



INFLUENCE OF IRON SOURCES IN THE NUTRIENT MEDIUM ON *IN VITRO* SHOOT MULTIPLICATION AND ROOTING OF MAGNOLIA AND CHERRY PLUM

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ABSTRACT

In this study, the effects of compounds providing Fe in chelated (NaFeEDTA and Fe(III)AC) and non-chelated (FeSO₄·7H₂O) forms as components of culture media, on *in vitro* shoot multiplication and rooting of *Magnolia soulangeana* 'Alexandrina', *Magnolia grandiflora* and *Prunus cerasifera* 'Nigra' were comparatively evaluated. Each of the tested chemicals was used as a single Fe source in the basal salt medium. In the stages of shoot multiplication and rooting plant response was scored by biometrical indices (number of shoots, leaves and roots, shoot and root length, percent of rooted plants and root hairs). The occurrence of physiological disorders was estimated by visual observations. In presence of FeSO₄, symptoms of chlorosis, hyperhydricity, early senescence and specific morphology of roots, suggesting Fe deficiency, were observed. These deteriorations were entirely prevented at the application of Fe chelates of which, in this experimental systems, Fe(III)AC was tested for the first time. The addition of Fe(III)AC positively affected the plant quality to extent comparable to that of NaFeEDTA. The obtained data suggest that both applied Fe chelates are more appropriate than non-chelated Fe form and can be alternatively used in the optimization of nutrient media for micropropagation of *Magnolia* and *Prunus cerasifera* genotypes.

Key words: Ammonium iron (III) citrate, NaFeEDTA, FeSO₄, *Magnolia grandiflora*, *Magnolia* × *soulangeana* 'Alexandrina', *Prunus cerasifera* 'Nigra'

INTRODUCTION

The clonal micropropagation is an established tool for obtaining large amounts of disease-free, genetically identical and high-quality planting material of various plants. This approach is also successfully applied for producing ornamental woody plants. Among the most attractive species, in demand for the purposes of landscaping and private gardens in subtropical and temperate climates, are magnolias (*Magnolia* spp., f. Magnoliaceae). Broadly addressed are also *Magnolia* spp. valuable for their pharmacological properties and for elaboration of systems for *in vitro* conservation of the gene pool of rare and endangered genotypes (Merkle & Wiecko 1990; Callaway 1994; Parris et al. 2012 and references therein). Cherry plum

(*Prunus cerasifera*, f. Rosaceae) is another popular ornamental deciduous, medium-sized tree preferred for its bright red colored leaves and abundant pale pink flowers and also known with the health-promoting ingredients of its fruits. Among the most fascinating is the purple-leaved black cherry plum 'Nigra'. Studies on *in vitro* regeneration response of *Prunus* sp. have focused on the type of primary explants and improvement of media compositions for establishment of cultures with high regeneration capacity (Quoirin & Lepoivre 1977; Morini et al. 1992; Emershad & Ramming 1994; Nowak et al. 2007; Liu et al. 2008; Sokolov et al. 2011, 2015).

The investigations on micropropagation of these plants have indicated that the growth and development of individual genotypes is strongly dependent on the basal salt medium, its supplements

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and the proper balance of the components. These include type, concentration and ratio of macro and microelements, particularly nitrogen, carbohydrate sources, phytohormones and vitamins jointly with the necessity for appropriate environmental conditions such as light and photoperiod (Quoirin & Lepoivre 1977; Biedermann 1987; Aier & Sharma 1990; Ambrozic-Turk et al. 1991; Morini et al. 1992; Emershad & Ramming 1994; Orlikowska & Gabryszewska 1995; Harada & Murai 1996; Kamenická & Valka 1997; Kamenická & Lanaková 2000; Shanjani 2003; Liu et al. 2008; Marinescu et al. 2008; Radomir & Radu 2008; George et al. 2008; Sokolov et al. 2014a,b, 2015; Paris et al. 2012; Radomir 2012; Wojtania et al. 2014).

Iron (Fe) is an essential element of tissue culture media. It is required for diverse processes related to the living status and survival such as photosynthesis, chlorophyll biosynthesis and respiration (Schmidt 2003). This metal is involved in the chemical structure of important molecules, for example metal-containing enzymes, Fe-sulfur cluster, heme and other Fe-binding sites (Rout & Sahoo 2015). Symptoms of Fe starvation impaired photosynthesis, chlorosis and various deteriorative changes at cellular and molecular levels. For avoiding Fe deficiency or toxicity, plants have created mechanisms for maintaining the homeostasis by controlling Fe uptake, utilization, and storage, depending on its environmental availability (Kobayashi & Nishizawa 2012 and references therein). Because Fe is hardly soluble, especially at aerobic conditions and high pH, it is more efficiently utilized in a form of Fe(III) chelates, but at extreme circumstances of Fe starvation, Fe(II) form can also be acquired (Robinson et al. 1999; Kobayashi & Nishizawa 2012).

