COMMUNICATION

Fe-EDDHA promotes rooting of rootstock GF-677 (*Prunus amygdalus* × *P. persica*) explants *in vitro*

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Abstract

The effect of organic (Fe-EDTA and Fe-EDDHA) and inorganic ($FeCl_3$) iron substances on rooting of the rootstock GF-677 (*Prunus amygdalus* × *Prunus persica*) *in vitro* was studied. Full rooting (100 %) was observed in explants nourished with Fe-EDDHA, while less rooting was found in the absence of iron or in the presence of $FeCl_3$. On the contrary, no root formation was observed in explants nourished with Fe-EDTA, which showed extremely lower chlorophyll and high iron contents at the end of the experiment.

Additional key words: chlorophyll, Fe content, iron, iron-chelates, micropropagation, peach rootstock.

The GF-677 (*Prunus amygdalus* × *P. persica*) is a peach rootstock tolerant to Fe deficiency (Gogorcena *et al.* 2000). Therefore, its micropropagation *in vitro* is important for commercial purposes. The induction of roots on explants from *in vitro* cultures is crucial in any micropropagation process. The ability of plant tissue to form adventitious roots depends on interaction of many exogenous and endogenous factors, including iron (Frenkel and Hess 1973). Although Fe is an essential micronutrient of plant tissue culture and involved in fundamental processes such as chlorophyll (Chl) and DNA synthesis and hormone formation (Briat and Lobréaux 1997), there is lack of knowledge concerning the role of Fe in the rooting process. According to Hewitt and Smith (1974) cell division and root tip meristems are especially sensitive to iron deficiency. Also, Fe deficiency is associated with morphological changes in roots, such as inhibition of root elongation, increase of the diameter of apical root zone, abundant root hair formation (Romheld and Marschner 1981, Chaney *et al.* 1992), and formation of rhizodermal transfer cells (Kramer *et al.* 1980). In addition to this, auxin has been implicated in the regulation of responses to Fe deficiency, whereas Fe-stress induced an increase of the synthesis of auxin (Romera *et al.* 1999).

Two of the standard media used in plant tissue culture are Murashige and Skoog (1962) (MS) and Lloyd and McCown (1980) (WPM) media, which contain iron in the chelated form of Fe-EDTA, although of all chelates known the most effective in acid and alkaline pH is Fe-EDDHA, the most effective form for correction of Fe-chlorosis for over fifty years. Many problems are connected with the use of Fe-EDTA *in vitro* (Dunlap and Robacker 1988, Stasinopoulos and Hangarter 1990, Hangarter and Stasinopoulos 1991). Also, there is evidence in the literature that the physiological effects of Fe-EDTA and Fe-EDDHA, as chelating agents, are different. For example, Chopra and Rashid (1969) found that the moss *Anoectangium thomsonii* did not form buds when grown on a medium containing Fe-EDTA, but did so when Fe-EDDHA was added to the medium. Haploid embryoids developed more freely from *in vitro* cultures of *Atropa belladonna* pollen microspores when Fe-EDDHA was incorporated into the medium, rather

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Abbreviations: Chl - chlorophyll; EDDHA - ethylenediamine-di-(*o*-hydroxyphenyl)-acetic acid; EDTA - ethylenediaminetetraacetic acid; MS medium - Murashige and Skoog medium; WPM medium - Lloyd and McCown medium.
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than Fe-EDTA (Rashid and Street 1973).

The objective of this study was to compare the effect of Fe-EDTA, Fe-EDDHA, and $FeCl_3$ at three concentrations on rooting of peach rootstock GF-677 (*Prunus amygdalus* × *P. persica*) explants.

