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ANALYSIS OF GENETIC STABILITY IN THE *ex vitro* ROOTED MICROCUTTINGS OF BLUEBERRY (*Vaccinium corymbosum* L.)

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ABSTRACT

During plant propagation changes in genetic stability may occur, not only during the *in vitro* multiplication but also in the *ex vitro* conditions in response to the sum of stress factors affecting plants. It is therefore necessary to evaluate planting material in terms of its genetic stability. The aim of the work was to assess by the RAPD method genetic stability of the *ex vitro* rooted blueberry (*Vaccinium corymbosum* 'Bluecrop' and 'Duke') microcuttings. Also an effect of biostimulator Goteo (0.2%) on rhizogenesis was tested as compared to the auxin indole-3-butyric acid IBA (50 mg·Γ^{1}) applied as water solution and a commercial rooting powder Rhizopon AA containing 1% IBA. All the rooting stimulators improved the percentage and degree of rooting in blueberry microcuttings. The biostimulator gave results only slightly poorer than the auxin. Both cultivars were genetically stable and no DNA polymorphism was found between the traditionally propagated stock plants growing in a nursery and those rooted *in vivo*, regardless of the rooting enhancer used.

Key words: ericaceous plants, RAPD, rhizogenesis, growth regulators, genetic stability

INTRODUCTION

There is a growing interest in growing blueberry (*Vaccinium corymbosum* L.) because of high dietetic and health values of its fruits. Because of showy bunches composed of blue berries plants are also praised as ornamental shrubs. As it was intensively bred in USA the species is often named the American berry. First, it has been grown mostly in North America but recently the plant is gaining popularity in many countries and its cultivation area is rapidly increasing [Strik 2006].

Commercially, blueberry is propagated by stem cuttings – softgreen and semi lignified or *in vitro* [Meiners et al. 2007, Masari 2009, Ružic et al. 2012]. Micropropagation is nowadays the most popular method being the most efficient relative to other techniques, and providing healthy and uniform planting material. The supportes of this method are convinced that blueberry shrubs from the *in vitro* cultures grow more vigorously [Read et al. 1989, El-Shiekh et al. 1996]. There are as well the oponents who think that even if initialy shrubs grow strong they start cropping later and their fruits are smaller as compared to the traditionally propagated plants [Litwińczuk et al. 2005, Ostrolucká et al. 2007]. However, the most important problem related to the *in vitro* propagation of blueberry is genetic variability as there are reports on genetic instability in micropropagated plants of this species [Jain 2001].

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Genetic variabilty occurring during micropropagation is often interpreted as plant effort to reduce stress [Burg et al. 2007]. Similar changes appear after moving plants to the *ex vitro* conditions. They are mostly due to drought stress as air humudity is lower in the new growth conditions while light stress results in oxidative stress [Hazarika 2003]. Genetic diversity is also the basis for preserving the adaptability of plants to changing environmental conditions. It helps to maintain the stability and durability of entire populations under changing climatic conditions and the occurrence of environmental stresses [Kruk et al. 2012].

A gradual withdrawal of auxin preparations, till now commonly used in nursery production, impel the growers to replace the synthetic auxins with safer biopreparations highly efficient in plant growth stimulation [Dobrzański et al. 2008, Przybysz et al. 2010, du Jardin 2015]. One of them is biostimulator Goteo (Arysta LifeScience Polska), a liquid preparation stimulating formation and restoration of rhizogenous layer. It contains GA 142 – biologically active filtrate from seaweed *Ascophyllum nodosum*, phosphorus – 13% as P_2O_5, and potassium – 5% as K_2O. The preparation is used in concentration 0.2% as recommended by the producer and confirmed in the earlier experiments including biostimulators based on seaweed extracts [Matysiak et al. 2010, Gajc-Wolska et al. 2012].

