

## Micropropagation of *Vaccinium arctostaphylos* L. via lateral-bud culture

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**Abstract:** The aim of this study was to determine the most suitable growth medium for rapid and efficient micropropagation of *Vaccinium arctostaphylos* L. (Ericaceae) via lateral-bud cultures. According to preliminary studies, the best explant collection time was late April and May. Accordingly, cuttings were taken from naturally growing *V. arctostaphylos* populations, and lateral shoot buds were used as explant sources. Anderson's rhododendron medium (AN), McCown's woody plant medium (WPM), and Murashige and Skoog (MS) basal medium, each supplemented with zeatin/indole-3-butyric acid (IBA) (1.0/0.1 mg L<sup>-1</sup>), were tested to determine the best basal medium for multiple shoot formation; WPM was the most effective basal medium for this purpose. Subsequent shoot multiplication and development studies were carried out with WPM supplemented with an auxin, IBA (0.1 mg L<sup>-1</sup>), and 2 different cytokinins, zeatin and thidiazuron (TDZ), in various concentrations (0.5, 1.0, and 2.0 mg L<sup>-1</sup>, respectively). After a 6-week photoperiod (16/8 h, light/dark) application, zeatin/IBA combinations were found to be the most suitable for shoot multiplication and growth. The highest shoot length (106.53%) was obtained from the medium supplemented with 1.0/0.1 mg L<sup>-1</sup> zeatin/IBA, while the highest increases in leaf number (141.89%) and multiple shoot proliferation (10.55-fold) were obtained from medium supplemented with 2.0/0.1 mg L<sup>-1</sup> zeatin/IBA. WPM was also employed as a rooting medium supplemented with different concentrations of IBA (from 0.5 mg L<sup>-1</sup> to 8.0 mg L<sup>-1</sup>) and a 16/8 photoperiod or a completely dark regime. At this stage, the medium supplemented with 0.5 mg L<sup>-1</sup> IBA was the best rooting growth regulator with 100% rooting success (16/8 photoperiod). Rooted plantlets transplanted into peat and perlite (2:1) substrate were subsequently acclimatized under climate chamber conditions.

**Key words:** Indole-3-butyric acid, shoot-bud culture, thidiazuron, *Vaccinium arctostaphylos*, whortleberry, zeatin

### 1. Introduction

The genus *Vaccinium*, a member of the family Ericaceae, includes about 450 species throughout the world. This genus is represented in Turkish flora by 4 species: *Vaccinium myrtillus* (bilberry), *Vaccinium vitis-idaea* (cowberry or lingonberry), *V. uliginosum*, and *Vaccinium arctostaphylos* (whortleberry). *Vaccinium* species are economically and biologically important plants. Their fruits contain vitamins, antimicrobial and antitumoral substances, and anthocyanin pigments, and are an efficient source of antioxidants. Because of these biological benefits, there is a growing interest in *Vaccinium* species, and many researchers study the commercial cultivation of these species (Ostrolucká et al. 2004; Meiners et al. 2007). Plant tissue culture techniques have recently been employed for the propagation of these species as this approach offers a number of advantages, including a controlled environment, effective clonal propagation, a shortened growth cycle, and production of disease-free plants (Ostrolucká et al. 2004). As far as large-scale propagation of *Vaccinium* species is concerned, this

technique is also suitable for rapid mass production of high quality planting material (Jaakola et al. 2002).

A number of techniques concerning in vitro propagation of *Vaccinium* species have been reported elsewhere, including axillary and adventitious shoot regeneration, isolated meristem culture, and plant regeneration via organogenesis and somatic embryogenesis. For in vitro propagation of various *Vaccinium* species, different growing media and plant growth regulators have been studied (Marcotrigiano et al. 1996; Debnath and McRae 2001). Cytokinins such as N<sup>6</sup>-[2-isopentenyl] adenine (2iP), zeatin [6-(4-hydroxy-3-methylbut-trans-2-enylamino) purine], and thidiazuron (TDZ) and auxins such as indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) have also been reported for shoot proliferation or rooting (Debnath and McRae 2005). Zeatin was found to be more effective for shoot initiation in *Vaccinium* species (Reed and Abdelnour-Esquivel 1991) and shoot proliferation in highbush blueberry (*V. corymbosum* L.) (Éccher and Noe 1989) and lingonberry (*V. vitis-idaea* L.) (Debnath and