Under *in vitro* conditions, an important factor related to bioavailability of Fe is the chemical compound through which this microelement is provided in the nutrient medium (Van der Salm et al. 1994; Shibli et al. 1997; Christensen et al. 2008). As such, in some culture media, especially in previous years, FeSO₄ has been used (Gamborg et al. 1976; Huang & Murashige 1977). However, it may react with other ingredients of the medium forming insoluble salts or ferrous oxides. The Fe from FeSO₄ in plant tissue cultures is available at relatively narrow pH

(around 5.5) of the medium (Huang & Murashige 1977; Huda et al. 2009 and references therein). Most of basic media for *in vitro* culturing, especially the ready-to-use commercial ones, contain the chelated Fe form ethylenediaminetetraacetic acid iron (III) sodium salt hydrate (NaFeEDTA) or FeSO₄ in combination with Na₂EDTA (Huang & Murashige 1976; Huda et al. 2009). In the recent works of Sokolov et al. (2014, 2015) it was demonstrated that different basal salt media comprising NaFeEDTA are appropriate for micropropagation of *Magnolia* spp. and for *Prunus* spp. It is considered that Fe ions released from NaFeEDTA are more available as compared to FeSO₄ (Murashige & Skoog 1962; Dörschug & Miller 1967; Nitsch 1969; Huda et al. 2009). The activity of this chelate is to a large extent dependent on its concentration. For some species, low concentrations of NaFeEDTA may exert negative effect possibly by causing Fe deficiency whereas high doses of the chemical might be toxic (Albert 1958; Whelan & Alexander 1986) due to release of undesirable high levels of Fe ions thus provoking various metabolic disturbances. From the other side, an excessive chelate amounts can form complexes with other metallic ions unavailable for plants (Hill-Cottingham & Lloyd-Jones 1961; Tomaszewski & Thimann 1966).

Depending on the type of chelate the *in vitro* plants may show improved physiological appearance and increased productivity or the influence might be negative (Christensen et al. 2008). It is suggested that the diversity of the effects is related to chemical characteristics and stability of the chelating agent and of the complex both of which can be subjected to, for example, photo- and thermodegradation. It has been suggested that *in planta* chelators such as EDTA and sodium diethyldithiocarbamate (DIECA) may interact with the metabolism and transport of the natural auxin indole-3-acetic acid (IAA) (Tomaszewski & Thimann 1966).

A chelated Fe form used in some nutrient media is ammonium iron (III) citrate [Fe(III)AC]. In plants, Fe-citrate complex may serve as Fe transporter through the xylem sap (Briat et al. 2007; Relán-Álvarez et al. 2010), which may presume that Fe(III)AC complex could be suitable Fe provider for *in vitro* cultures. For micropropagation of

M. × soulangeana some authors have recommended Catalano S medium, which by its original recipe comprises Fe(III)AC (Valova et al. 1996; Kamenická et al. 2001) but in these publications the Fe impact was not separated. Harvais (1982) tested various modifications of basal salt media containing Fe(III)AC and established that the effect of this chelate, as well as of FeEDTA, on seed germination, quality, and survival of plantlets of *in vitro* propagated orchids differed depending on pH and on the other elements of nutrient medium, including the type of cytokinin and auxin supplements. Our literature search did not reveal information about Fe(III)AC incorporation in media for *in vitro* propagation of *P. cerasifera*. However, based on the chemical properties of Fe(III)AC and its potential to serve as Fe-releasing compound in tissue culture media for other plants, we hypothesized that this chelate may appear efficient also as individual Fe provider in the nutrient media for *Magnolia sp.* and *P. cerasifera*.

The aim of present study was to comparatively assess the influence of chelated and non-chelated Fe forms, used as a single Fe source in the composition of basal salt media, on the growth and development of two *in vitro* cultured magnolia species and black cherry plum in the stages of proliferation and rooting.