Genetic stability of a final product should be verified at the end of a production cycle, by comparing the rooted cuttings with a stock plant. Recently, evaluation of genetic stability using molecular markers is getting frequent in production of many plant taxons. Usually, such techniques as: RFLP (Restriction Fragments Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), AFLP commonly referred to as Amplified Fragment Length Polymorphism, or SSR (Simple Sequence Repeats) are applied. They allow to detect differences in DNA sequences in specimen and to obtain their characteristics on the level of nucleotides, genes or the whole genomes. Analysis of sequences specific for particular specimen and characteristics of sequence within a population or species allows to obtain a picture of genetic variability on each of these levels [Zakaria et al. 2005, Zagalska-Neubauer and Dubiec 2007].

Genetic polymorphism understood as presence of more than one allel in a given *locus* may be identyfied by molecular markers [Dodgson et al. 1997, Sunnocks 2000], with different method based on the well known PCR (Polymerase Chain Reaction). In the popular RAPD method arbitral primers of 10 nucleotides are used [Welsh and McClelland 1990, Williams et al. 1990]. Random sequence of the short primer and relatively low temperature of its incorporation (usually 35–40°C) diminish the reaction specifics allowing the starter to incorporate in many sites of a genome [Williams et al. 1990, Bowditch et al. 1993]. This generates a considerable number of polymorphic DNA fragments [Gu et al. 1997], what is desirable while studying polymorphism [Zagalska-Neubauer and Dubiec 2007]. What more, this method does not need earlier studies on DNA sequences, is efficient, quick and does not require large amounts of DNA [Rodriguez et al. 1999].

The aim of this work was to determine genetic stability of the *ex vitro* microcuttings rooted plant material in two blueberry cultivars using the RAPD method. Plant material was obtained by micropropagation and compared with the shrubs propagated traditionally by stem cuttings. Moreover, the effect of biostimulator Goteo on rhizogenesis during the *ex vitro* rooting of blueberry microcuttings was evaluated as compared to the rooting enhancers based on the auxin IBA.

MATERIAL AND METHODS

The *in vivo* rooting of blueberry microcuttings (propagation method developed by combining tissue cultures with the *ex vitro* propagation) was carried out in July 2016. Microcuttings of two blueberry cultivars (*Vaccinium corymbosum* L. 'Bluecrop' and 'Duke') were used. Both cultivars are widely planted in commercial plantations in Poland and elsewhere. The experimental material consisted of the tip one-nodal cuttings taken from the stock plants cultured *in vitro*, already after acclimatization *ex vitro*. They were rooted in plastic trays filled with the mixture of peat and perlite (2 : 1 v/v), pH 5.0. They were kept in the phytotrone under controlled but not sterile conditions: temp. 25°C and 16 h light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

There were 6 treatments, each in triplicate, each containing 100 microcuttings (tab. 1). The rooting powder Rhizopon AA (1% IBA) was applied to the cuttings' bases before cuttings were inserted into the medium while two other preparations were given as foliar applications on inserted cuttings. The water-sprayed cuttings were the control treatment. The water IBA solution was used (50 mg·l^{-1}) while the biostimulator Goteo was sprayed in 0.2% concentration. The cuttings were sprayed until the whole leaf surface was covered with a solution. The treatments were performed once, immediately after placing cuttings in the boxes filled with the rooting medium.

A hand pressure sprayer of 1 l^{-1} volume was used. In the treatment 5 and 6 the application of Goteo was combined with IBA, either in the form of the powder Rhizopon AA (5) or the spraying with the IBA water solution (6).

Percentages of rooted cuttings and the degree of rooting were determined 3 weeks after the start of the experiment. The degree of rooting was evaluated on a 5-point scale rating the development of the root ball (tab. 2). Percent of rooted cuttings was also calculated – only the cuttings with root system within the scale range of 2–5 were regarded as rooted and counted.

