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REGENERATIVE CAPACITY OF LEAVES AND STEM SEGMENTS OF SIX GENOTYPES OF *Vaccinium corymbosum* L.

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Abstract

The aim of the present study is to observe the capacity of adventitious organogenesis of leaves and stem segments of six cultivars of highbush blueberry. The effect of WPM cultural medium enriched with 3 mg/l zeatin, 3 mg/l zeatin ribozoid and 2 mg/l 2-iP on the regeneration potential, average and total regeneration rates was assessed. Direct organogenesis is different for individual genetic types of genus Vaccinium. The regenerative response in most of the variants is in favour of the stem segments as explant sources. The highest average number of regenerants was recorded in leaf segments of 'Bluejay' genotype - 5.6 numbers/explant at A_{zr} medium.

Key words*: adventitious organogenesis, in vitro, Vaccinium sp., zeatin, zeatin ribozoid, 2-iP.*

INTRODUCTION

Highbush blueberry belongs to anthocyanin-coloured small berry species that are extremely rich in valuable biologically active substances (Häkkinen et al., 1999; Häkkinen et al., 2000; Puupponen-Pimiä et al., 2002; Hung et al., 2004; Puupponen-Pimiä et al., 2005) necessary for the normal functioning of physiological processes in the human body. It is a significant commercial and biological species (Debnath, 2007).

Blueberry is a relatively new crop for Bulgaria. It finds favourable soil and climate conditions for cultivation in the mountaine and hilly regions of Bulgaria. Traditional propagation methods - through mature and green cuttings are not effective enough to provide a sufficient amount of healthy planting material. The development of modern biotechnological methods for accelerated propagation is of use in this fruit species. Many blueberry cultivars have been studied for their regenerative capacity by direct organogenesis from leaf and stem segments (Rowland and Ogden, 1992; Cao et al., 2000; Ostrolucká et al., 2002; 2009; Cao et al., 2003; Gajdošová et al., 2006).

The purpose of the present experimental work is to develop a system for *in vitro* adventitious shoot regeneration from leaf and stem segments of some highbush blueberry leaves and stem segments using different cytokinins.

MATERIALS AND METHODS

Planting material

The following genotypes were the objective of the present study: 'Bluecrop', 'Bluegold', 'Bluejay', 'Spartan', 'Patriot', and 'Toro', which have scientific and commercial interest. The selected cultivars are fertile, with a different ripening period, which makes them attractive to the market.

Cultural mediums

Stem and leaf segments were isolated from 28 daily intact *in vitro* plants cultivated on WPM cultural medium (McCown and Lloyd, 1981), with half reduced salt concentration, supplemented with 1 mg/l IAA. The leaf explants were taken from the middle layer of the clonal plants, placed horizontally, with their abaxial side to the culture medium. Both types of explants were 5-8 mm in size. The primary explants were cultured in petri dishes, each containing 10 explants and 4-5 ml cultural medium.

Cultural mediums for direct organogenesis of blueberries

In the present scientific experiment, two nutrient variants (abbreviation with A_z or A_{zr}) were used: WPM, enriched with 3 mg/l zeatin and 2 mg/l 2-iP (abbreviation with A_z), in the second case zeatin was replaced with 3 mg/l zeatin ribozoid (abbreviation with A_{zr}), containing 20 g sucrose and 6 g agar. The

acidity of the medium was adjusted to 4.2 before autoclaving.

The following indicators were evaluated to study the regeneration capacity: % regeneration, average and total number of primary explant regenerants. The regeneration response was reflected after 50 days of cultivation without subculturing the explants in fresh cultural medium.

***In vitro* cultivation**

The adventitious regenerants were grown in growth chambers at 22 ± 2°C, a photoperiod of 16/8 day/night and a light intensity of 2000-3000 lx.

Statistical methods of analysis

The data were processed by a variational-statistical method (Lidanski, 1988).

RESULTS AND DISCUSSIONS

The scientific experiment conducted shows that the studied cultivars of highbush blueberry can be successfully propagated by direct organogenesis of leaf and stem segment. Adventitious shoot regeneration depends on the cultivar features, the type of explant, and the type of cytokinin included.

Figures 1 and 2 present the regenerative potential of the tested cultivars of the presented cultural media. All cultivars included in the study successfully manifest their morphogenetic potential. 'Spartan' responded with higher rates of regeneration than other genotypes included in the study. The lowest regenerative capacity *in vitro* was observed in leaf explants with 'Bluecrop' genotype - 10% and 15%. The intensity of adventitious shoot regeneration is different for the different cultivars of *Vaccinium corymbosum* L.

