Screening of different Fe(III) and Fe(II) chelate complexes in clonal micropropagation technology of highbush blueberry (*Vaccinium corymbosum* L.)

Ivan Nechiporenko[1,2,*], *Svetlana* Akimova[1,2], *Pavel* Kazakov[1,2], *Elena* Nikulina[3], *Nina* Tsirulnikova[3]

[1]Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Timiryazevskaya street, 49, 127434, Moscow, Russia
[2]All-Russian Phytopathology Research Institute, St. Institute, 143050, Big Vyazyomy, Russia
[3]National Research Center 'Kurchatov Institute', Square Akadimica Kurchatov, 1, 123182, Moscow, Russia

Abstract. The element iron plays a key role in plant physiology. For plant nutrition, chelated forms of iron are preferred. Therefore, in clonal micropropagation media, $FeSO_4$ x $7H_2O$ is usually introduced together with Na_2EDTA. At the same time, other effective iron chelates with both carboxyl and phosphorus chelates are known. So far, there are few scientific studies on the effect of iron chelates complexes on blueberry plants under *in vitro* conditions. Therefore, studies were conducted with different iron chelate complexes in 4 concentrations on highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue in clonal micropropagation technology. The best results were obtained using Fe(III)-DTPA in increased (×1.5) and doubled (×2.0) concentrations, where the multiplication factor was 4.59-4.65.

1 Introduction

With the development of the nursery industry, a lot of research continues has been going on in the field of clonal micropropagation witch at optimizing and quicken getting the production of planting material of fruit, forestry and ornamental crops [1, 2, 3, 4, 5]. In particular, so far one of the key factor successful technology in micropropagation of plants under *in vitro* conditions, largely depends on the correct composition of the culture medium and cultivation conditions due to the specific reaction of plants [6, 7, 8].

The nutrient medium includes macro- and microelements, vitamins, growth regulators, organic compounds, as well as a jelly-forming binding component (agar-agar, phytagel, *etc*.). The main element used in clonal micropropagation, along with nitrogen, phosphorus, sulfur and zinc, is iron – related to enzymes, is considered as one of the vitally important elements of nutrition, necessary for optimal growth, development and productivity of plants

*Corresponding author's e-mail: vannechiporenko@gmail.com

[9]. Iron plays a key function in various oxidation-reduction processes of plants, so iron deficiency can lead to disruption of chlorophyll synthesis and maintaining the structure and function of chloroplasts in leaves [10].
Chemically, the element iron is in two oxidation states: the oxidized form of Fe^{3+} (unavailable) and the reduced form of Fe^{2+} (available). Divalent iron oxidizes very quickly to trivalent iron, so rapid oxidation (which occurs in aerated soils) and the insolubility of the oxidized form are the cornerstones of the problem of iron-deficient chlorosis [11].

In iron deficiency, *in vitro* culture, tissue necrosis and leaf surface chlorosis can occur, which can slow down growth and development. It is also known that copper, nickel, zinc and phosphorus can cause iron deficiency. At the same time, excess iron can cause zinc deficiency [12].

Since iron salts are not well soluble, the sodium salt of ethylenediaminetetraacetic acid (EDTA) is commonly used to chelate iron, making it more soluble and usually supplied as (Na_2EDTA) commercially known also as Trilon B. In addition, there are reports that iron deficiency can be caused by a high acidity (pH) environment, which can cause iron to precipitate out. It is known that well aerated acidic soils show higher soluble iron content than calcareous soils [13]. And increasing EDTA content while maintaining normal iron concentrations can lead to iron toxicity. In addition to chelating cations, EDTA – has other effects in tissue culture. It can mimic the activity of auxin, affect various metabolic processes, and sometimes stimulate embryogenesis and organogenesis. There is also evidence that EDTA can decrease the acidity of the medium [14, 15].