Molecular analysis of plants genetic stability

Genetic stability was checked in blueberry plants coming form the above treatments and from the 3-year old blueberry shrubs propagated traditionally by stem cuttings and growing in the nursery. DNA for molecular analyses was isolated from young leaves (taken at the time of liquidation of the experience and evaluation rooting), using the GeneMATRIX Plant & Fungi DNA Purification Kit in accordance with the procedure recommended by the manufacturer (EURx). The genetic material was suspended in 30 µl H_2O and stored at –20°C. PCR reactions were performed in a total volume of 25 µl reaction mix containing: 16.88 µl deionized water, 2.5 µl of 10× Pol Buffer (containing $MgCl_2$, KCl and the load-

Table 1. A list of treatments in the experiment

No. of treatment	Methods of cuttings treatment
1	Control '0'1 spraying with distilled water
2	Rhizopon AA (1% IBA) powder
3	1 × spraying with IBA 50 mg·l^{-1}
4	1 × spraying with Goteo 0.2%
5	1 × spraying with Goteo 0.2% and Rhizopon AA (1% IBA) powder
6	1 × spraying with Goteo 0.2% and IBA 50 mg·l^{-1}

Table 2. Evaluation scale of the root development

Characteristic of the degree of rooting	Score
Cutting without visible roots	1
A few (1–3) short roots (over 1 cm long)	2
4–5 roots, some of them branched, no root ball formed (2–4 cm long)	3
Medium sized root system composed of 6–10 branched roots forming a root ball (4–5 cm long)	4
Well developed, branched root system forming a root ball (over 10 roots)	5

ing dye), 2.5 µl dNTP, 2 µl primer, 0.125 µl of Taq DNA polymerase (EURx), 1 µl DNA and in a pre-heated thermocycler Mastercycler (Eppendorf). PCR was initiated by a denaturation step at 94°C for 40 s and then the reaction was subjected to 35 cycles of 35°C for 60 s, 72°C for 60 s with a final elongation step of 2 min at 72°C. The amplification products were resolved by electrophoresis on a 1.5% agarose gel (agarose BASICA LE, ABO) with 14 mg·dm^{-3} ethidium bromide and visualized under UV [Zakaria et al. 2005, Anatala et al. 2014]. Twenty RAPD starters were analyzed and 12 or 5 were chosen for 'Duke' and 'Bluecrop', respectively, as they generated stable band patterns. PCR reactions with a given primer were repeated three times.

Statistical analysis

Arcsine transformation was performed for all experimental data taken in percentages before subjecting them to statistical analysis [Snedecor and Cochran 1967]. To compare the means, percentages of rooted cuttings were transformed according to Bliss. All data – percentages of rooted cuttings were subjected to the one-factorial ANOVA followed by Newman-Keuls test at α = 0.05 [Wójcik and Laudański 1989].

RESULTS

All the rooting enhancers used in the experiment significantly affected the degree and percentage of rooting in both blueberry cultivars (tabs 3 and 4). The poorest root system was developed in control untreated cuttings (evaluated as 2.1 in 'Bluecrop' and 2.6 in 'Duke'). Application of the water solutions of IBA and Goteo improved the degree of rooting (tab. 3). In cv. 'Bluecrop' the best result was obtained after the use of Rhizopon AA (3.4) while in 'Duke' microcuttings rooted best due to the joint application of the powder and spraying with Goteo what increased the parameter value to 3.9, i.e. by 0.3 and 0.6 as compared to the treatments with Rizopon AA or Goteo, respectively.