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McRae 2001). A very high concentration of zeatin (45–91  $\mu\text{M}$ ) was most effective for shoot proliferation in highbush blueberry (Eccher and Noe 1989). A low concentration of an auxin [5.7  $\mu\text{M}$  indole-3-acetic acid (IAA)] is beneficial when added to the induction medium (Morrison and Smagula 2000). Different concentrations of zeatin (0.5–2.0  $\text{mg L}^{-1}$ ) and indole-3-butyric acid (0.5–8.0  $\text{mg L}^{-1}$ ) have been used in the present study for shoot proliferation and rooting of *V. arctostaphylos* on a single medium, McCown's woody plant medium (WPM).

Nevertheless, as far as our survey of the literature ascertained, no report is available for in vitro propagation of *V. arctostaphylos*. Therefore, the aim of this study was to propagate *V. arctostaphylos* in vitro using lateral buds as an explant, multiply shoots in WPM, and root the plantlets.

## 2. Materials and methods

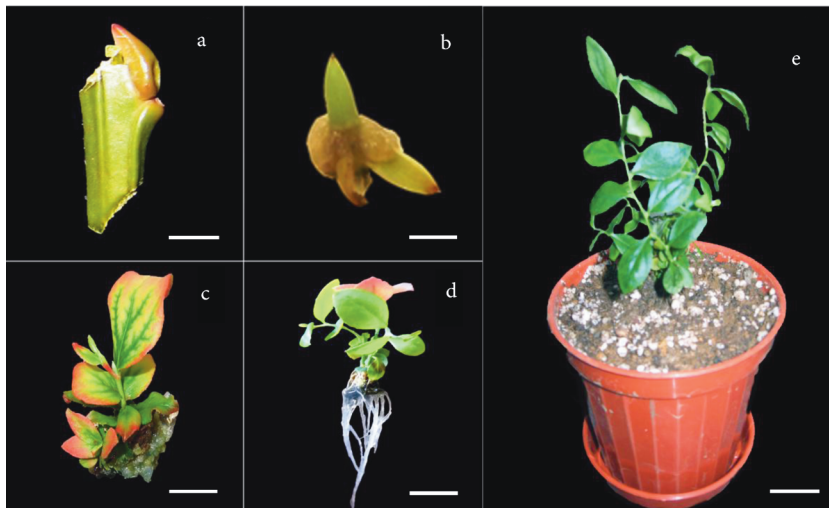
### 2.1. Source of explants

Young, soft, and actively growing shoots were collected from indigenous natural populations of *V. arctostaphylos* plants, from Sis Dağı, Beşikdüzü, Trabzon (40°51'16.45"N, 39°09'44.10"E; 1693 m) in May 2011. Soon after bringing the samples to the laboratory, nodal segments with buds were washed with tap water for 1 h, surface sterilized with 70% ethanol for 1 min, incubated for 15 min in 3% sodium hypochlorite (NaOCl), and washed 3 times for 15 min in sterile distilled deionized water.

### 2.2. Experimental

Lateral buds (Figure 1a) were aseptically dissected (about 5 mm) and cultured on approximately 40 mL of nutrient media in 98.5 × 59 mm glass containers. Initially, Anderson's rhododendron medium (AN), WPM, and Murashige and Skoog basal medium (MS), each supplemented with zeatin/IBA (1.0/0.1  $\text{mg L}^{-1}$ ), were tested to determine the best basal medium for multiple shoot formation; WPM was the most effective basal medium for this purpose.