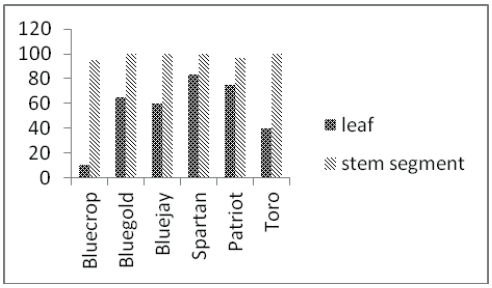

Figure 1. Percent regeneration of leaf and stem segments of different genotypes of highbush blueberries in medium A_z (%)

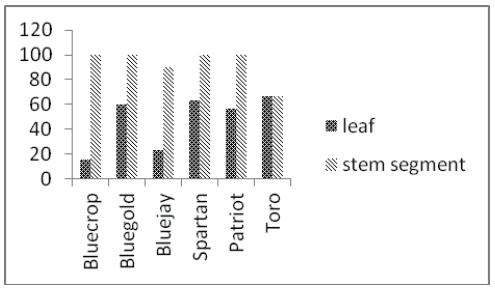

Figure 2. Percent regeneration of leaf and stem segments of different genotypes of highbush blueberries in A_{zr} medium (%)

The plant response to the regeneration rate obtained using the stem segments as explant sources was clearly better than all the variants (regardless of cytokinin used) of the study (except for 'Toro' in medium A_{zr} leaf and stem segments 66.7%) than the leaves. The observed difference was probably due to differences in the morphological structure and physiological state of both explants.

In our previous studies, we found that stem segments of 'Brigita blue' achieved their regenerative capacity *in vitro* - 92.9% in WPM enriched with 4 mg/l zeatin and 5 mg/l 2-iP (Georgieva and Kondakova, 2008).

Successful *in vitro* regeneration is dependent on the choice of the appropriate cytokinin in addition to the cultivar response. Zeatin is an effective cytokinin for the induction of adventitious organogenesis in genus *Vaccinium* and *V. vitis idaea* (Rowland and Ogden, 1992; Gonzales et al., 2000; Debnath and McRae 2002; Ostrolucká et al., 2002; Gajdošová et al., 2006; Meiner et al., 2007; Cappelletti et al., 2016). Its amount in the culture medium is fundamental to the efficiency of the regeneration process in highbush blueberry. Gajdošová et al. (2006) investigated adventitious organogenesis in 5 cultivars of highbush blueberry and reported 39.1 primary explant shoots for 'Brigitta' genotype, enriching the culture medium with 0.5 mg/l zeatin. Authors such as Ostrolucká et al. (2004) proved that the same concentration of zeatin works best for regeneration of highbush blueberry from leaf segments. Meiner et al. (2007) reported that 20 µM zeatin was more effective than TDZ and meta-topolin for induction of adventitious shoots in leaves cut from 'Ozarkblue' cultivar. Other studies have shown

that high concentrations of zeatin provoke callus formation in leaf explants (Shibli and Smith, 1996). In our study, the hormonal combination of 3 mg/l zeatin and 2 mg/l 2-iP in four genotypes ('Bluegold', 'Bluejay', 'Spartan', 'Toro') induced maximum regenerative capacity *in vitro* (100%) - in stem explants (Figure 1). The lowest regeneration capacity was observed in leaf segments of 'Bluecrop' (10%) and 'Toro' (40%) in medium A_z Similar to our experiment, other researchers such as Gonzales et al. (2000); Cappelletti et al. (2016) include 2-iP in the culture medium for blueberry morphogenesis. Zeatin is more effective than 2-iP for adventitious shoot regeneration in highbush blueberries (Rowland and Ogden, 1992; Ostrolucká et al., 2002; Gajdošová et al., 2006).

The stimulating effect of the linked cytokinin zeatin ribosoid on direct leaf organogenesis in the leaves of *Vaccinium* has been demonstrated in the scientific work of Rowland and Ogden, (1992), Cao et al. (2000; 2003). In 'Sunrise' cultivar, experimenters found that zeatin ribosoid increased shoots 2 or 5 times compared to zeatin and 2-iP (Cao et al., 2003).

