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EVALUATION OF GENETIC FIDELITY OF *IN VITRO* GROWTH PLANTS OF HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* L.) CULTIVARS USING SCoT MOLECULAR MARKERS

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Abstract

The aim of this research was to investigate the influence of cultivar upon in vitro multiplication rate of highbush blueberry (Vaccinium corymbosum L.) and to evaluate the genetic fidelity of in vitro-propagated plants using molecular markers. Four varieties of blueberries were studied: 'Bluecrop', 'Blueray', 'Brigitta', and 'Duke'. For the in vitro multiplication, the Woody Plant Medium (WPM) basal medium was used, supplemented with 1 mg/L zeatine, 100 mg/L Sequestrene 138 and Plant agar 4 g/L, pH = 5. After ten weeks of in vitro culture, the four highbush blueberry varieties had average proliferation rates between 2.98 ± 0.25 and 9.35 ± 0.50 and the average length of the shoots varied between 2.79 ± 0.15 cm and 3.29 ± 0.13 cm. Clonal fidelity has been checked by twelve Start Codon Target Polymorphism (SCoT) primers. No polymorphism was detected, that proving that the regenerated plants showed high clonal fidelity.

Key words*: clonal fidelity; photosynthetic pigments; polymorphism; shoot proliferation; Start Codon Target Polymorphism.*

INTRODUCTION

In vitro culture or micropropagation is a method of vegetative multiplication successfully applied to highbush blueberry and the commercial use of tissue culture technology could be a profitable way to produce high-quality propagating material (Debnath & Goyali, 2020). However, maintaining *in vitro* subcultures for a long time can often lead to somaclonal variations due to chromosomal rearrangements and mutations in plant cells. Therefore, it is very important to establish the genetic uniformity of micropropagated plants in order to confirm the quality of propagated plants and their commercial utility by various methods, including morphological, biochemical, and molecular techniques (Muraseva et al., 2018). In this context, DNA-based molecular techniques could now be considered valuable tools used to evaluate the genetic fidelity of *in vitro* propagated plants (Martins et al., 2004).

A number of molecular markers, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), arbitrary primed polymerase chain reaction (AP-PCR), DNA amplified fingerprinting (DAF), simple (short) sequence repeat (SSR), short tandem repeat (STR), sequence characterized amplified region (SCAR), sequence-tagged sites (STSs), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), expressed sequence tag (EST)-PCR and cleaved amplified polymorphic sequences (CAPS) derived from EST-PCR markers were used for genetic analysis of *in vitro* propagated plants (Debnath, 2010). In addition, the start codon targeted polymorphism (SCoT) markers are valuable PCR-based molecular markers used to analyze the genetic fidelity of *in vitro* grown plants. These molecular markers were developed based on data mining for short conserved amino acid sequences in proteins and designing polymerase chain reaction (PCR) primers based on the corresponding DNA sequence (Collard & Mackill, 2009).

The recent studies have shown that SCoT molecular markers have been used to test the clonal fidelity of *in vitro* grown plants in various species: *Bauhinia racemosa* Lam. (Sharma et al., 2019), *Annona reticulata* L.

(Kudikala et al., 2020), *Bambusa balcooa* Roxb. (Rajput et al., 2020), *Santalum album* L. (Manokari et al., 2021), *Crocus sativus* L. (Gautam & Bhattacharya., 2021), *Solanum khasianum* Clarke (Chirumamilla et al., 2021), *Prunus salicina* (Thakur et al., 2021) and *Dioscorea pentaphylla* L. (Manokari et al., 2022).

In this study, the genetic uniformity of *in vitro* regenerated plants of four varieties of highbush blueberry (*Vaccinium corymbosum* L.) was analyzed, after the 15th subculture. According to Ružić et al., 2012, this species is considered the most commercially important and biologically valuable species of the genus Vaccinium and the production of high-quality plants necessary for the establishment of highbush blueberry plantations involves the adoption of modern propagation techniques. Thus, numerous studies have focused on *in vitro* cultures of *V. corymbosum* varieties (Reed & Abdelnouresquivel, 1991; Ružic et al., 2012; Clapa et al., 2018; Wang et al., 2019; Georgieva & Kondakova, 2021), but there are only a few reports on the evaluation of clonal fidelity of *in vitro* raised plants (Gajdosova et al., 2006; Nowakowska & Pacholczak, 2017; Chen et al., 2018; Clapa et al., 2019).

