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Micropropagation *in vitro* of highbush blueberry (*Vaccinium corymbosum* L.)[1]

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Abstract. Murashige and Skoog medium (MS) and modified Anderson's Rhododendron medium (mAN) were compared for *in vitro* shoot multiplication of three highbush blueberries 'Berkeley', 'Bluecrop' and 'Goldtraube'. All media contained 0.5 mg l^{-1} zeatin applied either alone or combined with 0.1, 1 and 5 mg l^{-1} IBA. *In vitro* rooting was induced using mAN medium supplemented with 0.8 mg l^{-1} IBA and 4 g l^{-1} activated charcoal. The results obtained showed that mAN medium is more suitable for *in vitro* multiplication of the selected highbush blueberry cultivars than MS medium. Low concentration of IBA ($\leq$1 mg l^{-1}) added in zeatin-supplemented mAN medium increases shoot multiplication efficiency of highbush blueberries *in vitro* and can be recommended for large-scale propagation of high-quality plants. MS medium induced partial or full necrosis of stems and leaves, which was more pronounced on media containing zeatin combined with increasing concentration of IBA. Rooting capacity of shoots varied widely among the tested blueberry cultivars. The highest rooting and acclimatization rates were achieved in 'Goldtraube' (82.8% and 91.8% respectively), and the lowest (10% and 66.7% respectively) were in 'Berkeley'.

Keywords: Highbush blueberry, nutritive media, multiplication, rooting, acclimatization

1. Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is most commonly cultivated, commercially important and biologically valuable species of the genus *Vaccinium* L. Fruits contain high level of vitamins, anthocyanins and other bioactive organic substances possessing antioxidant, anti-tumour and anti-inflammatory activities [1]. In recent years, due to the high nutritional value and health benefits of blueberries, there has been an increased interest in their production both among growers and consumers throughout the world. The production of high-quality plants required for the establishment of plantings presupposes the adoption of modern propagation techniques. Although generally successful in blueberries, conventional methods of vegetative propagation by softwood and hardwood cuttings are slow and labour intensive, and the results are variable among genotypes, age of stock plant and growing seasons. Also, traditional propagation methods are not particularly efficient as regards the number of propagules generated and production of healthy, pathogen-free planting material [2]. These limitations can be overcome by the application of *in vitro* techniques. Furthermore, tissue culture-propagated blueberries have greater plant spread (bearing area) and consequently higher yields in comparison with conventionally propagated plants from softwood, single-node cuttings [3]. Debnath [4] argued that enhanced vegetative growth of tissue culture blueberry plants may prove to be

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beneficial to growers through establishing plants for an early fruit production and thus achieving improved return on investment.

In vitro propagation of blueberries through axillary branching system has been investigated over the past three decades. Most of the studies were concerned with the effect of various cytokinins and/or different basal media on shoot multiplication efficiency [5–11]. Systems of adventitious shoot regeneration from leaf explants of blueberry have also been reported for a number of cultivars [2, 12–15]. There were also few reports on application of different auxins in *in vitro* axillary and adventitious shoot development of blueberries [8, 15–18]. However, the results are not broadly applicable, since morphogenic response is highly genotype-dependent on plant growth regulators and media used for culturing [4].

The aim of this investigation was to develop an efficient protocol for micropropagation of different blueberry cultivars so as to ensure sufficient supply of healthy, pathogen-free planting material essential for the establishment and further development of intensive cultivation of blueberry in Serbia. Strictly, studies were conducted to compare two basal nutritive media of different hormonal composition used for axillary shoot multiplication of three highbush blueberry cultivars introduced from Slovakia.

2. Materials and methods

2.1. Plant material

Blueberry cultivars 'Berkeley', 'Bluecrop' and 'Goldtraube' were introduced from Slovakia (Institute of Plant Genetics and Biotechnology SAS, Nitra) to Serbia within the framework of bilateral project titled '*In vitro* regeneration of highbush blueberry *(Vaccinium corymbosum)*, determination of the genetic variability and development of *in vitro* transformation protocols'. Aseptic culture of these cultivars was established in Slovakia according to the procedure previously described by Ostrulucka et al. [14]. Shoots obtained in multiplication stage were brought into the Tissue Culture Laboratory of Fruit Research Institute, Čačak for further experiments.