The lowest percentage was found in control treatments (83.1% in 'Bluecrop' and 89.9% in 'Duke'). It was increased by 8% due to the foliar application of Goteo (in both cultivars) and the auxin (in 'Bluecrop') (tab. 4). In 'Bluecrop' the effect of

Table 3. The degree of rooting in cuttings of *Vaccinium corymbosum* 'Bluecrop' and 'Duke'

Cultivar	Control	Rh. AA 1% IBA (powder)	Spray 50 mg·l^{-1} IBA	1 × spray Goteo 0.2%	1 × spray Goteo 0.2% + Rh. AA (powder)	1 × spray Goteo 0.2% + 50 mg·l^{-1} IBA
Bluecrop	2.1 a	3.4 c	3.0 b	3.0 b	3.2 bc	3.0 1b
Duke	2.6 a	3.6 c	3.3 b	3.3 b	3.9 d	3.6 c

Means in a row ± SD followed by the same letter do not differ significantly at α = 0.05

Table 4. The percentage of rooting in cuttings of *Vaccinium corymbosum* 'Bluecrop' and 'Duke'

Cultivar	Control	Rh. AA 1% IBA (powder)	Spray 50 mg·l^{-1} IBA	1× spray Goteo 0.2%	1× spray Goteo 0.2% + Rh. AA (powder)	1× spray Goteo 0.2% + 50 mg·l^{-1} IBA
Bluecrop	83.1 a	95.2 c	88.9 b	90.0 b	92.1 bc	90.5 b
Duke	88.9 a	98.5 c	97.4 c	95.3 b	96.5 bc	95.6 b

Means in a row ± SD followed by the same letter do not differ significantly at α = 0.05

the water IBA solution was comparable to that of Goteo while in 'Duke' both forms of IBA gave comparable results, significantly better than that obtained with the biostimulator. The highest increase in percentage relative to the control – 10% on the average – resulted from the application of Rhizopon AA. This result was not further improved by the joint application of the powder and then biostimulator.

Molecular analysis of genetic stability

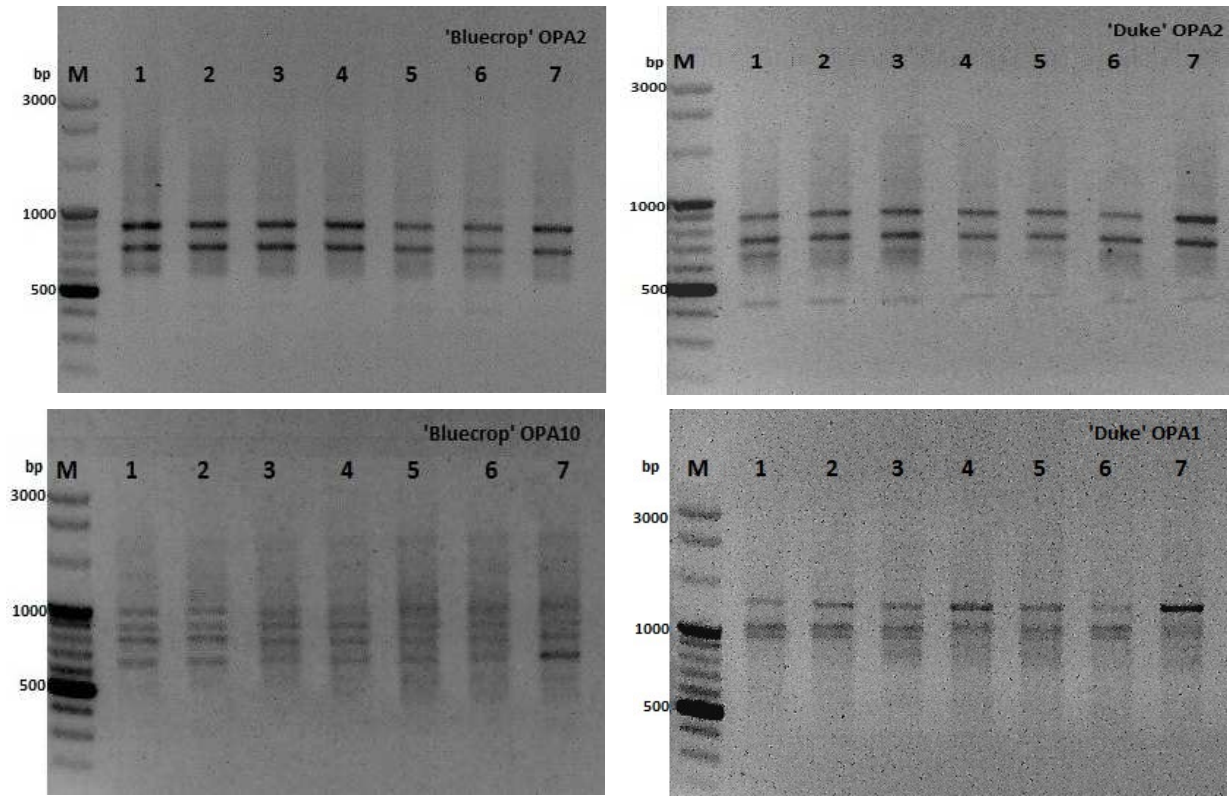
Using RAPD method, the authors did not find DNA polymorphism in microcuttings of two blueberry cultivars, irrespective of the applied preparations or the propagation method. For 'Duke' 203 products were obtained while for 'Bluecrop' 84 (tab. 5). In none of the reactions the polymorphic bands were obtained (phot. 1). On the average, for 'Duke' a single starter generated 2.4 monomorphic fragments for a single probe, of the length equal to 400 to 1500 bp and similar for 'Bluecrop' 2.4 fragments for a single probe, of 300–1500 bp. No polymorphism was found between microcuttings rooted *in vivo* after being treated with any rooting enhancer, or those propagated in the nursery by the traditional stem cuttings.