MATERIALS AND METHODS

Plant material

The experiments were carried out with *in vitro* cultured *Magnolia × soulangeana* 'Alexandrina', *M. grandiflora* and *P. cerasifera* 'Nigra'. Magnolia explants were multiplied on Chée & Pool C2D Vitis Medium (Chée & Pool 1987), previously established as highly efficient for *in vitro* culturing of *Magnolia sp.* (Sokolov et al. 2014a), supplemented with 0.5 mg·dm⁻³ 6-benzylaminopurine (BAP) for *M. × soulangeana* 'Alexandrina' and 1.0 mg·dm⁻³ BAP for *M. grandiflora* (Sokolov et al. 2014 a,b; 2015). For both magnolia species, 0.5 mg·dm⁻³ indole-3-butyric acid (IBA), 30 g·dm⁻³ sucrose and 7 g·dm⁻³ agar for solidification were added. For multiplication of the explants of *P. cerasifera* 'Nigra', Woody Plant Medium (WPM) basal salt (Lloyd & McCown 1980) in combination with

0.5 mg·dm⁻³ BAP, 30 g·dm⁻³ fructose and 7 g·dm⁻³ agar was used (Sokolov et al. 2015). The basal salt media C2D and WPM used for initial multiplication of the shoots originally contained NaFeEDTA as single Fe-releasing compound.

Testing the effect of different Fe sources on shoot multiplication

Nodal segments, approximately 4 to 5 mm long, from the middle part of shoots with one leaf, were separated from multishoot clumps obtained on the above media and transferred onto the media comprising different Fe sources. On these media, the explants were subjected to two consecutive subcultures, 60 days each. To evaluate the influence of different Fe sources, the basal salt media for subculture were manually prepared containing macro- and micro elements, and vitamins according to the original formulations (Lloyd & McCown 1980; Chée & Pool 1987; Sokolov et al. 2014, 2015), except the Fe compound: chelated Fe forms NaFeEDTA or Fe(III)AC or non-chelated Fe in the form of FeSO₄·7H₂O, in amounts ensuring an equimolar mass to Fe present in 36.7 mg NaFeEDTA; it means 33 mg·dm⁻³ Fe(III)AC or 27.8 mg·dm⁻³ FeSO₄·7H₂O. Because in the chemical formula of Fe(III)AC the number of Fe ions is variable, the final Fe content in the media was calculated based on approximately 17.5% Fe in the chelate.

Testing the effect of different Fe sources on rooting of shoots

In this experiment, the shoots isolated from multishoot clumps, initially produced on the above described multiplication medium, in which Fe was present as NaFeEDTA, were used. They were directly placed onto the media used for subculturing but without BAP and containing 4.0 mg·dm⁻³ IBA for both magnolia species or 0.1 mg·dm⁻³ IBA for *P. cerasifera* 'Nigra', and with either FeSO₄, FeNaEDTA or Fe(III)AC.

Growth conditions

In all the trials, media pH was adjusted to 5.8 with 0.1 N NaOH or 0.1 M HCl and autoclaved at 121 °C at 1.04 kg·cm⁻² pressure for 20 min. The explants were grown in 400 ml glass vessels, tightly covered with metal screw caps. The vessels were maintained under room temperature 25 ± 2 °C, pho-

toperiod day/night 16/8 and $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity provided by 40 W cool white fluorescent tubes (Philips, Bulgaria). The chemicals used in the experiments were purchased from Duchefa, The Netherlands.

Measurements and observations

Shoot length and shoot and leaf number were evaluated at the end of second 60-day period. The occurrence of physiological disorders was visually evaluated recording symptoms of hyperhydricity, chlorosis and senescence. Senescence was distinguished by loss of turgor of the plant organs, yellowing, wilting and dieback of leaves and apical area. For evaluation of the long term effect of Fe sources on the occurrence of senescence, after the second subculture the explants were kept at the same medium for additional forty days. In the rooting stage after 60 days culturing the percentage of rooted shoots, root length, and the development of lateral roots were estimated.

Statistical analysis

Data were statistically processed by one-way analysis of variance (ANOVA) and Duncan's multiple range test at probability level $p < 0.05$ (SPSS, IBM) was used to separate treatment means. The experiments were repeated twice with 50 microshoots per variant of the media for proliferation or rooting (magnolias – 10 vessels each containing 5 microshoots and cherry plum – 5 vessels with 10 microshoots each). The presented values in the tables are means \pm SD of the two independent sets of experiments.