Furthermore, as we know, the clonal fidelity of plants regenerated by tissue culture using ScoT molecular markers has not yet been assessed in *V. corymbosum*.

Therefore the aim of this research was to evaluate the genetic stability of micropropagated highbush blueberry plants to reveal the applicability of Start Codon Target Polymorphism (SCoT) molecular marker system.

MATERIALS AND METHODS

In vitro culture

In vitro shoot cultures of four highbush blueberry varieties (Bluecrop, Blueray, Brigitta and Duke) were established from axillary buds and maintained by regular sub-culturing after every 10 weeks, for three years, respectively fifteen sub-cultures on Woody Plant Medium (WPM) (Lloyd & McCown, 1980) supplemented with 1 $mg \cdot L^{-1}$ zeatine, 100 $mg \cdot L^{-1}$ Sequestrene 138 (FeNaEDDHA 6%) and 3% (w/v) of sugar. The culture media was solidified with 0.4 % (w/v) plant agar. The pH of the media was adjusted to 5.0 before autoclaving. The autoclave program used for sterilizing the culture media was run at 0.11 MPa at 121°C for 20 min. All the components were purchased from Duchefa Biochemie BV.

In each vessel (720 mL glass jar, 9 cm diameter, 13.5 cm high) with screw cap and ventilation holes (4 mm) 100 mL culture medium was dispensed. Ten explants per jar with a length of 1.5 to 2 cm were inoculated in such way that two-thirds to three-fourths of the basal part of the explants was immersed in the culture media.

The *in vitro* cultures were incubated in the growth room for a 16-h photoperiod with 32.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity (Cool white fluorescent lamps, 36 W; Philips, Amsterdam, The Netherlands) at 23 ± 1°C and 50% ± 2% humidity.

The length of the shoots and the rate of proliferation were recorded and calculated after fifteen subcultures. Five jars/each variety were measured for each treatment. The recorded data for proliferation rate represents the number of shoots with a length greater than one cm obtained/inoculum.

To determine the content of the photosynthetic pigment and to extract total genomic DNA, fresh leaves were collected from both *in vitro* three-year-old shoot cultures and mother plants of all varieties.

Photosynthetic pigments

Chlorophylls content. Levels of chlorophyll a (Chl a) and chlorophyll b (Chl b) were determined by spectrophotometry, using *in vitro* grown shoots. Samples of fresh vegetal material from shoots were weighed, homogenized, and extracted with 90% acetone in water using a magnetic stirrer until the residue became colorless.

The following formulas were used to quantify the chlorophyll content:

Chl a = (11.75 × A663 - 2:35 × A645) × V/g and Chl b = (18.61 × A645 – 3.96 × A663) × V/g), where A645 and A663 represent the optical density at a specific wavelength, V represents the volume of the extract (mL), and g represents the weight of the samples (mg). The recorded data were expressed as mg/g FW.

Carotenoids content. The extraction of carotenoids (Caro) was carried out with acetone. After the separation phase, the organic

phase was dried over anhydrous sodium sulfate until the solvent was evaporated. Finally, the residue was dissolved in a known volume of hexane and the measurements were recorded at an absorbance of 450 nm level using the Perkin Elmer Lambda 25 spectrophotometer. The concentration of total Caro was calculated according to the following formula: X mg carotenoids = (A × V × 1000)/(A 1% 1cm × 100), where A represents absorbance at 450 nm, V represents volume (mL), and A 1% 1 cm = 2500 and expressed as mg Caro/g fresh material (Britton et al., 1995).

Genetic fidelity evaluation of in vitro grown plants using SCoT markers

In order to verify the genetic homogeneity of high bush vitroplants for each analyzed variety, DNA was extracted from both the mother plant and the vitroplants propagated after the fifteen successive subcultures. Therefore, 6 plantlets from the 15th subculture were randomly selected to harvest the leaves for DNA extraction, before passing the plant material into the acclimatization stage.

The harvested leaves were dried, ground into a fine powder (TissueLyser II, Qiagen, Germany), and kept at 4°C until ScoT genetic analysis was performed.

DNA extraction

Total genomic DNA (g DNA) was extracted using the CTAB (cetyl trimethylammonium bromide) method following the protocol reported by Lodhi et al. (1994) and improved by Pop et al. (2003) and Bodea et al. (2016). DNA purity and concentration were assessed with a NanoDrop-1000 spectrophotometer (ThermoFisher Scientific, USA). Prior to analysis with SCoT markers, DNA samples were diluted to 50 ng μL^{-1} using sterile double distilled water.