In our previous studies, 2 mg/l zeatin ribozoid gave better results (96.67% regeneration in leaf explants and 100% regeneration of stem segments) compared to the same amount of zeatin in low bush blueberry (Georgieva, 2013). In the present scientific experiment, the intensity of the regeneration process is in the range of 15% ('Bluecrop') to 66.7% ('Toro') - for leaf explants in A_{zr} cultural medium (Figure 2). Analysis of the research data confirms the stimulating effect of the three types of cytokinins - zeatin and zeatin ribozoid at a concentration of 3 mg/l, combined with 2 mg/l 2-iP on the morphogenesis of *Vaccinium corymbosum*.

The results of our experimental protocol indicate that the average number of explant regenerants in most of the cultivars (regardless of the type of parent explant) is in favor of media enriched with 2-iP and zeatin ribozoid (except 'Spartan' genotype - leaf segments, 'Patriot' - stem segments and 'Toro' leaf and stem segments) (Figures 3a, 3b). The highest average number of explant regenerants was recorded for 'Bluejay' (leaf, A_{zr} medium) - 5.5 numbers/explant. The lowest values of this indicator were again registered in 'Bluecrop' - 1.5 -1.7 numbers/leaf explant. The studies of Cao et al. (2003) are in agreement with our results for the genotypic specificity of this cultivar. Similar to our results, Cao et al. (2003) received 11 shoots/leaf explant with 'Bluecrop', and 'Rowland' and 'Ogden' (1992) registered 20 shoots/leaf explant with 'Sunrise'.

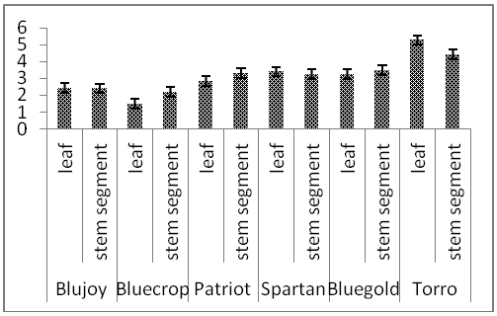

Figure 3a. Average number of regenerants in medium A_z from leaf and stem segments of different genotypes of highbush blueberries

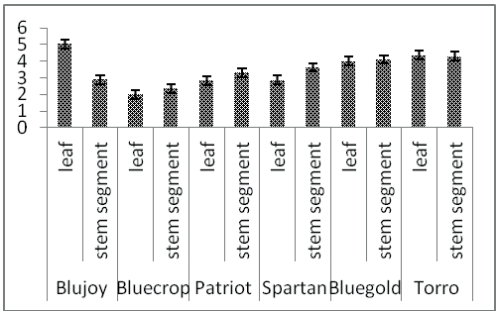

Figure 3b. Average number of regenerants of medium A_{zr} from leaf and stem segments of different genotypes of highbush blueberries

The total number of regenerants from the studied six cultivars of highbush blueberry is in favor of 'Toro' genotype - 128 numbers (stem segments, medium A_z), and 'Spartan' - 109 numbers (stem segment, medium A_{zr}). A higher total number of regenerants was found using the stem segments as explant sources (Figures 4a, 4b). In four cultivars - 'Bluecrop', 'Bluegold', 'Spartan' and 'Patriot', a positive correlation between the highest values of the average and the total number of regenerants was observed (Figures 3a, 3b, 4a, 4b). The included cytokinins had a positive effect on the adventitious organogenesis of the six varieties of *Vaccinium corymbosum* (Figures 5 and 6).

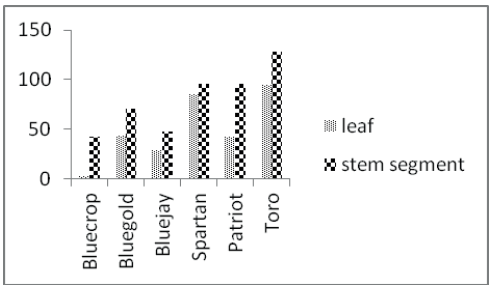

Figure 4a. Total number of regenerants of medium A_z from leaf and stem segments of different genotypes of highbush blueberries

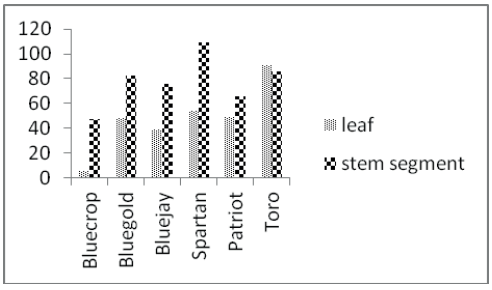

Figure 4b. Total number of regenerants of medium A_{zr} from leaf and stem segments of different genotypes of highbush blueberries