Earlier studies have already been conducted in the study of iron chelate complexes on fruit and ornamental crops *in vitro* [16, 17, 18, 19, 20]. There have also been studies on blueberry plants [21, 22], but we decided to expand the number of chelate complexes tested.

Therefore, the purpose of this work was to study the effect of adding various chelated forms of iron to standard nutrient medium according to the WPM protocol (Lloyd & McCown) [23] at the multiplication stage on microplants of highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue.

2 Materials and methods

Experiments were carried out at Russian State Agrarian University—Moscow Timiryazev Agricultural Academy, Department of biotechnology and berry crops of Edelstein Educational Scientific and Production Center for Horticulture and Vegetable Growing in 2022–2023

The objects of the study were microplants of highbush blueberry cultivar Brigitta Blue (*Vaccinium corymbosum* L.). For multiplication we used Woody Plant Medium (WPM) nutrient medium enriched with the following substances (mg/L): thiamine hydrochloride (B1), pyridoxine hydrochloride (B6), nicotinamide (PP) – 0.5; mesoinositol - 100, sucrose - 30.000, with addition of 2-iP (2-isopentenyladenine) at a concentration of 2.5 g/L. The acidity of the medium is pH 4.5, agar-agar 8 g/L [24].

Five microcuttings, at least 0.8-10 mm long, were planted in each culture vessel on the multiplication stage. *In vitro* microplants were subcultured in a light room with an illuminance of 500 lux under mixed lighting (phytolamps – PPFD 18.9 umol/s^{-1}/m^{-2} and fluorescent lamps – PPFD 43.0 umol/s^{-1}/m^{-2}) with a 16-h photoperiod and a room temperature of 20-22 ºC.

In the experiment scheme included various iron chelates provided by the "Laboratory of Technology of Complexons and Complex Compounds of the Kurchatov Institute Research Center". – Institute of Chemical Reagents and Highly Pure Chemicals" in 4 concentrations.

Instead of the traditionally used $FeSO_4 \times 7H_2O$ + Na_2EDTA (control), five iron complexons were introduced into the composition of the nutrient medium:

1. **Carboxyl ligands:**
   a. Fe(III)-EDTA (ethylenediaminetetraacetic acid – 13,4%);
   b. Fe(III)-DTPA (ethylenediaminetetraacetic acid – 9,81%);
   c. Fe(III)-EDDHA (ethylenediamine-bis-hydroxyphenylacetic acid – 6%).
2. **Phosphorus-containing complexones:**
   a. Fe(III)-HEDP (hydroxyethylidene diphosphonic acid – 0,65%);
   b. Fe(II)-HEDP (exciethylidene diphosic acid – 0,52%).

In the nutrient medium prescribed by Woody Plant Medium, $FeSO_4 \times 7H_2O$ is used in a quantity equal to 27.8 mg/L. The molar mass of $FeSO_4 \times 7H_2O$ is 278.05 g, in which the molar mass of iron (Fe) is 55.85 g, indicating that 27.8 mg of $FeSO_4 \times 7H_2O$ contains 5.58 mg of iron. The amount of Fe in the experimental variants was calculated depending on the initial amount in the MS recipe, 5.58 mg, and the following concentrations were used: ×0.5-half reduced (2.79 mg/L), ×1.0-standard (5.58 mg/L), ×1.5-increased by 1.5 times (8.37 mg/L), and ×2.0-doubled (11.16 mg/L) (Table 1).

Table 1. Experimental concentrations of iron chelates in the modified nutrient media.