ScoT PCR - analysis

Twelve primers were used for SCoT analysis to assess the genetic uniformity of vitroplants with the mother plant as shown in Table 1.

For SCoT analysis, PCR reactions were performed with a total volume of 15 µL: 3 µL g DNA, 5.6 µL distilled H2O for the PCR reactions, 2.5 µL GoTaq Flexi Green buffer (Promega, USA), 2.5 µL MgCl2 (Promega, USA), 0.25 µL dNTP mix (Promega, USA), 1 µL SCoT primer (GeneriBiotech, Czechia), and 0.15 µL of GoTaq polymerase (Promega, USA). The PCR temperature cycling conditions were: (i) initial denaturation at 94°C for 5 min, (ii) 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, and (iii) the final elongation step of 5 min at 72°C.

Table 1. Sequences of SCoT primers used to evaluate the genetic uniformity of *in vitro* plants and mother plants

Primer Code	Primer sequence (5'-3')
SCOT1	CAACAATGGCTACCACCA
SCOT2	CAACAATGGCTACCACCC
SCOT3	CAACAATGGCTACCACCG
SCOT4	CAACAATGGCTACCACCT
SCOT5	CAACAATGGCTACCACGA
SCOT6	CAACAATGGCTACCACGC
SCOT7	CAACAATGGCTACCACGG
SCOT8	CAACAATGGCTACCACGT
SCOT9	CAACAATGGCTACCAGCA
SCoT10	CAACAATGGCTACCAGCC
SCoT11	AAGCAATGGCTACCACCA
SCoT12	ACGACATGGCGACCAACG

Separation of the amplified PCR products was performed by electrophoresis on 1.6% agarose gels (Promega, USA) stained with RedSafeTM Nucleic Acid staining solution (iNtRON Biotech, South Korea) in 1X TAE (Tris-acetate-EDTA buffer), at 100V and 98mA for 2.5-3 hours. DNA fingerprints were visualized in UVP Biospectrum AC Imaging System (UVP BioImaging Systems, Germany).

Data analysis

One-way ANOVA was performed to analyze the data and comparisons between the mean values of treatments were done according to Tukey's HSD test ($p<0.05$). The values shown are means ± S.E. Principal component analysis (PCA) and a dendrogram based on agglomerative hierarchical clustering (AHC) were built using the XLSTAT software. A heat map was drawn based on Pearson's correlation showing the relationships between varieties, *in vitro* growth parameters and photosynthetic pigments with OriginPro 2021 software.

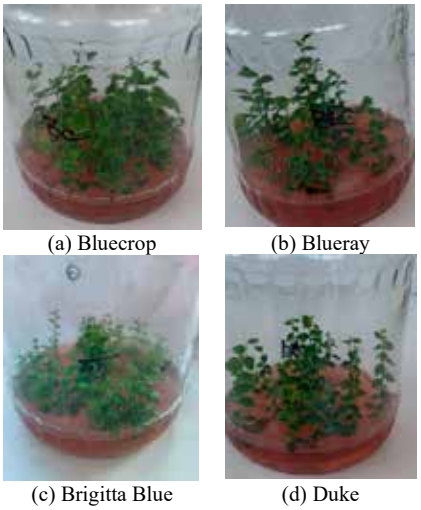
ScoT gel images were analyzed using TotalLab TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the number and the range size of the amplified

bands. Intensity of the bands were not considered while scoring.

RESULTS AND DISCUSSIONS

In vitro culture

The results of this study showed that the four highbush blueberry varieties analyzed reacted differently to long-term *in vitro* cultivation, especially in terms of proliferation rate (Figure 1, Figure 2).



(a) Bluecrop (b) Blueray

(c) Brigitta Blue (d) Duke

Figure 1. *In vitro* culture of *V. corymbosum*: (a) Bluecrop; (b) Blueray; (c) Brigitta Blue; (d) Duke. The *in vitro* shoot cultures of highbush blueberry were established from axillary buds and maintained by regular subculturing after every 10 weeks, for three years, respectively fifteen sub-cultures on Woody Plant Medium (WPM + 100 mg/l Se-questren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod

Thus, the highest proliferation rate was obtained in Brigitta (6.76 ± 0.41) with statistically significantly higher differences than the other varieties. The lowest proliferation rates were obtained in Duke (2.98 ± 0.25) and Blueray (3.02 ± 0.27), but the differences between them were statistically insignificant (Figure 2). Contrary to our findings, Ostrolucká et al. (2004) reported that on the WPM culture medium supplemented with 2 mg/L zeatin, the Duke variety (5.28) had a higher proliferation rate than Bluecrop (3.94) and Blueray (1.71).