2.2. Effects of nutrient media and hormonal composition on shoot multiplication

For the multiplication phase, two basal nutrient media, Murashige and Skoog (MS) medium [19] and modified Anderson's Rhododendron medium (mAN) were tested. Anderson's Rhododendron medium – AN medium [20] was modified as follows: 37.3 mg l^{-1} NaEDTA and 27.8 mg l^{-1} $FeSO_4 \times 7\ H_2O$ were used instead of 73.40 mg l^{-1} FeNaEDTA. The media contained 0.5 mg l^{-1} zeatin applied alone or combined with different concentrations (0.1, 1 and 5 mg l^{-1}) of indole-3-butyric acid (IBA) (Table 1). All the media contained 30 g l^{-1} sucrose and 8 g l^{-1} agar, pH value being adjusted to 4.8 before autoclaving. Zeatin was filter sterilised (Millipore filter, 0.22 μm) and added to the media upon autoclaving.

Uniform single shoots excised from the established cultures were used in multiplication experiments. Shoots were subcultured twice on each medium, and multiplication parameters were determined upon second subculture (35 days subculture interval). The multiplication parameters monitored were multiplication index and length of axial and lateral shoots. The multiplication index was defined as number of newly formed shoots (>0.5 cm) per initial shoot tip recorded after the stated subculture interval.

Cultures were maintained in growth chamber at $23 \pm 1^{\circ}$C, under 16-h photoperiod. Light intensity, supplied by cool white fluorescent tubes, was 54 μmol m^{-2} s^{-1}.

2.3. Rooting and acclimatization

Shoots of all the cultivars were rooted on modified AN medium supplemented with 0.8 mg l^{-1} IBA and 4 g l^{-1} activated charcoal. Percentage of the rooted plants as well as the other rooting parameters, such as number and length of roots and height of the rooted plants, were determined after 28 days. Both rooted and non-rooted shoots were removed from culture vessels, washed carefully with water to remove adhering medium, transferred to plastic pots containing sterile soil substrate and acclimatized on a 'mist' bench in greenhouse for two weeks.

Table 1
Effect of basal medium and hormonal composition on multiplication parameters in different blueberry cultivars after 35 days of culturing

Cultivar	Basal medium	Growth regulator combination (mg l^{-1})	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
'Berkeley'	mAN	zeatin (0.5)	2.00 b[1]	2.01 b	0.98 bc
		zeatin (0.5) + IBA (0.1)	2.21 a	2.32 a	1.14 b
		zeatin (0.5) + IBA (1)	1.92 b	2.23 ab	1.61 a
		zeatin (0.5) + IBA (5)	1.00 e	1.75 c	–
	MS	zeatin (0.5)	1.42 c	1.38 d	0.81 c
		zeatin (0.5) + IBA (0.1)	1.22 d	1.33 d	0.85 c
		zeatin (0.5) + IBA (1)	1.09 de	1.51 d	0.70 c
		zeatin (0.5) + IBA (5)	1.00 e	1.46 d	–
'Bluecrop'	mAN	zeatin (0.5)	2.12 b	1.77 b	1.04 ab
		zeatin (0.5) + IBA (0.1)	2.50 a	1.69 b	0.89 bc
		zeatin (0.5) + IBA (1)	1.62 c	2.08 a	1.20 a
		zeatin (0.5) + IBA (5)	1.25 d	1.77 b	0.77 c
	MS	zeatin (0.5)	1.88 bc	1.29 c	0.91 bc
		zeatin (0.5) + IBA (0.1)	1.79 c	1.36 c	0.95 bc
		zeatin (0.5) + IBA (1)	1.26 d	1.46 c	0.83 bc
		zeatin (0.5) + IBA (5)	1.00 e	1.37 c	–
'Goldtraube'	mAN	zeatin (0.5)	2.37 ab	1.77 c	0.96 bc
		zeatin (0.5) + IBA (0.1)	2.21 ab	2.30 a	1.19 ab
		zeatin (0.5) + IBA (1)	2.42 a	2.41 a	1.31 a
		zeatin (0.5) + IBA (5)	1.70 c	2.03 b	1.22 ab
	MS	zeatin (0.5)	2.05 b	1.77 c	1.04 abc
		zeatin (0.5) + IBA (0.1)	1.50 c	1.54 cd	0.88 c
		zeatin (0.5) + IBA (1)	1.00 d	1.50 d	–
		zeatin (0.5) + IBA (5)	1.00 d	1.32 d	–

[1]Mean values of multiplication parameters within each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); mAN – modified Anderson's Rhododendron medium; MS – Murashige and Skoog medium.

2.4. Statistical analysis

In the multiplication experiment, each treatment included 6 culture vessels ×5 uniform shoots ×2 replications. The data were analysed by ANOVA and subsequently by the Duncan's Multiple Range Test for mean separation.