RESULTS

Effect of Fe sources on shoot multiplication and physiological disorders

The results showed that for the two magnolia genotypes, the lowest number of lateral shoots developed on media containing FeSO_4 , whereas for cherry plum this compound exerted slight positive effect in comparison to chelated Fe. Leaf number in *P. cerasifera* did not significantly differ between the evaluated media but in both *Magnolia* spp. the

number of leaves was higher in microshoots grown on medium containing NaFeEDTA or Fe(III)AC. In all cases, shoots were substantially longer on the media with chelated Fe (Table 1).

Physiological disorders resembling Fe deficiency (initial symptoms of chlorosis and hyperhydricity) were noticed on most of the microshoots of the three species propagated on media containing FeSO_4 and a premature senescence was initiated before the end of the regular subculturing period. Representative images of plants expressing Fe deficiency during proliferation stage of *P. cerasifera* 'Nigra' are shown in Fig. 1. In the chlorotic leaves, a yellowing and loss of chlorophyll were best visible in the inter-vein areas and particularly in the upper half of the leaf, more clearly distinguishable close to the leaf top. The chlorosis resulting from severe Fe starvation, expecting as dark green coloration of the veins and yellow to white inter-nerve leaf tissue, were not strikingly expressed. These observations indicate that the Fe chlorosis was at an initial stage and that for the duration of subculturing the Fe stress occurred probably at relatively mild level. The lack of chlorophyll might be due to impairment of chlorophyll synthesis at insufficient Fe supply and the leaf yellowing due to early senescence process.

An interesting phenomenon was observed on medium containing FeSO_4 . The leaves of microshoots remained green and did not develop red coloration (Fig. 1a) whereas in the presence of Fe chelates the red-purple foliage appeared, typical for this species (Fig. 1b, c). Chelated Fe forms promoted the development of microshoots of excellent quality (Fig. 1b, c). At the addition of Fe(III)AC, on some of the older shoots of *P. cerasifera* symptoms of stem lignification were observed, indicating accelerated development at the particular *in vitro* conditions (Table 1). Senescence of plantlets in presence of Fe(III)AC was substantially delayed in comparison to medium with non-chelated Fe. During the prolonged subculturing, in reference to media with NaFeEDTA, at Fe(III)AC the plant aging of studied species was delayed by approximately 20–25 days.

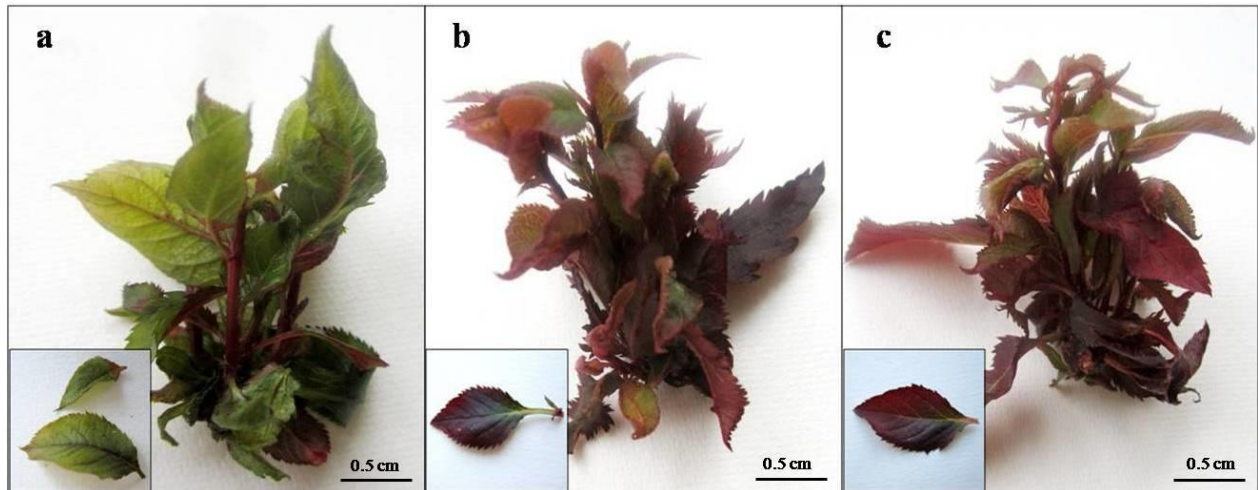


Fig. 1. Effect of Fe sources on development of *in vitro* cultured *P. cerasifera* 'Nigra' in proliferation stage

a. FeSO_4 ; b. NaFeEDTA; c. Fe(III)AC

The explants were multiplied on WPM basal salt medium containing NaFeEDTA, hormones and carbohydrate as described in Material and Methods. Thereafter the explants derived from this medium were subcultured on medium comprising Fe source in a single chelated (NaFeEDTA or Fe(III)AC) or non-chelated ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) form. Photos are taken at day 60 of 2nd subculture