Basal medium WPM (Duchefa, the Netherlands) (Lloyd and McCown 1980) contains 2% sucrose and 0.8% agar, and is supplemented with cytokinin/cytokinin-like zeatin and TDZ in different concentrations (e.g., 0.5  $\text{mg L}^{-1}$ , 1.0  $\text{mg L}^{-1}$ , and 2.0  $\text{mg L}^{-1}$ ) together with IBA (0.1  $\text{mg L}^{-1}$ ) for shoot regeneration. IBA and  $\alpha$ -naphthaleneacetic acid (NAA) were independently employed for rooting in various concentrations (0.5  $\text{mg L}^{-1}$ , 1.0  $\text{mg L}^{-1}$ , 2.0  $\text{mg L}^{-1}$ , 4.0  $\text{mg L}^{-1}$ , and 8.0  $\text{mg L}^{-1}$ ). Zeatin and TDZ were filter-sterilized and added to the cooling media at 40 °C. The medium pH was adjusted to 5.5 before autoclaving at 121 °C for 15 min. Cultures were maintained in the growth chamber at 24 ± 2 °C, with a photoperiod application (16 h of light and 8 h of dark) and light intensity of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under white fluorescent lamps. The subculturing protocol was carried out every 6 weeks as woody plants gave a better response under this application after 4 weeks, due to their slow growth rate. The regeneration ability of cultures was then evaluated on the basis of mean number



**Figure 1.** Shoot and root formation on lateral bud explants of whortleberry, *V. arctostaphylos*. (a) Lateral bud as an explant of *V. arctostaphylos*. (b) Shoot initiation after 6 weeks on WPM basal medium supplemented with 1.0  $\text{mg L}^{-1}$  zeatin. (c) Shoot proliferation and callus formation at the base of shoot after 6 weeks on culture medium supplemented with 2.0  $\text{mg L}^{-1}$  zeatin. (d) Effect of IBA on rooting of whortleberry from shoot-bud-culture-derived seedlings. Roots were developed in vitro from nodal segments after 8 weeks on 1.0  $\text{mg L}^{-1}$  IBA. (e) Rooted shoots grown in the climate chamber for 4 months. Bar: a = 2.5 mm; b = 5 mm; c = 6.5 mm; d = 12 mm; e = 17 mm.

of shoots per explant, length of shoots emerging from each explant, and mean number of leaves. Isolated microshoots were then rooted. Rooting percentage was also evaluated using the aforementioned medium. Each experiment was performed in triplicate.

### 2.3. Statistical analysis

SPSS 17.0 was used in this study for statistically analyzed data. Analysis of variance (ANOVA) was used to calculate statistical significance, and the means  $\pm$  standard errors (SE) that differed significantly were determined using Duncan's multiple range test at  $P < 0.05$ . At least 9 replications were performed for shoot organogenesis and in vitro rooting.

## 3. Results

### 3.1. Effects of the basal media and zeatin concentrations on initiation of shoot multiplication

In order to determine the best basal medium for multiple shoot formation, AN, WPM, and MS, each supplemented with 1.0/0.1 mg L<sup>-1</sup> zeatin/IBA, were tested as basal media (Table 1). In all tested media, shoots of *V. arctostaphylos* were successfully initiated from lateral buds with 95% survival.

In WPM supplemented with 1.0 mg L<sup>-1</sup> zeatin and 0.1 mg L<sup>-1</sup> IBA, 74% of the explants developed multiple shoots of 14 mm (Figure 1b). Therefore, this medium was chosen for further shoot multiplication. In AN and MS media supplemented with 1.0 mg L<sup>-1</sup> zeatin and 0.1 mg L<sup>-1</sup> IBA, 59% and 54% of the explants developed multiple shoots of 10 mm and 8 mm, respectively (Table 1). Shoots supplemented with these media were much smaller, showed stunted growth, and did not develop further. Based on these results, WPM was the most effective basal medium for shoot multiplication.