Complex	Iron Content in Powder/in Solution, %	Concentration of Fe Solution, mg/L			
		Reduced (×0.5) Fe-2.79	**Standard (×1.0) Fe-5.58**	**Increased (×1.5) Fe-8.37**	**Reduced (×0.5) Fe-2.79**
Fe(III)-EDTA (mg/L)	In powder 13.4	0.02	0.04	0.06	0.08
Fe(III)-DTPA (mg/L)	In powder 9.81	0.03	0.06	0.09	0.12
Fe(III)-EDDHA (mL/L)	In solution 0.015	18.6	37.2	55.8	74.4
Fe(III)-HEDP (mL/L)	In solution 0.65	0.43	0.86	1.29	1.72
Fe(II)-HEDP (mL/L)	In solution 0.52	0.54	1.07	1.61	2.15

On the 45th and 60th days of subcultivation, the morphometric indices of the microplants were measured. The number of microshoots, average length of microshoots, and multiplication rate were counted.

Statistical processing of two-way analysis of variance (AVONA) was performed using Microsoft Excel 2016 computer programs and the method of Isachkin A.V. Data are presented as mean values and standard deviations (M±SD). Repetition of experiments was three times, 15 plants per repetition.

3 Results and discussions

As a result of observations of the experiment, it was found that iron chelate (factor a) and the interaction of factors – iron chelate and concentration (ab) significantly affect the morphometric parameters of blueberry microplants, but the concentration (factor b) did not significantly affect.

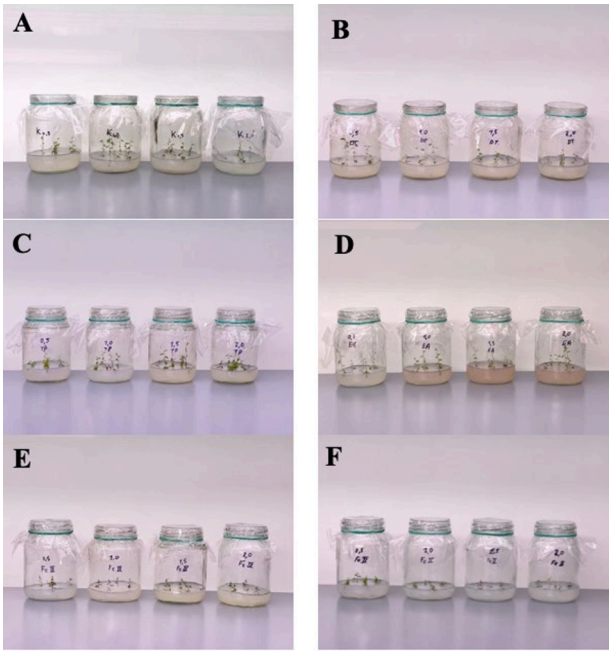
On the 45th day of subcultivation of blueberry microplants the greatest quality of microshoots were reliably revealed (factor a) in the variants with addition of chelate form of iron with carboxyl ligand Fe(III)-DTPA to nutrient medium and was 2.13±1.30 pcs. (in concentration ×1.5) vs. 1.47±0.64 pcs. in the controls and 2.20±2.01 pcs. (in concentration

×2.0) vs. 1.47±0.64 pcs. in the controls, and Fe(II)-HEDP 2.40±1.40 pcs. (in concentration ×0.5) vs. 1.87±0.99 pcs. in the controls. The Fe(III)-DTPA chelated form of iron significantly affected the length of the microshoots at double concentration (×2.0) and was 2.82±2.40 cm vs. 2.04±0.67 cm in the controls (Table 2, Figure 1).

Table 2. Effect of different sources and concentrations of iron during the multiplication stage of the highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue on day 45th of subcultivation.