Consistent with our results, Sedlaka and Paprstein (2009) reported that the *in vitro* plants proliferation rate of Blueray, Bluecrop and Berkeley varieties grown on different culture media and supplemented with different concentrations of zeatin depends on the variety. Moreover, our results are in agreement with the studies of other authors (Tetsumura et al., 2008; Sedlaka and F. Paprstein, 2009), who showed that the number of newly formed shoots also depends on the variety.

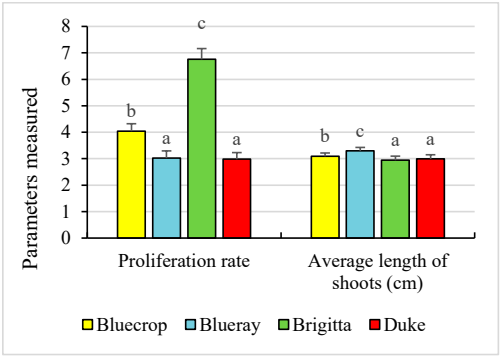


Figure 2. Proliferation rate and average lenght of shoots (Bluecrop, Blueray, Brigitta Blue and Duke) after fifteen *in vitro* sub-cultures on Woody Plant Medium (WPM + 100 mg/l Sequestren 138 + 1mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod

Regarding the length of *in vitro* grown shoots, the longest ones were obtained for the Blueray variety (3.30 ± 0.13 cm) with statistically significantly larger differences compared to the other varieties. The shortest shoots were recorded in the Brigitta (2.94 ± 0.15 cm) and Duke (2.99 ± 0.16 cm) varieties, but the differences between them were statistically insignificant (Figure 2).

In a previous report, Mohamed & Alsadon (2010) stated that plantlets with higher chlorophyll content may have a better chance of survival during the acclimatization stage due to photosynthetic competence and carbon allocation. In this study, the chlorophyll content of the analyzed highbush blueberry varieties was determined due to the fact that photosynthetic pigments are important indicators of the physiological state of the plants. Brigitta was the variety with the lowest content of chlorophylls (Chla; Chlb) and carotenoids (caro). The highest content of photosynthetic pigments was determined in the shoots of the Bluecrop variety (Figure 3).

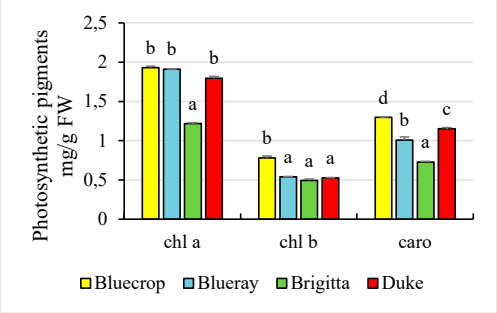


Figure 3. Photosynthetic pigments mg/g FW (Bluecrop, Blueray, Brigitta and Duke) after fifteen *in vitro* sub-cultures on Woody Plant Medium (WPM + 100 mg/l Sequestren 138 + 1mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod

Principal component analysis (PCA) was performed in order to explore the relationships between the *in vitro* growth parameters and the content of photosynthetic pigments of analyzed varieties (Figure 4). The PCA biplot revealed a very clear clustering of the photosynthetic pigments (Chla, Chlb, Caro) in the third and fourth quadrant of the plot compared to the growth parameters: length of shoots and proliferation rate (LS, PR).

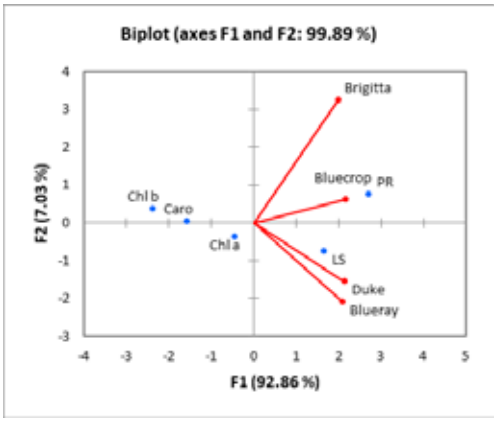


Figure 4. Principal component analysis (PCA) of all variables in four highbush blueberry cultivars (Bluecrop, Blueray, Brigitta and Duke) after fifteen *in vitro* sub-cultures on Woody Plant Medium (WPM + 100 mg/l Sequestren 138 + 1mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod. The tested variables included average length of the shoots (LS), the proliferation rate (PR), chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Caro)