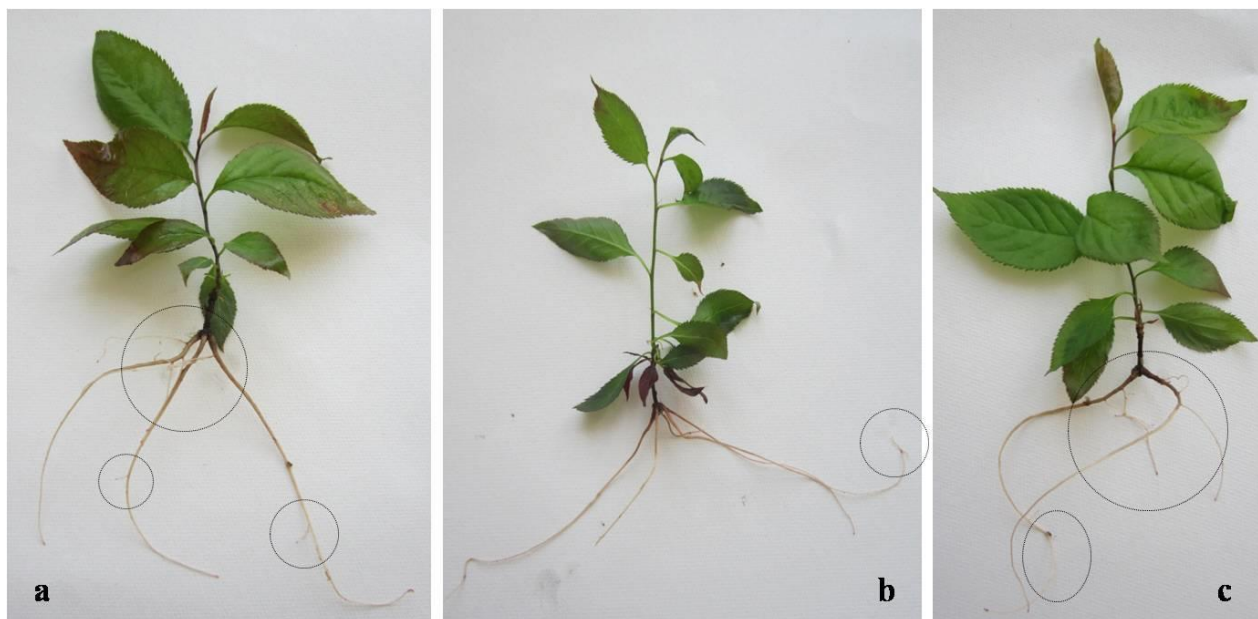


Fig. 2. Effect of Fe sources on *in vitro* rooting of *P. cerasifera*

a. FeSO_4 ; b. NaFeEDTA; c. Fe(III)AC

The explants were rooted on WPM basal salt medium supplemented with the relevant single Fe source, $0.1 \text{ mg} \cdot \text{dm}^{-3}$ IBA, $30 \text{ g} \cdot \text{dm}^{-3}$ fructose, and $7 \text{ g} \cdot \text{dm}^{-3}$ agar. Photos are taken at day 60 after initiation of rooting stage.

a: visible are clustered lateral roots close to the base of main roots, symptoms of chlorosis and senescence appear on some leaves – indicated by arrows; **b:** note the single small lateral root; leaves are well developed without physiological disorders; **c:** normally distributed lateral roots are formed, leaves are well developed, no physiological disorders are observable. The positions of lateral roots are indicated with circles.

Table 1. Effect of Fe source in the nutrient medium on growth and development of microshoots in proliferation stage of two *in vitro* cultured *Magnolia* sp. and *P. cerasifera* 'Nigra'