The explants were shoot tips (15 mm in length) preserved from previous *in vitro* cultures and maintained in a growth chamber. Each explant was transferred aseptically onto 10 cm^3 full strength MS medium (Murashige and Skoog 1962) in 25 × 100 mm test tubes. The MS nutrient medium was supplemented with 30 g dm^{-3} sucrose, 7 g dm^{-3} agar, 0.6 mg dm^{-3} benzyladenine (BA), 0.2 mg dm^{-3} gibberellic acid (GA_3) and 0.05 mg dm^{-3} 3-indolebutyric acid (IBA), and contained three types of iron ($FeCl_3$, Fe-EDTA, Fe-EDDHA). Each form was supplied at three iron concentrations (0.05, 0.1, and 0.2 mM Fe), while the control was MS medium minus iron (-Fe). Their pH was adjusted to 5.2 before autoclaving at 121 °C for 15 min. In each treatment, 25 replicates (tubes) were included, arranged randomly in the growth chamber, and maintained at 22 ± 1 °C and 16-h photoperiod (*Philips TLD 54/36W* fluorescence tubes, irradiance of 45 μmol m^{-2} s^{-1}), for a total period of 24 d. At the end of the experiment, number, length, and fresh and dry mass (DM) of shoots and roots were recorded. The date of first root emergence, visible to the naked eye, was recorded for each treatment. Rooting was recorded as the percentage of explants producing roots after 24 d on the rooting medium.

Chl content was estimated at the end of the experiment by extraction from leaf disks (0.77 cm^2) with 96 % ethanol in water bath at 78 °C, until complete discoloration of the disks. The absorbance of the extracts was measured in a spectrophotometer (*Spectronic 20D*, Milton Roy, USA) at 665 and 649 nm. Chl *a* and *b* concentrations were calculated by the equations given by Wintermans and Mots (1965) and expressed on the basis of dry mass.

At the end of the experiment shoots (stems plus leaves) and roots from each plantlet were harvested, rinsed with distilled water, dried at 68 °C for 48 h, and ground to pass a 30-mesh screen. After ashing at 530 °C for 16 h, the residue was dissolved in 3 cm^3 of 6 M HCl and brought to 50 cm^3 with distilled water. Fe was measured by atomic absorption spectroscopy (*Perkin Elmer 2340*) (Franson 1985).

The explants supplied with Fe-EDDHA had the highest rooting percentage (100 %), as well as the greatest number of roots per explant, root length, and root fresh mass (Table 1, Fig. 1). The number of roots and their length was significantly increased in explants treated with 0.2 mM Fe as Fe-EDDHA, compared to the other Fe treatments. The inorganic iron treatments ($FeCl_3$) resulted in an 81 - 86 % rooting, while the -Fe treatment resulted in a rooting close to 50 % (Table 1). Furthermore, MS medium containing Fe-EDDHA advanced root emergence by 1 - 3 d, while rooting medium without Fe or containing $FeCl_3$ delayed root emergence by 3 or 1 - 2 d, respectively (Table 1). No significant differences in stem number, length, or fresh and dry mass were found among the various treatments (values not presented).

In explants treated with Fe-EDTA leaf chlorosis was noticed, while slight chlorosis was observed in -Fe treatment. Foliar symptoms of chlorosis did not occur in plants treated with Fe-EDDHA or $FeCl_3$. No-rooted explants had extremely low contents of Chl *a* and *b* (Table 1). On the contrary, in the rooted explants (treated with Fe-EDDHA or $FeCl_3$) high contents of Chl were observed. Chl content in the rooted explants increased in relation to the rise of Fe concentration in the medium.

The non-rooted explants (treated with Fe-EDTA) exhibited extremely high Fe content (Table 1). No significant differences among the various treatments were recorded in the contents of the rest essential elements (values not presented).