Evaluation of the association of growth parameters with the content of photosynthetic pigments using PCA revealed a total variability of 99.89 % explained by two components (F1 and F2). The first component (X-axis) alone explains 92.86 % of the variance. In addition, as shown in Figure 4, there was a positive correlation between Brigitta and Bluecrop varieties and the proliferation rate.

These varieties were grouped in the first quadrant with the proliferation rate, and our results show that these two varieties had the highest proliferation rates (6.75 and 4.04, respectively).

The agglomerative hierarchical grouping (AHC) using Ward's method highlighted the difference between the four blueberries varieties based on their *in vitro* growth parameters and photosynthetic pigments contents (Figure 5).

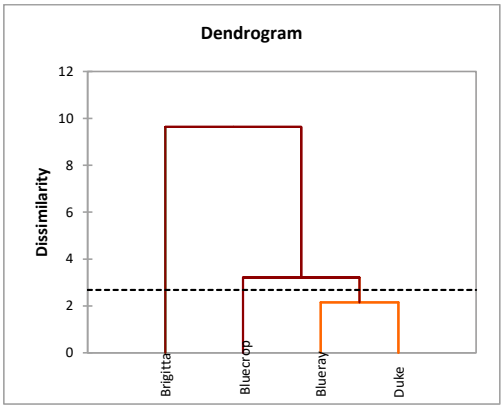


Figure 5. Dendrogram of hierarchical clustering based on *in vitro* growth parameters and photosynthetic pigments of four highbush blueberry cultivars (Bluecrop, Blueray, Brigitta and Duke) after fifteen *in vitro* sub-cultures on Woody Plant Medium (WPM + 100 mg/L Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod

The AHC dendrogram grouped the varieties into three distinct classes (C1, C2 and C3). Class C1 included Bluecrop which had the highest content of photosynthetic pigments, class C2 including Duke and Blueray varieties with the lowest proliferation rates. The class C3 included the Brigitta variety with the highest proliferation rate. Similarly, the built heat map (Figure 6) grouped the four varieties into two main clusters: the first represented by Brigitta with the highest proliferation rate recorded, and the second main cluster which included two subgroups: Duke and Blueray varieties and, respectively, Bluecrop.

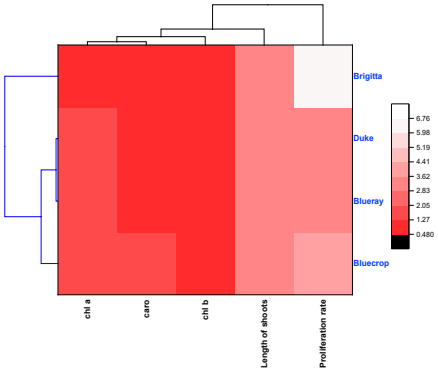


Figure 6. Heat map of Pearson correlation analysis of all variables in four highbush blueberry cultivars (Bluecrop, Blueray, Brigitta and Duke) after fifteen *in vitro* sub-cultures on Woody Plant Medium (WPM + 100 mg/l Sequestren 138 + 1mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5) at 22 ± 1°C, 32.4 mmol·m^{-2}·s^{-1}, 16-h photoperiod

Genetic fidelity assessment using ScoT markers

Assessing the genetic uniformity of plants grown *in vitro* is very important and is an important objective for mass propagation. In this context, various molecular techniques have been successfully used to check genetic stability and lack of somaclonal variation in micropropagated plants (Goto et al., 1998; Tiwari et al., 2013; Butiuc-Keul et al., 2016; Thakur et al., 2016; Sharma et al., 2019; Tikendra et al., 2019).

Therefore, SCoT markers which are reproducible markers that are based on the short conserved region in plant genes surrounding the ATG translation start codon (Collard and Mackill, 2009) have been used in this study.