### 3.2. Effect of TDZ and zeatin concentrations on shoot multiplication and growth

Various concentrations of zeatin and TDZ (0.5, 1.0, and 2.0 mg L<sup>-1</sup>) were individually tested in the presence of IBA (0.1 mg L<sup>-1</sup>). Shoot cultures of *V. arctostaphylos* were successfully subcultured in all tested media with a 90% viability rate, and mean number of shoots, leaf numbers, and shoot lengths per explant were determined at the end of 6 weeks. The numbers for multiple shoot proliferation were 6.22, 5.11, and 10.55 on media supplemented with 0.5 mg L<sup>-1</sup>, 1.0 mg L<sup>-1</sup>, and 2.0 mg L<sup>-1</sup> zeatin, respectively; in media containing 0.5 mg L<sup>-1</sup>, 1.0 mg L<sup>-1</sup>, and 2.0 mg L<sup>-1</sup> TDZ the numbers were 2.67-, 4.67-, and 3.67-fold, respectively. A statistically significant difference was observed between zeatin and TDZ in terms of shoot proliferation per explant ( $P \leq 0.05$ ). WPM containing 2.0 mg L<sup>-1</sup> zeatin is the best medium for multiple shoot proliferation (Table 2) (Figure 1c). Variability in leaf numbers was observed in the same media containing 0.5 mg L<sup>-1</sup>, 1.0 mg L<sup>-1</sup>, and 2.0 mg L<sup>-1</sup> zeatin and TDZ. Zeatin (2.0 mg L<sup>-1</sup>) gave the best leaf number increase (141.89%) (Table 2). At the end of 6 weeks, multiple shoots of about 20 mm were then subcultured on media supplemented with 0.5 mg L<sup>-1</sup>, 1.0 mg L<sup>-1</sup>, and 2.0 mg L<sup>-1</sup> zeatin or TDZ (in the same concentrations) to determine the growth rate of shoots. Results obtained from the aforementioned media were 44.12%, 106.53%, and 83.10% and 22.83%, 35%, and 38.85%, respectively. The highest shoot length value was achieved on medium with 1.0 mg L<sup>-1</sup> zeatin (Table 2). In this study, zeatin was found to be very effective for shoot multiplication, leaf formation, and the development of shoot length.

**Table 1.** Effects of different basal media supported with cytokinin/auxin combination on initiation culture of *V. arctostaphylos* lateral buds.

		MS		WPM		AN	
PGRs		Rates of shoot (%)	Shoot length (mm)	Rates of shoot (%)	Shoot length (mm)	Rates of shoot (%)	Shoot length (mm)
(mg L <sup>-1</sup> )	Zeatin/IBA (1.0/0.1)	54	8	74	14	59	10

Data were recorded 8 weeks after culture and represent a total of 27 replicates per treatment. PGR: plant growth regulator.

**Table 2.** Effects of zeatin and TDZ on shoot proliferation, shoot length, and leaf numbers in the presence of IBA (0.1 mg L<sup>-1</sup>).

PGRs	PGR concentrations (mg L <sup>-1</sup> )	Starting material			Six weeks after culture		
		Number of shoots per explant	Shoot length (mm)	Leaf numbers per shoot	Number of shoots per explant	Shoot length (mm)	Leaf numbers per shoot
Zeatin/ IBA	0.5/0.1	1.00 a	27.15 b	6.22 c	6.22 b	41.17 c	9.78 c
	1.0/0.1	1.00 a	26.92 b	6.11 c	5.11 bc	55.61 a	12.00 b
	2.0/0.1	1.00 a	26.76 b	6.66 c	10.55 a	49.01 b	16.11 a
TDZ/ IBA	0.5/0.1	1.00 a	26.19 b	6.11 c	2.67 d	32.18 e	8.22 d
	1.0/0.1	1.00 a	27.60 b	6.66 c	4.67 bc	37.26 d	8.33 d
	2.0/0.1	1.00 a	26.56 b	6.88 c	3.67 cd	36.89 d	10.89 bc

Data were recorded 6 weeks after culture and represent a total of 9 replicates per treatment. Different letters in any 2 columns indicate that these 2 means are statistically different at  $P \leq 0.05$  according to Duncan's multiple range test from lateral bud explants of *Vaccinium arctostaphylos*. PGR: plant growth regulator.

### 3.3. Rooting

For rooting, 6-week-old in vitro shoots of 30–40 mm were transferred to WPM (Lloyd and McCown 1980). In Caucasian whortleberry, rooting was by IBA at various concentrations (0.5, 1.0, 2.0, 4.0, and 8.0 mg L<sup>-1</sup>).