Species	Fe source	No. of lateral shoots	Plant height (mm)	Leaf No.	Physiological inadequacies
<i>M. grandiflora</i>	FeSO ₄ ·7H ₂ O	4.6 ± 0.9 ^a	16.2 ± 1.8 ^a	12.8 ± 1.1 ^a	Symptoms of chlorosis, hyperhydricity and senescence
	NaFeEDTA	6.5 ± 0.4 ^b	22.2 ± 2.4 ^b	17.3 ± 2.7 ^b	-
	Fe(III)AC	6.1 ± 0.3 ^b	21.5 ± 1.8 ^b	16.4 ± 1.5 ^b	Rarely symptoms of senescence
<i>M. × soulangeana</i> 'Alexandrina'	FeSO ₄ ·7H ₂ O	4.3 ± 0.5 ^a	14.3 ± 1.9 ^a	13.0 ± 1.2 ^a	Symptoms of chlorosis, hyperhydricity and senescence
	NaFeEDTA	5.9 ± 0.9 ^b	25.0 ± 1.3 ^b	15.9 ± 1.8 ^b	-
	Fe(III)AC	5.6 ± 0.8 ^b	23.4 ± 0.5 ^c	15.2 ± 1.1 ^b	Rarely symptoms of senescence
<i>P. cerasifera</i> 'Nigra'	FeSO ₄ ·7H ₂ O	6.8 ± 0.4 ^b	18.6 ± 0.7 ^a	20.9 ± 1.7 ^a	Symptoms of chlorosis, hyperhydricity and senescence
	NaFeEDTA	6.3 ± 0.2 ^a	20.1 ± 1.1 ^b	19.7 ± 1.5 ^a	-
	Fe(III)AC	6.1 ± 0.3 ^a	19.7 ± 0.9 ^b	20.3 ± 1.8 ^a	Rarely symptoms of senescence, lignification of some older shoots

Values with different letters in the same sub-column corresponding to the relevant species are significantly different from each other at $p < 0.05$, according to Duncan multiple range test.

Table 2. Effect of Fe source in the composition of rooting medium on root development of two *in vitro* propagated *Magnolia* sp. and *P. cerasifera* 'Nigra'

Species	Fe source	% rooted plants	Root no. per plant	Root length (mm)	Development of lateral roots
<i>M. grandiflora</i>	FeSO ₄ ·7H ₂ O	62.5 ± 6.6 ^a	1.9 ± 0.2 ^a	22.5 ± 0.4 ^a	-
	NaFeEDTA	78.4 ± 8.5 ^b	2.3 ± 0.1 ^b	24.7 ± 0.6 ^c	-
	Fe(III)AC	79.0 ± 7.4 ^b	2.5 ± 0.1 ^b	23.3 ± 0.3 ^b	-
<i>M. × soulangeana</i> 'Alexandrina'	FeSO ₄ ·7H ₂ O	66.4 ± 8.9 ^a	1.2 ± 0.2 ^a	27.7 ± 0.7 ^b	-
	NaFeEDTA	83.9 ± 5.5 ^b	1.6 ± 0.1 ^b	26.3 ± 0.8 ^a	-
	Fe(III)AC	82.4 ± 4.3 ^b	1.7 ± 0.2 ^b	25.7 ± 1.3 ^a	-
<i>P. cerasifera</i> 'Nigra'	FeSO ₄ ·7H ₂ O	72.5 ± 13.2 ^a	3.5 ± 0.5 ^c	32.5 ± 1.3 ^a	+++
	NaFeEDTA	94.1 ± 3.8 ^b	2.8 ± 0.1 ^a	44.4 ± 5.7 ^c	+
	Fe(III)AC	96.5 ± 3.5 ^b	3.0 ± 0.1 ^b	36.2 ± 1.6 ^b	++

(-) Lateral roots have not developed or are formed on single plants; (+) lateral roots on 10-30% of the roots; (++) lateral roots on 40-60% of the roots; (+++) lateral roots on more than 60% of the roots. Values with different letters in the same sub-column corresponding to the relevant species are significantly different from each other at $p < 0.05$ according to Duncan multiple range test.

Effect of Fe sources on the rooting of microshoots

In the media containing chelated Fe, the number of rooted shoots of the three species was higher by approximately 20-24% as compared to media with FeSO₄ (Table 2). For *Magnolia* spp. the number of roots developed per microshoot grown on media with Fe in chelated form was higher than the number of roots in the presence of FeSO₄. An increase of root length in media containing Fe chelates was observed for *M. grandiflora* and *P. cerasifera*. The roots of *M. × soulangeana* in medium with FeSO₄ were slightly longer than the roots of plantlets in media with Fe chelates. In contrast to magnolias, the number of *P. cerasifera* roots per microshoot was higher at FeSO₄ (Table 2). On cherry plum, the lateral roots were developed in the presence of each Fe source (Table 2, Fig. 2a, b, c). In the medium with FeSO₄, more than 60% of the roots were branched. However, in this case the lateral rootlets appeared short and positioned in clusters close to the base of the main roots (Fig. 2a). Only 10-30% of microplants with lateral roots were observed in media containing NaFeEDTA (Table 2, Fig. 2b). On the microplants grown on Fe(III)AC containing medium, the percentage of those with lateral roots, the latter normally distributed along the length of roots, was intermediate, that is, was higher than in presence of NaFeEDTA and lower in FeSO₄ (Table 2, Fig. 2c). No lateral roots developed on magnolia microplants.