Iron chelate (Factor A)	**Concentration (Factor B)**				Factor average B
	×0.5	**×1.0**	**×1.5**	**×2.0**	
Number of microshoots, pcs.					$LSD_{05}b = F_e<F_t$
Control	1.87 ± 0.99	1.60 ± 0.83	1.47 ± 0.64	1.47 ± 0.64	1.60
Fe(III)-EDTA	1.47 ± 0.83	1.47 ± 0.74	1.40 ± 0.74	1.53 ± 0.74	1.47
Fe(III)-DTPA	2.27 ± 0.96	1.87 ± 1.06	**2.13 ± 1.30[a]**	**2.20 ± 2.01[a]**	2.12
Fe(III)-EDDHA	1.07 ± 0.26	1.00 ± 0.00	1.33 ± 0.62	1.40 ± 0.63	1.20
Fe(III)-HEDP	1.27 ± 0.59	1.40 ± 0.51	1.20 ± 0.41	1.47 ± 0.64	1.33
Fe(II)-HEDP	**2.40 ± 1.40[a]**	1.93 ± 1.10	1.33 ± 0.72	1.53 ± 0.92	1.80
Factor average A $LSD_{05}a = 0.47$	1.73	1.55	1.48	1.60	
$LSD_{05}ab = F_e<F_t$					
Length of microshoots, cm					$LSD_{05}b = F_e<F_t$
Control	2.99 ± 1.81	2.90 ± 1.51	2.07 ± 1.11	2.04 ± 0.67	2.50
Fe(III)-EDTA	2.28 ± 1.26	1.98 ± 1.20	1.49 ± 0.93	1.64 ± 1.11	1.85
Fe(III)-DTPA	2.23 ± 1.07	1.91 ± 1.31	2.47 ± 1.22	**2.82 ± 2.40[a]**	2.36
Fe(III)-EDDHA	1.41 ± 0.79	1.39 ± 1.03	1.87 ± 1.07	1.93 ± 1.13	1.65
Fe(III)-HEDP	1.17 ± 0.40	1.15 ± 0.39	1.14 ± 0.40	1.01 ± 0.29	1.12
Fe(II)-HEDP	1.23 ± 0.46	0.76 ± 0.44	0.82 ± 0.37	0.59 ± 0.44	0.85
Factor average A $LSD_{05}a = 0.56$	1.89	1.68	1.64	1.67	
$LSD_{05}ab = F_e<F_t$					

Least significant difference $P<0.05$ was calculated by two-way analysis of variance: * the results were expressed as mean ± standard deviation (M±SD); ** «a, b, ab» - are the differences between the averages when the recording is significant based on the comparison of the differences between the mean with an LSD at a 5% significance level: “a” by factor “a” (iron chelates), “b” by factor “b” (concentration), and “ab” by the interaction of factors.



Experimental *in vitro* blueberry microplants on different iron chelates at different concentrations (from left to right: ×0.5; ×1.0; ×1.5; ×2.0): **A** – Control; **B** – Fe(III)-EDTA; **C** – Fe(III)-DTPA; **D** – Fe(III)-EDDHA; **E** – Fe(III)-HEDP; **F** – Fe(II)-HEDP.

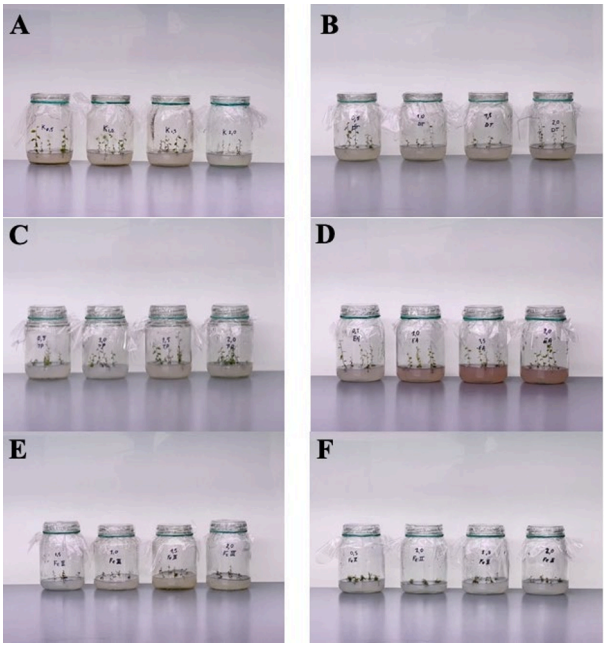
Fig. 1. Microplants of highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue on the 45th day subcultivation