Shoots rooted on media supplemented with IBA showed concentration-dependent callus formation. Root initiation did not occur at the shoot base, but often originated from a callus or the leaves that touched the medium surface. At the end of 8 weeks there was a higher rooting percentage with 0.5 mg L<sup>-1</sup> IBA (Figure 1d), at 100% and 66% under photoperiod (16/8) or dark application, respectively (Table 3).

### 3.4. Acclimatization

Rooted plantlets transplanted into a peat and perlite (2:1) substrate were subsequently acclimatized under climate chamber conditions with a special light treatment (50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and regular irrigation once every 15 days. At the beginning (4 weeks) they were kept in containers covered by lids; the lids were later opened. Plantlets of 80–100 mm (after 3 months; Figure 1e) were replanted into bigger containers (120 mm in diameter) and placed in open-air conditions. Transfer of regenerants from in vitro to ex vitro conditions and their acclimatization was successful; almost 80%–90% of transferred plants survived.

**Table 3.** Effects of IBA concentrations and illumination on in vitro rooting of *Vaccinium arctostaphylos* shoots.

IBA concentrations (mg L <sup>-1</sup> )	Photoperiod 16/8)		Dark application	
	Rooting rates (%)	Callus rates (%)	Rooting rates (%)	Callus rates (%)
0.5	100	-	66.6	-
1.0	11.1	44.4	-	33.3
2.0	11.1	-	-	22.2
4.0	11.1	33.3	33.3	11.1
8.0	11.1	-	33.3	11.1

Data were recorded 8 weeks after culture and represent a total of 9 replicates per treatment on WPM.

#### 4. Discussion

Numerous studies regarding blueberry in vitro cultures have been published. In general these studies are aimed at improvement of shoot multiplication efficiency or elaboration of the method of adventitious shoot development. Meiners et al. (2007) studied *V. corymbosum* and *V. vitis-idaea* for initiation cultures and reported that zeatin ( $2.0 \text{ mg L}^{-1}$ ) in combination with IAA ( $1.0 \text{ mg L}^{-1}$ ) significantly enhanced shoot multiplication (72%) in the woody plant medium. Zeatin was also found to be effective for shoot initiation (Debnath and McRae 2001) and proliferation (Debnath and McRae 2001, 2005) of *Vaccinium* species. For initiation cultures, we used IBA ( $0.1 \text{ mg L}^{-1}$ ) instead of IAA with zeatin ( $1.0 \text{ mg L}^{-1}$ ) and enhanced shoot multiplication by 74%. This result supports our study significantly. Several reports concerning in vitro propagation of *Vaccinium* species, with the exception of *V. arctostaphylos*, can be found in the literature. To some extent, previous reports are in accordance with our findings. For instance, Meiners et al. (2007) investigated the effects of zeatin and TDZ together with NAA on shoot multiplication and elongation using nodal segments. The same researchers reported that zeatin ( $2 \text{ mg L}^{-1}$ ) in combination with NAA ( $0.1 \text{ mg L}^{-1}$ ) significantly enhanced shoot multiplication and elongation in *V. corymbosum* and *V. vitis-idaea*. In our study, NAA was replaced by IBA.

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Ostrolucká et al. (2004) reported that AN supplemented with various concentrations of zeatin, 2iP, and TDZ is the best basal medium for shoot proliferation in *V. corymbosum* and *V. vitis-idaea*. However, according to our results, WPM was the best medium for shoot proliferation. Our results are also in agreement with the previous study (Ostrolucká et al. 2004), which showed that zeatin is suitable for the stimulation of shoot multiplication.

There is no report concerning in vitro root formation of *V. arctostaphylos*. However, a number of studies dealing with in vitro rooting of other *Vaccinium* species are available in the literature. For instance, Meiners et al. (2007) used various concentrations and combinations of NAA and IBA for in vitro rooting of *V. corymbosum* and *V. vitis-idaea* shoots and reported that IBA was the most suitable auxin. This result is in accordance with our rooting studies.

Meiners et al. (2007) reported the highest rooting percentage (98%) with  $2.0 \text{ mg L}^{-1}$  IBA. According to these results, lower concentrations of IBA were the most appropriate for rooting.

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