At the rooting stage, similar disorders (chlorosis, senescence and hyperhydricity) were observed as at the shoot multiplication stage.

Taken together, the data from the experiments addressing the rooting indicated that chelated Fe forms more efficiently stimulated the root formation of the two magnolia genotypes and of black cherry plum, mainly by increasing the amount of rooted microplants. Root elongation was stimulated at NaFeEDTA for *M. grandiflora* and slightly reduced for *M. × soulangeana* 'Alexandrina' as compared to FeSO₄. For *P. cerasifera*, Fe(III)AC increased the portion of rooted microplants over FeSO₄ and NaFeEDTA but the number of microplants developing lateral roots was expressed at intermediate level. However, the structure of root system appeared of best quality. These results show that Fe provided

from Fe(III)AC exerts overall positive influence on rooting and can successfully be used as alternative of NaFeEDTA.

DISCUSSION

Our data showed that for the three studied woody ornamental species, both chelated Fe forms ensure effective growth and development of plantlets in the stages of shoot multiplication and rooting, and prevented the physiological deteriorations observed at application of FeSO₄. For *in vitro* grown *M. × soulangeana* 'Alexandrina' and *M. grandiflora*, NaFeEDTA and Fe(III)AC exerted similar positive effects on all aspects of proliferation and rooting. For *P. cerasifera* 'Nigra' Fe chelates potentiated only shoot and root length and the percent of rooted microshoots. However, it should be taken into account that for the purpose to comparatively assess the influence of Fe sources we have applied FeSO₄ and Fe(III)AC only in concentrations corresponding to the molar mass of Fe ions present in NaFeEDTA, the most commonly used Fe chelate. Therefore, it should not be ignored that the occurrence of symptoms of Fe deficiency in presence of FeSO₄ might be related to the inappropriate dose of this chemical.

The reported data substantiated the findings of other authors that the regeneration rate *in vitro* is potentiated, if Fe is provided as a chelate (Murashige & Skoog 1962; Doerschug & Miller 1967; Nitsch 1969; Huang & Murashige 1976; Harvais 1982; Van der Salm et al. 1994; Shibli et al. 1997; Christensen et al. 2008; Huda et al. 2009). Our current experiments, however, cannot explain the mechanisms of plant reaction to the tested Fe sources, which requires other physiological and biochemical analysis.

Observation of root architecture revealed straight roots without lateral rootlets on magnolia and the formation of lateral roots on *P. cerasifera* what resembled the structure of root systems of these species *in planta*. Nonetheless that the number of lateral roots of *P. cerasifera* in the media with FeSO₄ was highest, the appearance of cluster rootlets indicated eventual Fe or P deficiency. It has been suggested that Fe from FeSO₄ may form insoluble

salts with P, thus reducing the level of both elements (Doerschug & Miller 1967). Another possibility is that at conditions of Fe starvation, both Fe and P may induce development of cluster rootlets as established by Meng et al. (2012) in lupine. Additionally, at limited Fe availability in the soil, the development of more root hairs can increase the absorption surface of the root and improves the affinity of Fe uptake system (Schmidt 2003; Séguéla et al. 2008). Such regulation of Fe uptake at Fe deficiency might, at least in part, explain the increased number of lateral roots developed on cherry plum microplants grown on medium with FeSO₄. The mentioned findings suggest that for the here studied species, FeSO₄ presumably does not provide sufficient amount of available Fe ions, thus leading to some changes of the rooting process.

In several works, the mechanisms of Fe acquisition *in planta* are discussed as partially attributed to chelators involvement in the regulation of auxin metabolism and action (Thimann & Takahashi 1958; Heath & Clark 1960; Tomaszewski & Thimann 1966). EDTA has been shown to prevent the basipetal auxin transport by replacing calcium, which plays a role in auxin transport system across the cellular membranes. It has been found that EDTA may also counteract the action of natural auxin indole acetic acid (IAA) but failed to affect the rooting effects of the synthetic auxin analogs IAA, IBA and 2,4-dichlorophenoxyacetic acid (2,4-D) (Tomaszewski & Thimann 1966). On the other hand, EDTA might potentiate the action of IAA by repressing its degradation through inhibiting IAA decarboxylation, but this synergism-like mechanism has not been confirmed at replacement of naphthalene acetic acid (NAA) and 2,4-D with EDTA (Tomaszewski & Thimann 1966). Additionally, the chelating agent DIECA has been shown to suppress the destruction of IAA by inhibiting IAA oxidase, but without causing substantial growth enhancement, possibly because its decomposition products may repress the respiration (Carr & Ng, 1959; Janssen 1971; Tomaszewski & Thimann 1966). These findings may to some extent explain the positive or negative influence of different chelators. Moreover, it should be taken into consideration