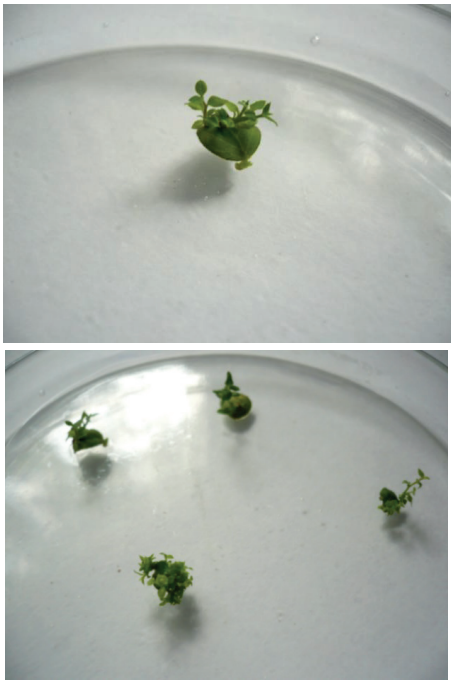

Figure 5. Adventitious shoot regeneration from leaf segment of 'Spartan' cultivar after 50 days cultivation in A_{zr} medium

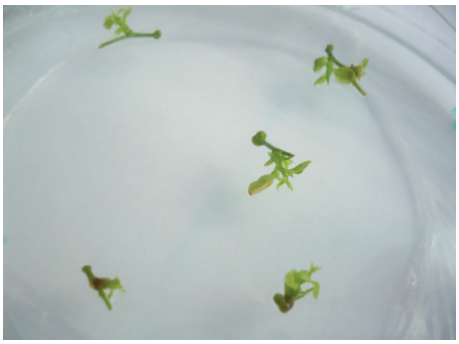

Figure 6. Adventitious shoot regeneration from stem segment in 'Bluecrop' cultivar after 50 days cultivation in medium A_z

Tables 1 to 4 present data from the mathematical processing of the results.
According to the analysis of the variant, there are no statistically proven differences among the cultivars in terms of the regenerative potential of leaf explants of both media (Table 1). With respect to stem explants, cultivar differences in regeneration potential caused by differences in environments were statistically proven (Table 2).
Differences ($P < 0.05$) in the regeneration potential between leaf and stem segments in various genotypes in zeatin media were demonstrated (Table 3). The medium enriched with zeatin ribizoid
(Table 4) is with a tendency ($P < 0.10$) to prove the differences for both types of explants of the genotypes, but without any statistical significance.

Table 1. Statistical analyzes among cultivars, media and explants

A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}
leaf		leaf		leaf		leaf		leaf		Leaf	
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	5.0	1.5	2.0	2.8	2.8	3.4	2.9	3.3	4.0	5.3	4.3
LSD 0.05	n.s										

Table 2. Statistical analyzes among cultivars, media and explants

A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}
stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	2.9	2.2	2.4	3.3	3.3	3.3	3.6	3.5	4.1	4.4	4.3
LSD 0.05	2.27										

Table 3. Statistical analyzes among cultivars, media and explants

A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z
leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	2.4	1.5	2.2	2.8	3.3	3.4	3.3	3.3	3.5	5.3	4.4
LSD 0.05	3.24										

Table 4. Statistical analyzes among cultivars, media and explants

A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}
leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
5.0	2.9	2.0	2.4	2.8	3.3	2.9	3.6	4.0	4.1	4.3	4.3
LSD 0.05	n.s										

CONCLUSIONS

The regenerative response is genotypically dependent. The highest percentage of regeneration was distinguished for 'Spartan' cultivar, irrespective of the type of explant sources and the type of cytokinin used (100% for stem segments, 83% leaf, medium A_z and 63% leaf, medium A_{zr}).

The regeneration potential of some cultivars of highbush blueberry ('Bluecrop', 'Bluegold', 'Bluejay', 'Spartan', 'Patriot' and 'Toro') at *in vitro* level was examined using leaf and stem segments as explants. The plant response favours the use of stem segments as explant sources.

Regarding the choice of cytokinin, a higher rate of regeneration was reported in most variants using zeatin ('Bluegold' - 100%, 'Bluejay' - 100%, 'Spartan' - 100%, 'Toro' - 100% stem segment), compared to zeatin ribozoid. The highest average number of regenerants was distinguished for 'Bluejay' genotypes - 5.6 numbers/explant, leaves of A_{zr} medium and 'Toro' - 5.3 numbers/explants, leaf of medium A_z. The highest total number of regenerants (128 numbers) was found in the stem segments of 'Toro' cultivated on culture medium A_z, followed by the stem segments of the 'Spartan' genotype (109 numbers) obtained on A_{zr} medium.