3. Results and discussion

The results obtained showed that mAN medium is more suitable for *in vitro* multiplication of selected highbush blueberry cultivars than MS medium (Table 1). Shoots multiplied on MS exhibited lower multiplication rate and poorer growth than those on the mAN media of the same hormonal composition. Debnath [21] also proved that media with low ionic concentrations are suitable for *Vaccinium* culture. The media most frequently used for highbush blueberry propagation are AN medium [2, 9, 14] and Woody Plant Medium – WPM [7, 8, 22]. The

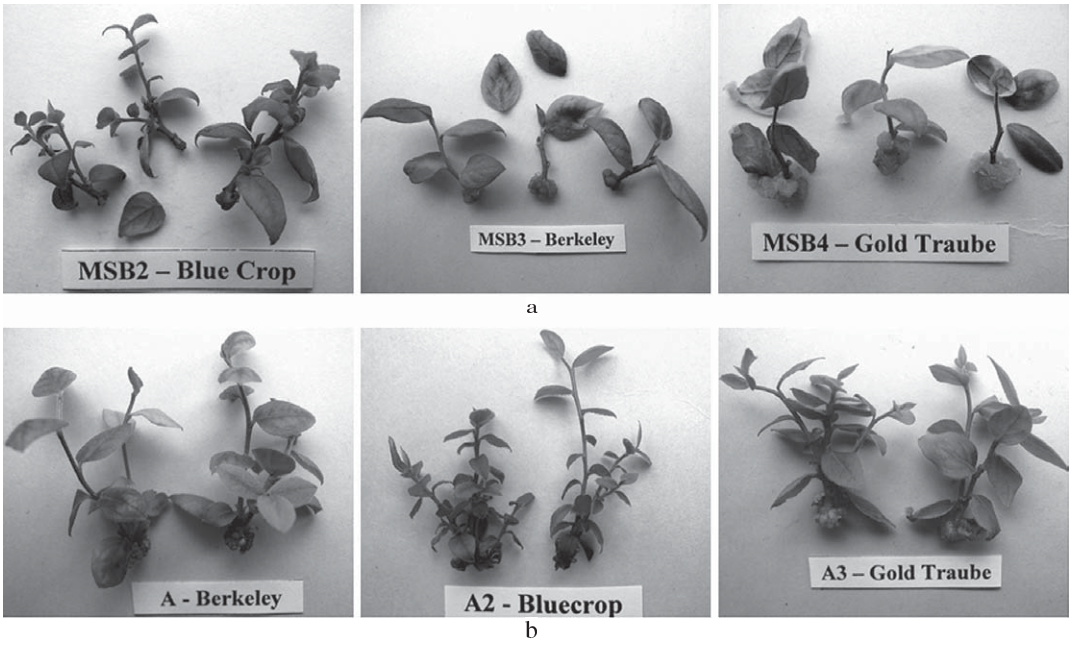

Fig. 1. Shoots of blueberry cultivars in multiplication stage on two basal nutritive media. (a) MS medium containing 0.5 mg l^{-1} zeatin combined with IBA at 0.1 mg l^{-1} (left), 1 mg l^{-1} (middle) and 5 mg l^{-1} (right). (b) Modified AN medium containing 0.5 mg l^{-1} zeatin alone (left) or combined with IBA at 0.1 mg l^{-1} (middle) and 1 mg l^{-1} (right).

latter proved to be more suitable for micropropagation of highbush blueberry cultivars 'Spartan', 'Bluecrop' and 'Berkeley' in comparison with AN medium and half-strength MS medium [23]. In contrast, Tetsumura et al. [10] showed that during the multiplication stage shoots on WPM medium showed poorer growth than those grown either on MS medium or on mixture of equal parts of MS and WPM media. In their experiment, shoots on MS medium grew well but tended towards hyperhydricity, probably due to a high concentration of ammonium ions in this medium [10]. Although no symptoms of hypehydricity were observed in the present experiment, partial or full necrosis of stems and leaves was detected on MS media, and was more pronounced on those containing zeatin combined with increasing concentrations of IBA (Fig. 1a). The highest percentage of necrotic shoots (80%) was noticed in 'Goldtraube' on MS medium containing 1 and 5 mg l^{-1} IBA combined with 0.5 mg l^{-1} zeatin (Fig. 1a). Additionally, higher concentrations of IBA in MS medium completely inhibited shoot multiplication and significantly decreased length of axial and lateral shoots in all the cultivars (Table 1). Viable shoots were yellowish green with appearance of reddish colouring along leaf edges and stems of lateral shoots. Although Tetsumura et al. [10] associated red shoots in blueberry cultivars grown on WPM medium with nitrogen deficiency we observed identical symptoms on shoots grown on MS medium which contains four times as much nitrogen as WPM. Blueberry shoots on mAN in our experiment also showed red colouring but it was less pronounced, especially in 'Goldtraube'.