On the 60th day of subcultivation of highbush blueberry microplants continued the trend in the number of microshoots on the variant with carboxyl ligand – Fe(III)-DTPA and was 2.20±1.42 pcs. at standard concentration (×1.0) vs. 1.60±0.83 pcs. in the controls, 2.27±1.67 pcs. at increased concentration (×1.5) vs. 1.60±0.74 pcs. in the controls and 2.60±2.41 pcs. at doubled concentration (×2.0) vs. 1.73±0.59 pcs. in the controls; in addition, in Fe(II)-HEDP variant the number microshoots was significantly 3.07±1.98 pcs. (in concentration ×0.5) vs. 2.13±1.06 in the controls and 2.53±1.25 pcs. (in concentration ×1.0) vs. 1.60±0.83 pcs. in the controls. The dynamics of carboxyl ligand Fe(III)-DTPA in increased (×1.5) and double (×2.0) concentrations were also maintained for the length of microshoots and were 3.67±2.62 cm vs. 2.50±1.41 cm in the controls and 3.72±3.19 cm vs. 2.38±0.80 cm in the controls, respectively (Table 3, figure 2).

Table 3. Effect of different sources and concentrations of iron during the multiplication stage of the highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue on day 60th of subcultivation.

Iron chelate (Factor A)	**Concentration (Factor B)**				Factor average B
	×0.5	**×1.0**	**×1.5**	**×2.0**	
Number of microshoots, pcs.					$LSD_{05}b = F_e<F_t$
Control	2.13 ± 1.06	1.60 ± 0.83	1.60 ± 0.74	1.73 ± 0.59	1.77
Fe(III)-EDTA	1.73 ± 1.10	1.67 ± 1.11	1.47 ± 0.74	1.53 ± 0.74	1.60
Fe(III)-DTPA	2.33 ± 0.82	**2.20 ± 1.42[a]**	**2.27 ± 1.67[a]**	**2.60 ± 2.41[a]**	2.35
Fe(III)-EDDHA	1.27 ± 0.46	1.00 ± 0.00	1.33 ± 0.62	1.47 ± 0.83	1.27
Fe(III)-HEDP	1.33 ± 0.62	1.53 ± 0.64	1.27 ± 0.46	1.60 ± 0.91	1.43
Fe(II)-HEDP	3.07 ± 1.98[a]	2.53 ± 1.25[a]	1.80 ± 0.86	1.80 + 0.94	2.30
Factor average A $LSD_{05}a = 0.56$	1.98	1.76	1.62	1.79	
$LSD_{05}ab = F_e<F_t$					
Length of microshoots, cm					$LSD_{05}b = F_e<F_t$
Control	3.95 ± 2.26	3.99 ± 1.99	2.50 ± 1.41	2.38 ± 0.80	3.21
Fe(III)-EDTA	2.89 ± 1.50	2.67 ± 1.61	2.11 ± 1.30	2.29 ± 1.67	2.49
Fe(III)-DTPA	3.01 ± 1.36	2.73 ± 1.93	**3.67 ± 2.62[a]**	**3.72 ± 3.19[a]**	3.28
Fe(III)-EDDHA	1.82 ± 1.08	1.63 ± 1.04	2.40 ± 1.38	2.43 ± 1.34	2.07
Fe(III)-HEDP	1.36 ± 0.65	1.45 ± 0.47	1.18 ± 0.54	1.19 ± 0.46	1.30
Fe(II)-HEDP	1.86 ± 0.63	1.43 ± 0.87	1.15 ± 0.50	0.95 ± 0.53	1.35
Factor average A $LSD_{05}a = 0.77$	2.48	2.32	2.17	2.16	
$LSD_{05}ab = F_e<F_t$					

Least significant difference $P<0.05$ was calculated by two-way analysis of variance: * the results were expressed as mean ± standard deviation (M±SD); ** «a, b, ab» - are the differences between the averages when the recording is significant based on the comparison of the differences between the mean with an LSD at a 5% significance level: “a” by factor “a” (iron chelates), “b” by factor “b” (concentration), and “ab” by the interaction of factors.