The explants treated with Fe-EDDHA had higher rooting percentage, whereas the explants supplied with

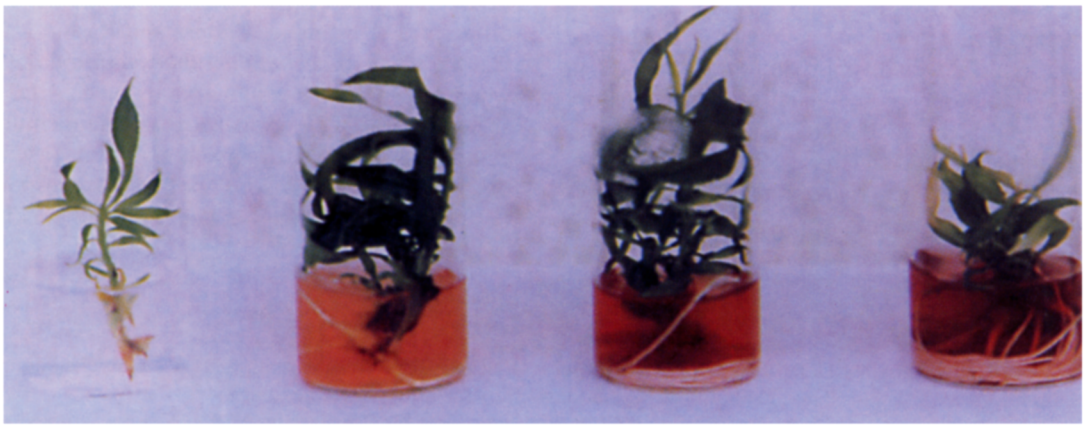

Fig. 1. Plantlets treated with different concentrations of Fe-EDDHA.

Table 1. Rooting parameters, chlorophyll (Chl) *a* and *b* contents, and Fe contents, of GF-677 explants, under different Fe sources and concentrations. Each value is the mean of 13 replications (for Chl of 3 replicates, each obtained from three explants). Values followed by the same letter do not differ significantly (Duncan's multiple range test at $P = 0.05$). Explants treated with Fe-EDTA did not form roots.

Fe-form	Conc. [mM]	Root emergence [d]	Rooting percentage [%]	Number of roots [explant^{-1}]	Root length [cm]	Root FM [mg]	Root DM [mg]	Chl *a* [μg g^{-1}(DM)]	Chl *b* [μg g^{-1}(DM)]	Fe	Fe [%]
-Fe		14-15	58	4.0a	3.9c	110a	16a	2.08b	1.25b	84	100
$FeCl_3$	0.05	12-13	85	5.6b	2.9b	215bc	26cd	3.01c	1.78c	132	157
	0.10	12-13	84	5.1ab	2.1a	131a	20ab	4.63d	2.22d	178	211
	0.20	13-14	81	4.2a	1.8a	126a	18a	5.24e	2.46d	138	164
Fe-EDDHA	0.05	11-12	100	5.6b	3.6c	207bc	23bc	3.18c	1.72c	138	164
	0.10	11-12	100	5.9b	3.1b	218bc	23bc	4.91de	2.58de	281	334
	0.20	11-12	100	7.3c	3.8c	249c	30d	5.18e	2.88e	305	363
Fe-EDTA	0.05	-	-	-	-	-	-	1.91ab	0.88a	747	889
	0.10	-	-	-	-	-	-	1.46a	0.76a	1059	1260
	0.20	-	-	-	-	-	-	1.54a	0.74a	876	1042

Fe-EDTA did not form roots. This disagrees with the suggestion for GF-677 rooting MS medium containing Fe-EDTA (Zuccherelli 1979). However, the beneficial effect of Fe-EDDHA on GF-677 rooting agrees with recent reports (Dimassi-Theriou 1989). Satisfactory rooting can take place on full strength MS medium, but it is very common practice to transfer shoots to be rooted from full strength MS medium to a dilution of the same medium used for proliferation (George 1993, 1996).