that pH optimum of the chelating agents is an important condition for the activity of chelates. For example, DIECA is very unstable in acidic pH, whereas EDTA pH optimum is around 4 (Carr & Ng 1959). In an earlier paper, it has been reported that citrate-chelating agents may dissociate substantial amount of metal ions at pH 6-8 and are not stable at acidic conditions (Bobtelsky & Jordan 1945). This suggests, that at lower pH their ability to release Fe ions might depend on the rate of breakdown of the citrate chelator. If assuming that Fe(III)AC possesses chemical properties of citrate-chelating agents, and having in mind the suggested activity of this Fe chelate in acidic pH range, this might at least hypothetically explain the found in our experiments efficiency of the compound due to potentially releasing satisfactory amount of Fe ions at pH 5.8 of the used growth media. Bobtelsky and Jordan (1945) also stated that the free citric acid does not form complexes with divalent ions such as Cu, Co and Zn. In this regard, assuming that the citric acid is a product of Fe(III)AC decomposition, it might not negatively affect the levels of important enzyme cofactors. This suggests that Fe(III)AC may express lower potential for immobilizing metal elements either in the nutrient medium or in the plant tissues. The chelators and their breakdown products may be involved also in other chemical processes (Thimann & Takahashi 1958; Heath & Clark 1960; Tomaszewski & Thimann 1966; Van der Salm et al. 1994; Shibli et al. 1997; Chen et al. 2010). For example, EDTA may react with mineral components of the nutrient medium such as potassium, calcium, and ammonium salts, thus converting the sources of these elements into other less acquirable compounds (Buszek 1965). Such interactions of the chemicals in the medium may be an eventual cause for lesser effect of NaFeEDTA on root branching of *P. cerasifera*. The lower percentage of cherry plum microshoots with lateral roots at the addition of NaFeEDTA may also suggest insufficient Fe utilization or could be due to other physiological events not addressed in this study.

In proliferation stage, the leaves of microshoots of *P. cerasifera*, propagated on medium with FeSO₄ did not develop reddish coloration and

in rooting stage the foliage remained green, independent on the Fe source. During adaptation of *in vitro* rooted plants in soil, the newly developed leaves occurred in dark purple-brown nuance disregarding Fe form used in *in vitro* culture medium. To the extent of our experiments, these observations are difficult to be explained. The red-purple color of *P. cerasifera* leaves is determined by the presence of anthocyanins (Kyparissis et al. 2007) in the generation of which Fe(III) ions play an important role (Tanaka et al. 2008). However, whether the availability and acquisition of Fe from chelated and non-chelated compounds might affect the process of anthocyanin synthesis in *in vitro* cultured cherry plum is not known. This opens a trend for further research.

CONCLUSIONS

1. Chelated Fe forms NaFeEDTA and Fe(III)AC efficiently promoted the growth, development and rooting of *in vitro* regenerated microplants of magnolia and cherry plum.
2. In presence of these two chelates, the symptoms of physiological deteriorations indicating Fe deficiency at culture medium comprising non-chelated Fe in the form of FeSO₄ were entirely prevented.
3. The tested chelated Fe compounds can be alternatively used in the composition of proliferation and rooting media for micropropagation of magnolia and cherry plum.

We believe, that the here reported analysis provide novel information about the influence of different Fe-releasing compounds on growth, developmental and physiological behavior of magnolias and cherry plum *in vitro*. The presented first experimental evidence about the possibilities for successful incorporation of Fe(III)AC in tissue culture media as a single Fe source is expected to contribute to elaboration of more efficient protocols for *in vitro* regeneration of these ornamental trees. Further studies involving, among others, determination of the content of Fe acquired in tissues of *in vitro* grown plants from the studied and other genotypes, and assessment of physiological and biochemical indicators of Fe stress will allow more profound understanding

of the effects of Fe comprising chelate complexes and their mechanism of action.

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