Modified AN medium was superior in shoot multiplication, elongation and quality of shoots in all the cultivars (Fig. 1b). Shoots multiplied on medium containing zeatin alone were green and had small, firm, light green callus, while adding IBA at 0.1 and 1 mg l^{-1} resulted in well developed, light green shoots which, however, exhibited more pronounced red colouring of leaves and stems. Significant differences in multiplication parameters were also observed among media with different hormonal composition (Table 1). As for 'Berkeley' and 'Bluecrop', the medium containing zeatin combined with 0.1 mg l^{-1} IBA gave higher shoot multiplication rate than the multiplication medium with zeatin alone. Multiplication capacity of 'Goldtraube' on media containing zeatin combined with 0.1 and 1 mg l^{-1} IBA was similar to that obtained on medium with zeatin alone. Also, adding 0.1 and 1 mg l^{-1} IBA significantly increased the length of axial shoots in all the cultivars. The same tendency was observed in length of

Table 2
Rooting parameters in different blueberry cultivars on modified AN medium supplemented with 0.8 mg l^{-1} IBA and 4 g l^{-1} activated charcoal

Cultivar	% of rooting	Average no. of roots per rooted shoot	Average length of roots (cm)	Average height of rooted shoots (cm)
'Berkeley'	10.0 c[1]	1.0 b	1.15 a	2.35 b
'Bluecrop'	39.1 b	1.3 b	1.04 a	2.87 a
'Goldtraube'	81.8 a	2.6 a	1.06 a	2.37 b

[1]Mean values of rooting parameters within each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$). Data presented in the form of percentage were subjected to arcsine transformation.

lateral shoots. IBA supplemented at 1.16 mg l^{-1} significantly enhanced elongation of axillary shoots of highbush blueberry *Vaccinium* x *covilleanum* But. et PI 'Herbert' [17]. On the other hand, further increase in IBA concentration to 5 mg l^{-1} in our experiments significantly decreased shoot multiplication rate in all the cultivars, bringing about the formation of low-quality shoots with chlorotic leaves and huge calli. The occurrence of shoot tip necrosis was also observed.

These results suggest that AN medium containing zeatin at 0.5 mg l^{-1} combined with low concentration of IBA ($\leq$1 mg l^{-1}) can be successfully used in micropropagation of the three highbush blueberry cultivars. Numerous studies have demonstrated that zeatin is an important plant growth regulator for efficient multiplication and growth in *Vaccinium* spp. Zeatin was found to be more effective than other cytokinins (e.g. 2iP) for shoot initiation in eight of twelve *Vaccinium* genotypes [7] as well as for axillary shoot proliferation and adventitious regeneration of highbush blueberry [2] and lingonberry (*V. vitis-idea*) [24]. Although very high concentration of zeatin (10–20 mg l^{-1}) was found most effective for shoot proliferation of highbush blueberry [6], Gajdošova et al. [2] pointed out the effectiveness of zeatin at low concentration (0.5 mg l^{-1}) for inducing multiple shoot formation in meristem cultures of *Vaccinium* spp. Also, low concentrations of zeatin (0.2–1.3 mg l^{-1}) were successfully applied to develop an efficient cranberry (*Vaccinium macrocarpon* Ait.) cloning protocol that enables shoot proliferation and rooting in one step [25]. However, the effect of different auxins on shoot multiplication of *Vaccinium* spp. has been given little attention. Gonzalez et al. [8] found that adding IAA at 0.65 mg l^{-1} into medium supplemented with 2iP did not significantly affect multiplication and elongation of shoots of highbush blueberry 'Berkeley'. On the other hand, the study of Litwinczuk and Wadas [17] revealed that IBA combined with cytokinin can facilitate micropropagation of highbush blueberry 'Herbert' through axillary shoots, as it reduces development of adventitious shoots. However, IBA was found to cause more frequent dying or callusing of explants and to occasionally weaken proliferation of axillary shoots, thus decreasing the efficiency of micropropagation [18], which was confirmed in our experiments only at high concentrations of IBA (5 mg l^{-1}).

