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Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'

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Abstract

In vitro propagation of the rose rootstock 'Moneyway' was investigated on the following media: Murashige and Skoog (MS), Quoirin and Lepoivre (QL) and Woody Plant (WP). Growth, which was measured as length of shoots after a 6-week period, was faster on MS and QL than on WP. In spite of the better growth, chlorosis of newly formed leaves occurred from the third week on and was correlated with a lower chlorophyll content of shoots. Replacement of FeEDTA by FeEDDHA in QL and MS resulted in the development of green shoots for more than 3 months. The occurrence of chlorosis was not pH directed since the pH of QL with FeEDTA or FeEDDHA had not changed after 6 weeks of growth. Addition of the light absorbing dye fast yellow 9 to QL with FeEDTA also resulted in green shoots with a higher chlorophyll content. It is suggested that FeEDDHA is a more photostable chelate than FeEDTA, resulting in a higher availability of iron for the rose shoots. The impact of the iron chelate formula on the micropropagation of plant species that are susceptible to iron deficiency is discussed.

Abbreviations: BA – 6-benzyladenine, fast yellow 9 – 4-amino–1,1'-azobenzene–3,4'-disulfonic acid, FeEDTA – ferric ethylenediamine tetraacetate, FeEDDHA – ferric ethylenediamine di(o-hydroxyphenylacetate), IAA – indole–3-acetic acid, IBA – indole–3-butyric acid, LSD – least significant difference, NAA – α-naphthaleneacetic acid, P – probability

Introduction

To establish vigorous growth and high flower yield, cut rose cultivars (*Rosa hybrida* L.) are generally grown on a rootstock. In vitro plant cultures of cut rose cultivars and rootstocks are used for micropropagation, for obtaining pathogen-free material and for the development of protocols to introduce new characteristics by genetic modification.

In general, growth and development of micropropagated plants depends on factors such as macroelement composition, the total salt strength of the medium, vitamin mixture used, sugar concentration, growth regulator composition and climatic conditions (light, day length, temperature). Techniques for the micropropagation of roses have been reviewed by Skirvin et al.(1990), Short & Roberts (1991) and Horn (1992). Rose shoots have been grown on WP (Lloyd & McCown 1981), SH (Schenk & Hildebrandt 1972), QL (Quoirin et al. 1977; Valles & Boxus 1987) or MS (Murashige & Skoog 1962). A high concentration of cytokinin (2–13 μM BA) and a relatively low concentration of auxin (0.02–0.5 μM NAA, IAA or IBA) have been used to promote the outgrowth of axillary buds. For adventitious root formation in vitro media with higher concentrations of auxin (0.5–5 μM NAA, IAA or IBA) and without cytokinin were used. Rooting was enhanced if the macroelements were reduced to a quarter or half of their original concentration (Skirvin & Chu 1979; Khosh-Khui & Sink 1982). This beneficial effect was attributed to reduced nitrogen levels (Hyndman et al. 1982).

Three standard media for the micropropagation of the rose rootstock 'Moneyway' were evaluated. None of these media supported adequate growth and yielded healthy leaves. Here, we describe the effects of two different iron chelates on the shoot length and chlorophyll content of leaves of the rose rootstock 'Moneyway'.

Material and methods

Plant material and plant cultivation

Tissue culture material of the rose rootstock 'Moneyway' was obtained from axillary shoots emerging from surface-disinfected nodes of greenhouse-grown plants. Shoots were subcultured every 6 weeks. For subculture several media were used: MS, QL and WP with 86 μM FeEDTA or FeEDDHA, 58.4 mM sucrose, 4.4 μM BA, 0.49 μM IBA and 0.8% (w/v) agar (Oxoid, bacteriological agar) at pH 5.6. Three 5–10 mm shoot tips were grown on 50 ml medium in 400 ml glass jars closed with polycarbonate screw lids and sealed with household plastic film. For each treatment, 3 or 5 jars were maintained in a growth chamber at 22 °C with 16 h light (Osram L58W/77 fluorescent tubes, 20 μmol m^{-2} s^{-1} at plant level). The experiments were performed with a randomized block design.

In three experiments, treatments with FeEDTA were compared to treatments with an equimolar concentration of FeEDDHA. First a qualitative comparison was performed between the micropropagation on MS, QL and WP with FeEDTA and QL with FeEDDHA. Next the influence of the iron chelate formula in QL was quantified. Finally the influence of the iron chelate formula on chlorophyll content was quantified for QL and MS. Additional protection of FeEDTA against photodegradation was accomplished by addition of 53 μM fast yellow 9 (Sigma, St. Louis, U.S.A.) (M. De Block, pers. comm.).

Measurements

The pH of the medium of each jar was measured with a flat surface-polymer body electrode (Sensorex). Shoots in each jar were measured from medium surface to apex after 6 weeks and mean shoot length per jar was calculated. A sample of 50–70 mg leaf material was taken from 3 shoots and frozen in liquid nitrogen, ground in an Eppendorf tube and subsequently incubated overnight at room temperature with 1 ml acetone for chlorophyll extraction. Chlorophyll a and b content was determined spectrophotometrically (Bruinsma 1963).