DISCUSSION

Vegetative propagation of blueberry by microcuttings is the simplest and most effcient method to obtain new plants, espaecially those mass-propagated. A modyfication of micropropagation protocol was used to obtain the rooted blueberries, aiming to shorten the whole procedure: the phase of rooting and acclimatization run simultaneously under controlled but not sterile conditions. This may shorten considerably the production cycle in blueberry: rooting and acclimatization may occur even in two [Vescan et al. 2012] or three weeks [Pacholczak and Nowakowska 2015]. Usually, these two phases

Table 5. Primer sequences and number of scored monomorphic bands produced by RAPD markers in *Vaccinium corymbosum* 'Bluecrop' and 'Duke'

Marker	Sequence (5′–3′)	Number of bands scored per plant	
		'Bluecrop'	'Duke'
OPA 01	CAGGCCCTTC	3	3
OPA 02	TGCCGAGCTG	2	4
OPA 03	AGTCAGCCAC		3
OPA 04	AATCGGGCTG		2
OPA 05	AGGGGTCTTG		3
OPA 07	GGTCCCTGAC	2	2
OPA 09	GGGTAACGCC		2
OPA 10	GTGATCGCAG	4	3
OPA 11	CAATCGCCGT		1
OPA 12	TCGGCGATAG	1	
OPA 15	TTCCGAACCC		2
OPA 17	GACCGCTTGT		3
OPA 18	AGGTGACCGT		1
Total		84	203
Average numer per primer		16.8	16.8
Average fragments for a single probe		2.4	2.4



Phot. 1. Examples of electropheregrams showing the reactions of RAPD amplification for blueberry cuttings: 'Duke' (with starters OPA1 i OPA2) and 'Bluecrop' (with starters OPA10 i OPA2) rooted *in vivo*: 1 – microcuttings not treateted with rooting enhancers, 2 – microcuttings treated with Rhizopon AA, 3 – microcuttings treated with IBA, 4 – microcuttings treated with Goteo, 5 – microcuttings treated with Goteo and Rhizopon AA (1 : 1 v/v), 6 – microcuttings treated with Goteo and IBA (1 : 1 v/v), 7 – cuttings propagated traditionally in a nursery; M – DNA marker

last twice as long: Ružic et al. [2012] needed 4 weeks for rooting of microcuttings in 3 blueberry cultivars ('Bluecrop' included), followed by 2 weeks of acclimatization.