In vitro rooting of blueberry has been little described so far. Eccher and Noe [6] used quick dipping treatment of highbush blueberry shoots in IBA solution followed by growth on hormone-free medium. Rooting can also be induced in the shoot proliferation medium without plant growth regulators [10] or in medium containing IBA or NAA [14, 15, 23]. In this study we examined the effect of IBA and active charcoal at constant concentration on induction of rhizogenesis in different cultivars. Rooting capacity of shoots varied greatly among the tested blueberry cultivars (Table 2). The modified AN medium supplemented with 0.8 mg l^{-1} IBA and 4 g l^{-1} activated charcoal was the most effective for root induction in 'Goldtraube'. The rooting rate in this cultivar (81.8%), along with most of the other rooting parameters, was significantly higher than in those obtained for 'Berkeley' and 'Bluecrop', being 10% and 39.1%, respectively. In all the cultivars, IBA promoted development of roots directly from the basal section of shoots, without callus formation (Fig. 2a, b). Sedlak and Paprstein [23] reported similar effect of this auxin on direct root induction, but much higher rooting rates in 'Berkeley' and 'Bluecrop' on WPM medium supplemented with 1 mg l^{-1} IBA.

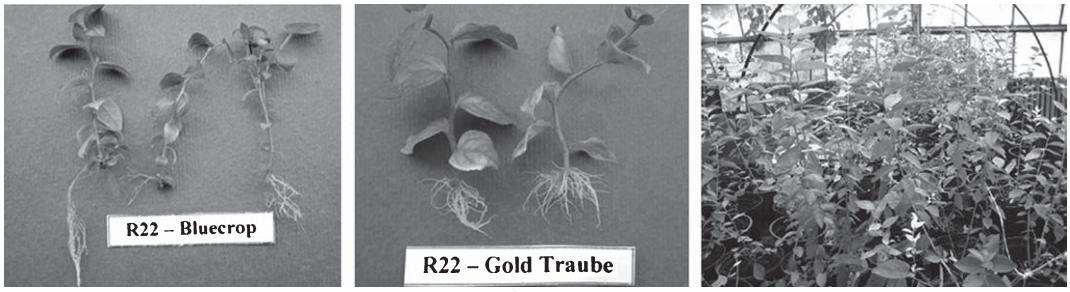

Fig. 2. *In vitro* rooted shoots of 'Bluecrop' (a) and 'Goldtraube' (b). Adapted blueberry plants in greenhouse one year upon acclimatization (c).

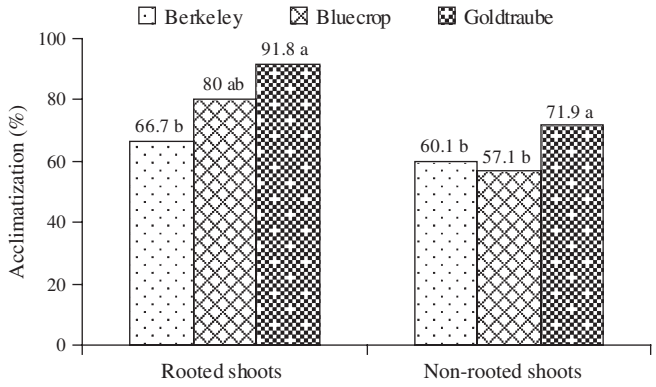

Fig. 3. The comparison of acclimatization rates in both rooted and non-rooted shoots of blueberry cultivars. Values within each category of shoots followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test (data were subjected to arcsine transformation).

The acclimatization was monitored two weeks upon transferring both *in vitro* rooted and non-rooted plants to *ex vitro* conditions (Fig. 2c and 3). As regards rooted shoots, the acclimatization rate was the highest in 'Goldtraube' (91.8%), and the lowest in 'Berkeley' (66.7%). This tendency was observed in acclimatization of non-rooted shots, although the acclimatization rates were considerably lower, especially in 'Bluecrop' and 'Goldtraube'.

4. Conclusions

These studies reveal that nutrient media have critical impact on multiplication and growth of highbush blueberries 'Berkeley', 'Bluecrop' and 'Goldrtaube'. MS medium cannot be used for blueberry micropropagation due to the low multiplication and high incidence of shoot necrosis. Low concentration of IBA (≤ 1 mg l^{-1}) added in zeatin-supplemented AN medium increases shoot multiplication efficiency of highbush blueberries *in vitro* and can be recommended for large-scale propagation of high quality plants. As regards rooting, further research is required to improve *in vitro* rooting potential of 'Berkeley' and 'Bluecrop' shoots.

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