Table 2 evidence the number and size range of amplified fragments generated by SCoT markers in *Vaccinum corymbosum* L. varieties. As can be seen in Table 2, the lowest number of bands (6) was recorded with SCoT6 and SCoT10 primers for Blueray and Duke varieries and the highest number of bands (18) was obtained with the SCoT12 primer for Brigitta variety. In addition, Brigitta variety showed the highest total number of PCR-amplified bands (133), and the lowest number (120) was recorded for the Blueray variety. However, the size range of PCR bands varied between 250 and 2500 for all four *V. corymbosum* varieties analyzed (Table 2).

To our knowledge, this study is the first report on the application of SCoT markers for the evaluation of genetic fidelity of micropropagated plants of *Vaccinum corymbosum* L. varieties: Bluecrop, Blueray, Brigitta and Duke.

As shown in Figure 7, the results of this study confirm that micropropagated highbush blueberry plants are true-to-type with the mother plants, according to the described protocol, and authenticate that they are not suscetible to somaclonal variation.

In practice, ensuring the genetic uniformity of *in vitro* grown plants at an early stage is a very important economical goal, especially for clonal multiplication and mass production which require a high degree of genetic uniformity between regenerated plants (Clapa & Hârţa, 2021).

Table 2. Number and size range of amplified fragments generated by SCoT markers in *Vaccinum corymbosum* L. varieties

Primer Code	No. of scorable bands				Size range of bands (bp)			
	Bluecrop	Blueray	Brigitta	Duke	Bluecrop	Blueray	Brigitta	Duke
SCOT1	9	10	11	7	600-2000	250-2500	750-2500	500-2000
SCOT2	11	11	13	12	500-2500	500-2000	500-2500	250-2000
SCOT3	10	10	14	11	500-2000	500-2000	500-2000	250-2000
SCOT4	9	9	12	13	250-1800	250-1900	250-2000	250-2500
SCOT5	11	8	11	7	500-2000	500-1800	500-1500	750-1800
SCOT6	9	8	8	6	250-1500	250-1500	500-1500	750-1500
SCOT7	10	11	10	8	250-1500	250-1500	500-1500	250-1500
SCOT8	12	10	12	11	500-2000	500-2000	500-2000	500-2000
SCOT9	11	10	8	10	250-1500	250-1500	250-1500	250-1500
SCoT10	7	6	11	9	500-2000	500-2000	500-2000	500-2000
SCoT11	9	10	13	13	250-2000	250-1800	250-1800	250-2000
SCoT12	15	16	18	14	250-2500	250-2500	250-2500	250-2500
Total bands	123	120	133	121	-	-	-	-

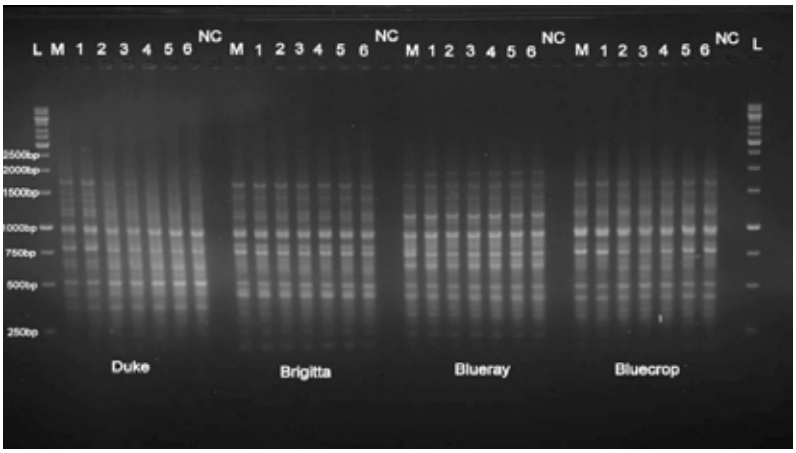


Figure 7. The assessment of genetic uniformity of micropropagated plants with their mother plants by SCoT markers. DNA fingerprinting profile obtained with SCoT-12 primer of *in vitro* plants. Lane L: 1 kb DNA Ladder (Fermentas, Leon-Rot, Germany); Lane M: PCR banding patterns of mother plants for each variety; Lane 1–6: PCR banding patterns of *in vitro* propagated plants for each analyzed variety

CONCLUSIONS

The experiments of this study confirmed that the length of the shoots, the proliferation rate and the photosynthetic pigment contents of the four highbush blueberry varieties grown *in vitro* depend not only on the culture medium but also on the response of the individual genotype. As far as we know, this is the first report to establish the clonal fidelity of micropropagated plants of *V. corymbosum* L. using SCoT markers.

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