In spite of the fact that Fe nutrition *in vitro* has been studied by many workers, its physiological functions are only partially understood. One possible explanation for the above results is that IAA degradation in plant tissue culture media is catalyzed by the photodynamic activity of Fe-EDTA (Dunlap and Robacker 1988, Hangarter and Stasinopoulos 1990). Light-induced IAA degradation can result in reduced growth of tissues that require auxin for growth (Dunlap and Robacker 1988, Stasinopoulos and Hangarter 1990). Because Fe-EDTA is the major light-absorbing component in plant culture media (Stasinopoulos and Hangarter 1990), it may be the most likely source of the light-induced growth reduction observed in *Arabidopsis* roots. In addition to this, Hangarter and Stasinopoulos (1991) have shown that Fe-EDTA is photochemically degraded in the MS medium. Photooxidation of Fe-EDTA results in formaldehyde formation, which is toxic to plant growth. In our experiment the explants treated with Fe-EDTA were chlorotic, with low Chl content and this fact lead possibly to inhibition of rooting.

Protein synthesis must occur before cell division of root initials can take place (Molnar and LaCroix 1972), therefore enzymatic activity is essential for root development in the *in vitro* explants (Gaspar *et al.* 1992). EDTA also inhibited the polyphenol oxidase activity of sunflower (*Helianthus annuus* L.) leaf tissue *in vitro* (Weinstein *et al.* 1951); this compound competitively removed metals essential for polyphenol oxidase activity. Frenkel and Hess (1973) found isoenzymatic changes of polyphenol oxidase during root initiation of mung bean (*Phaseolus aureus* Roxb.). Also, Fe is an essential component of peroxidase, which activity was studied thoroughly as a marker of the successive rooting phases (Gaspar *et al.* 1994, Saxena *et al.* 2000). In preliminary experiments in our laboratory, we found that Fe forms and concentrations can directly affect the peroxidase activity and its isoenzymatic pattern during adventitious root formation of GF-677 *in vitro* (values not presented).

We show that the physiological effects of Fe-EDTA and Fe-EDDHA were different. Thus, the addition of Fe-EDTA in the culture medium greatly improved the availability of the Fe. In comparison to -Fe treatments, iron content was 1.5 - 2 fold higher with $FeCl_3$, 3.5-fold greater with Fe-EDDHA, and 9 - 12 fold greater with Fe-EDTA. The fact that the no-rooted explants had high Fe content possibly indicates that the concentration of iron in the GF-677 explants could affect their rooting process. Under field conditions, the critical iron toxicity contents of leaves are above 500 μg g^{-1}(DM) (Marscher 1995). In our experiment, explants treated with Fe-EDTA had Fe content 747 - 1059 μg g^{-1}(DM), thus this chelating agent began to be toxic. Iron can participate in Fenton reactions and catalyze the generation of hydroxyl radicals and other toxic oxygen species. This oxidative stress may lead to metabolic disfunction and ultimately in DNA damage (Becana *et al.* 1998), leading possibly to inhibition of rooting. DNA synthesis is a prerequisite for cell division, which is one of the three basic processes of adventitious root formation (Molnar and LaCroix 1972). According to Barbieri *et al.* (1998), DNA is an excellent complexing agent for ferric iron and addition of Fe(III) increases the number of strand breaks of plasmid DNA in aqueous solution (Ambroz *et al.* 2001).

In our study the effect of Fe-EDDHA was beneficial

for rooting of GF-677 *in vitro*. Fe-EDDHA is a more stable form than Fe-EDTA and may suffer less hydrolysis at higher concentrations than Fe-EDTA. Another explanation of the effect of Fe chelates in the rooting process of GF-677 could be the differences in the configuration of Fe-EDTA and Fe-EDDHA, which could cause differences in their properties (Assaad and Awad 1981).

On the basis of this research we propose, at least for the peach rootstock GF-677, the substitution of Fe-EDTA by 0.2 mM Fe-EDDHA in the MS medium. Similar experiments should be conducted with other genotypes and other nutrient media using Fe-EDTA to find out if it is necessary to substitute this form with Fe-EDDHA.

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