The experimental unit was a jar. Data were submitted to analysis of variance. To compare treatment effects LSD was calculated at $P = 0.05$.

Results

Different media for rose micropropagation were compared. Shoots on WP were small with poorly developed leaves after 6 weeks. In contrast, shoots developed on MS were longer and had chlorotic leaves that turned necrotic and senescent within 6 weeks. Shoots on QL also grew fast, but from the third week on chlorosis occurred, which was most pronounced in the youngest leaves. Since chlorosis is indicative of iron deficiency, FeEDTA in QL was replaced by the more stable iron chelate FeEDDHA. This change resulted in shoots having green leaves after 6 weeks (Fig. 1). Even after 3 months newly formed leaves were green.

Subsequently, the effect of the iron chelate formula in the medium on pH, shoot length, chlorosis and chlorophyll content of shoots was further examined (Table 1). After 6 weeks, the pH of QL with FeEDTA or with FeEDDHA were similar and also similar to the initial pH. The length of shoots grown on QL with FeEDDHA was significantly higher ($P = 0.013$) whereas the chlorophyll content of shoots was 6 fold higher than on QL with FeEDTA.

Significant differences were not observed in length of shoots grown on QL or MS (Table 2). Addition of FeEDDHA instead of FeEDTA improved growth ($P = 0.049$) and resulted in a significantly higher chlorophyll content in shoots grown on QL or MS (3.3- and 1.7-fold, respectively). A medium × chelate interaction was responsible for the higher effect of the substitution on QL than on MS ($P = 0.008$).

Table 1. Effect of iron chelate on the pH of the medium, shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway' after 6 weeks (n=3 jars).

	QL-medium with		
	FeEDTA	FeEDDHA	$LSD_{0.05}$
pH	5.7	5.8	1.1
Shoot length (mm)	15	29	9
Chlorophyll a+b (mg g^{-1} FW)	0.27	1.83	0.37

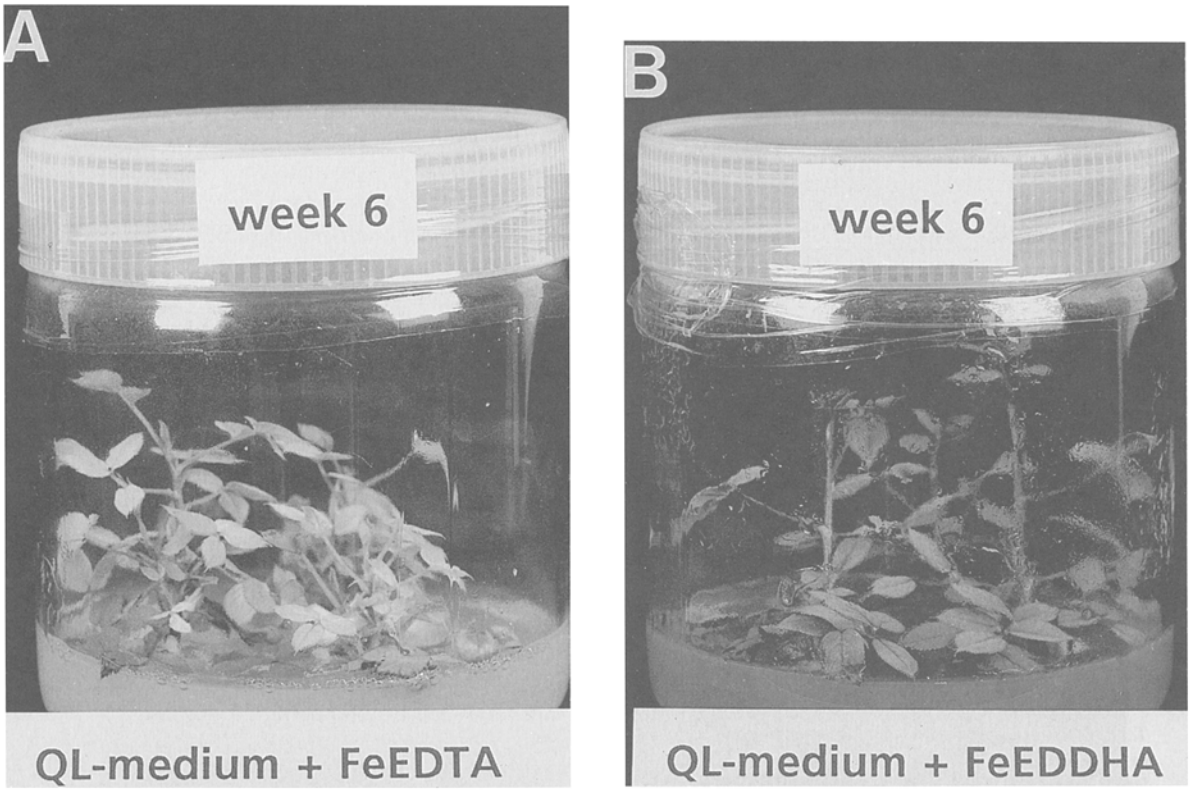

Fig. 1. Chlorotic shoots of *Rosa hybrida* L. 'Moneyway' on QL with 86 µM FeEDTA (*A*) and green shoots on QL with 86 µM FeEDDHA (*B*) after 6 weeks.