Experimental *in vitro* blueberry microplants on different iron chelates at different concentrations (from left to right: ×0.5; ×1.0; ×1.5; ×2.0): **A** – Control; **B** – Fe(III)-EDTA; **C** – Fe(III)-DTPA; **D** – Fe(III)-EDDHA; **E** – Fe(III)-HEDP; **F** – Fe(II)-HEDP.

Fig. 2. Microplants of highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue on the 60th day subcultivation

Ha On the 60th day of subcultivation of highbush blueberry the multiplication rate was also calculated. The highest multiplication factors was observed in the carboxyl ligand in the Fe(III)-DTPA variant in increased (×1.5) and double (×2.0) concentrations and were 4.59±3.27 vs. 3.13±1.76 in the controls and 4.65±3.98 vs. 2.98±1.00 in the controls, respectively. Apart from this, it must be said that the control variants with $FeSO_4 \times 7H_2O$ + Na_2EDTA at concentrations of reduced (×0.5) and standard (×1.0) also had high results and were 4.96±2.83 and 4.98±2.49, respectively (Table 4).

Table 4. Effect of different sources and concentrations of iron during the multiplication stage of the highbush blueberry (*Vaccinium corymbosum* L.) cv. Brigitta Blue on the multiplication rate (60th day of subcultivation).

Iron chelate (Factor A)	Concentration (Factor B)				Factor average B $LSD_{05}b = F_e<F_t$
	×0.5	×1.0	×1.5	×2.0	
Control	4.96 ± 2.83	4.98 ± 2.49	3.13 ± 1.76	2.98 ± 1.00	4.01
Fe(III)-EDTA	3.61 ± 1.87	3.34 ± 2.01	2.63 ± 1.62	2.86 ± 2.09	3.11
Fe(III)-DTPA	3.76 ± 1.70	3.42 ± 2.41	**4.59 ± 3.27[a]**	**4.65 ± 3.98[a]**	4.11
Fe(III)-EDDHA	2.28 ± 1.36	2.03 ± 1.30	3.00 ± 1.73	3.04 ± 1.67	2.59
Fe(III)-HEDP	1.70 ± 0.82	1.82 ± 0.59	1.48 ± 0.68	1.49 ± 0.58	1.62
Fe(II)-HEDP	2.33 ± 0.79	1.79 ± 1.08	1.44 ± 0.63	1.19 + 0.66	1.69
Factor average A $LSD_{05}a = 0.96$	3.10	2.89	2.71	2.70	
$LSD_{05}ab = F_e<F_t$					

Least significant difference P<0.05 was calculated by two-way analysis of variance: * the results were expressed as mean ± standard deviation (M±SD); ** «a, b, ab» - are the differences between the

averages when the recording is significant based on the comparison of the differences between the mean with an LSD at a 5% significance level: “a” by factor “a” (iron chelates), “b” by factor “b” (concentration), and “ab” by the interaction of factors.

4 Conclusions

1. At day 60 of subcultivation of cv. Brigitta Blue highbush blueberry microshoots at multiplication stage using carboxyl ligands the advantage of Fe(III)-DTPA complexons in concentrations ×1.5; ×2.0 with the application of which multiplication factor was 4.59-4.65 was revealed;
2. At day 60 of subcultivation of cv. Brigitta Blue highbush blueberry microshoots at multiplication stage when using phosphorus-containing ligands no advantage of Fe(II) and Fe(III) complexons (HEDP) on multiplication factor was found;
3. The research in this area should be continued further.

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