In has been widely known that changes in auxin and cytokinin concentrations affect many aspects of plant development and functioning [Wróblewska 2013]. Auxins are usually applied during rhizogenesis as they stimulate new root formation [Spethmann 1998, Khan et al. 2009, Kroin 2009]. Recently, using water auxin solutions have become widely used in nusery production as the water soluble IBA salts are easily acccessible for plants [Drahn 2007, Kroin 2009, 2010, 2014]. Though there is a general knowledge on commercial use of different auxin application methods the information on their effciency is rather scarce. Moreover, each application method should be tested and adjusted to particular taxons – tree and shrub cultivars [Kroin 2010]. Also the IBA concentrations recommended by producers may differ from the optimal ones what must be tested and veryfied during production, thus allowing to lower the production costs [Drahn 2007].

The results of this trial show that application of the IBA-containing rooting enhancers increased the rooting parameters – a degree and percentage of rooting in blueberry. The commercial powder containing 1% IBA was the most effective in both cultivars.

Similar results were obtained in two ninebark cultivars by Pacholczak et al. [2013] and in blueberry by Pacholczak and Nowakowska [2015].

The seaweed extracts affect synthesis of such hormones as indolil acetic acid (IAA) and abscisic acid (ABA) thus stimulating plant growth. Increase in fresh weight of shoots and roots in *Lolium perenne* due to the seaweed extract application was reported by Zaman et al. [2015]. Kumar and Sahoo [2011] proved a positive effect of alg preparations on root number of germinating plants in *Triticum aestivum.* Also Mugnai et al. [2008] report on a better root growth in *Vitis vinifera* treated with seaweed extracts. Here, the biopreparation Goteo – biologically active filtrate from the seaweed *Ascophyllum nodosum* – gave results comparable to the foliar application of IBA in water solution.

Molecular markers are used nowadays as universal research tools in many fields. The method of the random amplification of polymorphic DNA (RAPD) is very popular and applied for molecular analyses of many plant species. Its big advantage is a possibility to use in genetic studies without prior knowledge of a genome [Anatala et al. 2014]. Using this method the authors did not observe polymorphism in DNA in microcuttings of two blueberry cultivars, regardles of the propagation method or a rooting enhancer. Similar molecular analyses were done by Ahmad and Anis [2010] who checked genetic stability of microcuttings of *Vitex negundo* L., both propagated *in vitro* and acclimatized. Using the RAPD method they did not find any changes in genome of the analyzed plantles. Neither Gaafar and Saker [2006] using the same method, observed polymorphism in strawberry cuttings. Studies on genetic variability in 5 blueberry cultivars, including 'Duke' and 'Bluecrop', were carried out by Gajdošová et al. [2006]. Similarly, as in this trial, they used a ready kit for DNA isolation and ran PCR with 20 starters from Operon Technologies. They did not find differences in genetic profiles of the cultivars under study or in the initial material used to established the *in vitro* cultures. Similar results concerning two cultivars in common are reported in this work.

The above presented results may be a base for further research aiming to develop the most efficient propagation methods for blueberry (*Vaccinium corymbosum* L.) what will result in lowering production costs and the cuttings' prices. If needed, replacement of the auxin-based rooting enhancers with biostimulators such as Goteo seems possible. To confirm genetic stability of blueberry cultivars the research with a higher number of PCR starters would be recommended as well as application of other molecular methods.

CONCLUSIONS

1. All the rooting stimulators improved the percentage and degree of rooting in blueberry microcuttings.

2. Biostimulator Goteo proved a good rooting enhancer for blueberry giving only slightly poorer results than the auxin IBA.

3. Using the RAPD technique no DNA polymorphism was found in the traditionally propagated or *in vivo* rooted blueberry plants regardless of the rooting enhancer used.

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