REFERENCES

Cao, X., Fordham I., Douglass L., Hammerschlag, F. (2003). Sucrose level influences micropropagation and gene delivery into leaves from in vitro propagated highbush blueberry shoots. *Plant cell tissue organ culture*, 75, 255-259.

Cao, X., Hammerschlag, F. A. (2000). Improved shoot organogenesis from leaf explants of highbush blueberry. *Hortscience*, 35, 945-947.

Cappelletti, R., Sabbadini S., Mezzetti, B. (2016). The use of TDZ for the efficient in vitro regeneration and organogenesis of strawberry and blueberry cultivars, *Scientia Horticulturae*, 207, 117-124.

Debnath S. (2007). Strategies to propagate *Vaccinium* nuclear stocks for the Canadian berry industry, *Can J Plant Sci,* 87, 911-922.

Debnath, S. C. & McRae, K. B. (2002). An efficient adventitious shoot regeneration system on excised leaves of micropropagated lingonberry *(Vaccinium*

vitis-idaea L.*). J. Hort. Sci. and Biotech.*, 77, 744-752.

Gajdošová, A., Ostrolucká, M. G., Libiaková, G., Ondrušková, E., Simala. D. (2006). Microclonal propagation of *Vaccinium sp*. and *Rubus* sp. and detection of genetic variability in culture *in vitro*. *J. Fruit and Ornamental Plant Research*, 14(1), 61-76.

Georgieva, M. (2013). Micropropagation of lowbush blueberry (*Vaccinium angustifolium*), *Banat's Journal of biotechnology*, IV, (8), 42-47.

Georgieva, M., & Kondakova, V. (2008). Micropropagation of highbush blueberry /*Vaccinium corymbosum*/, International Scientific Conference- „Sustainable fruit growing: from plant to product", Jurmala-Dobele, 134-140.

Gonzalez, M. V., Lopez, M., Valdes, A. E., Ordas, R. J. (2000). Micropropagation of three berry fruit species using nodal segments from field-grown plants, Annals of Applied Biology, 137(1), 73-78.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., Törrönen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International*, 32, 345-53.

Häkkinen, Sari H., Riitta Törrönen, A. (2000). Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique. *Food Research International*, 33(6), 517-524.

Hung, H.-C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., Colditz, G. A., Rosner, B., Spiegelman, D., Willett, W. C. (2004). Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst.*, 96, 1577-1584.

Lidanski T. (1988). *Statistical methods in biology and in agriculture* (pp. 135-160). Sofia, Bulgaria.

McCown, B. N., Lloyd, G. (1981). Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant species. *HortScience,* 16, 453.

Meiners, J., Schwab, M., Szankowski, I. (2007). Efficient *in vitro* regeneration systems for *Vaccinium* species. *Plant cell, tissue and organ culture,* 89(2-3), 169-176.

Ostrolucká, M. G., Gajdošová, A., Ondrušková, E., Libiaková, G. (2009). *In vitro* propagation of several *Vaccinium corymbosum* L. and *Vaccinium vitis-idaea* L. cultivars, *Latvia-Agronomijas-Vestis* , 12, 75-80.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G. (2002). Influence of zeatin on microclonal propagation of *Vaccinium corymbosum* L. *Propag. ornam. Plants,* 2, 14-18.

Ostrolucká, M. G., Libiaková, G., Ondruková, E., Gajdosová, A. (2004). *In vitro* propagation of *Vaccinium* species. *Acta Universitatis Latviensis: Biology*, 676, 207-212.

Puupponen-Pimiä, R., Aura, A. M., Oksman-Caldentey, K. M., Myllärinen, P., Saarela, M., Mattila-Sandholm, T., Poutanen, K. (2002). Development of functional ingredients for gut health. *Trends in Food Science & Technology,* 13, 3-11.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K., Oksman-Caldentey, K.-M. (2005). Berry phenolics selectively inhibit the growth of intestinal pathogens. *J Appl. Microbiol.,* 98, 991-1000.

Rowland, L. J., Ogden, E. L. (1992). Use of a cytokinin conjugate for efficient shoot regeneration from leaf sections of highbush blueberry. *Hortscience,* 27, 1127-1129.

Shibli, R. A. & Smith, M. A. L. (1996). Direct shoot regeneration from *Vaccinium pahlae* (Othelo) and *V. myrtillus* (bilberry) leaf explants. *Hortscience*, 31, 1225-1228.