In the same experiment the light absorbing dye fast yellow 9 was supplemented to QL to analyze the differential influence of FeEDTA and FeEDDHA on shoot length and chlorophyll content. The addition of fast yellow 9 to QL did not affect shoot length (Table 3). However, it resulted in a two-fold higher chlorophyll content of the shoots grown on QL with FeEDTA. In contrast to this positive effect in QL with FeEDTA, fast yellow 9 had a negative effect on the chlorophyll content of shoots grown on QL with FeEDDHA.

Discussion

For the propagation of the rose rootstock 'Moneyway' three standard media, which differed mainly in their nitrogen content, were tested: MS, QL and WP. QL has a lower content of ammonium (5 mM) and a similar nitrate content (33 mM) as MS (20.6 and 39.4 mM, respectively), whereas WP has a lower content of both ammonium (5 mM) and nitrate (9.8 mM). Faster growth of shoots on MS and QL was attributed to the higher total nitrogen content of these media. However, on both standard media, leaves became chlorotic suggesting an iron deficiency. Chlorosis did not occur if FeEDTA was substituted by the more stable iron chelate FeEDDHA (Wallace et al. 1955; Halvorson & Lindsay 1972). The pH of the media had not changed after 6 weeks of growth, indicating that chlorosis of shoots was not caused by a low iron availability, which might be expected at higher pH values. The greening of the leaves was not only improved by replacement of FeEDTA by FeEDDHA, but also by addition of the light absorbing dye fast yellow 9 to medium with FeEDTA. This suggests that FeEDDHA is more photostable than FeEDTA resulting in a higher availability of iron to the rose shoots. Addition of fast yellow 9 to QL with FeEDDHA resulted in shoots with a significantly lower chlorophyll content. This strongly suggests that next to its protective function, fast yellow 9 also has some negative effect on the chlorophyll content.

Table 2. Effect of medium and iron chelate formula on shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway'. Means after 6 weeks of growth are given (n=5 jars).

	Iron chelate		
Medium	FeEDTA	FeEDDHA	$\bar{x}$
	Shoot length[y] (mm)		
QL	24	28	26 a
MS	17	24	21 a
$\bar{x}$[z]	20 b[w]	26a	
	Chlorophyll a+b[x] (mg g^{-1} FW)		
QL	0.66 c	2.19 a	
MS	1.43 b	2.40 a	

[z] Mean value of treatments;
[y] $LSD_{0.05}$ value for shoot length was 6 mm;
[x] $LSD_{0.05}$ value for chlorophyll content was 0.28 mg g^{-1} FW;
[w] Means followed by the same letter do not differ significantly at $p < 0.05$.

Table 3. Effect iron chelate formula and the presence (+) and absence (-) of fast yellow 9 in QL on shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway'. Means after 6 weeks of growth are given (n = 5 jars).

	Fast yellow 9		
Medium	-	+	$\bar{x}$
	Shoot length[y] (mm)		
QL + FeEDTA	24	21	22 a
QL + FeEDDHA	28	23	25 a
$\bar{x}$[z]	26 a[w]	22 a	
	Chlorophyll a+b[x] (mg g^{-1} FW)		
QL + FeEDTA	0.66 c	1.54 b	
QL + FeEDDHA	2.19 a	1.60 b	

[z] Mean value of treatments;
[y] $LSD_{0.05}$ value for shoot length was 6 mm;
[x] $LSD_{0.05}$ value for chlorophyll content was 0.28 mg g^{-1} FW;
[w] Means followed by the same letter do not differ significantly at $p < 0.05$.

Chlorosis has also been reported for in vitro cultured shoots of *Pyrus* (Dolcet-Sanjuan et al. 1990), *Rhododendron* (Anderson 1984) and *Rosa* (Horn 1992) grown on FeEDTA-containing medium. In addition, iron deficiency is often observed in calcifugous plant species such as *Azalea*, *Citrus*, *Rhododendron*, *Vaccinium corymbosum* L., *Vitis* and deciduous fruit trees grown in the field or in the greenhouse (Mengel & Kirkby 1978).

In summary, iron deficiency in 'Moneyway' cultures can be overcome either by addition of fast yellow 9 or by the replacement of FeEDTA by FeEDDHA. The use of FeEDDHA is more effective. It is tempting to assume that the replacement of FeEDTA by FeEDHHA in media will also improve the in vitro culture of other plant species that are susceptible to iron deficiency, such as the above mentioned plants.

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