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Micropropagation of *Vaccinium myrtillus* L. (Bilberry) naturally growing in the Turkish flora

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Abstract: This study was designed to micropropagate *Vaccinium myrtillus* L., a naturally growing bilberry, in the Turkish flora. In order to determine the most effective basal medium, lateral buds were initially selected as explants, which were then individually cultured in Murashige and Skoog medium, Anderson's rhododendron medium, and McCown's woody plant medium (WPM) supplemented with 1.0 mg L^{-1} zeatin in combination with either 0.1 mg L^{-1} indole-3-butyric acid (IBA) or 0.1 mg L^{-1} α-naphthalene acetic acid. WPM, supplemented with zeatin and IBA, was found to be the most effective basal medium tested. As shoot regeneration ability highly depends on the medium and cytokinin concentration, WPM basal media containing various concentrations (0.5, 1.0, and 2.0 mg L^{-1}) of 3 different cytokinins, namely zeatin, thidiazuron, and N^6-[2-isopentenyl] adenine, were employed together with IBA (0.1 mg L^{-1}) and were compared with basal media containing none of the growth regulators. In terms of shoot multiplication, 2.0 mg L^{-1} zeatin was found to be superior to the other tested growth regulators when combined with 0.1 mg L^{-1} IBA. WPM was also used as a basal medium for rooting and was supplemented with different concentrations of IBA (0.25–2.0 mg L^{-1}) with or without activated charcoal (AC). WPM supplemented with 0.5/1.0 mg L^{-1} IBA/AC was the best culture medium for rooting with 60% root formation.

Key words: Micropropagation, *Vaccinium myrtillus,* indole-3-butyric acid, N^6-[2-isopentenyl] adenine, thidiazuron, zeatin

1. Introduction

Vaccinium myrtillus L. (Bilberry), a member of the family Ericaceae, is economically the most important wild berry growing in the Turkish flora. Owing to its relatively high anthocyanin content (Stark et al., 1978), its berry fruits can be considered as nutraceuticals (Liu et al., 2010) and good sources of antioxidants (Wang et al., 1997). Therefore, they are considered to have great importance for the prevention of various human degenerative diseases.

Vaccinium species have been studied by diverse research groups, with the research being focused on ecological, physiological, and genetic biotechnological studies (Jaakola et al., 2001). Based on its biological importance, some reports concerning the breeding and domestication purposes of *Vaccinium* species have been published in the literature (Ostrolucká et al., 2004; Meiners et al., 2007).

In terms of plant biotechnology, in vitro approaches have recently been introduced to propagation of the *Vaccinium* species in order to provide several advantages, such as using a controlled environment, supplying effective clonal propagation, shortening the growth cycle, and producing 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).

Several publications concerning in vitro propagation techniques using axillary and adventitious shoot regeneration, isolated meristem culture, and plant regeneration via organogenesis or somatic embryogenesis can also be found in the literature for other *Vaccinium* species (Marcotrigiano et al., 1996; Debnath and McRae, 2001a).

Although many studies have been carried out concerning the physiological effects of secondary metabolites isolated from the berries of *V. myrtillus* in particular, only a few micropropagation studies can be found in the literature (e.g., Jaakola et al., 2001), and micropropagation of native species grown in the Turkish flora has not been studied before. Moreover, environmental conditions, physiological states of the plants, sources of explants, effects of different nutrient media, and concentrations of plant growth regulators (PGRs) have not been studied comprehensively. In the present study, an efficient micropropagation system has been proposed for *V. myrtillus* using lateral bud explants. As emphasized

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elsewhere (Cüce et al., 2013), both basal media and PGRs had dramatic impacts on shoot regeneration of *Vaccinium arctostaphylos*. Hence, it is necessary to investigate this for other economically valuable *Vaccinium* species through more comprehensive and detailed experiments.

2. Materials and methods

2.1. Source of explants

Young, soft, and actively growing shoots with lateral buds were collected from indigenous natural populations of *V. myrtillus* from Zigana Dağı, Maçka, Trabzon (40°40′017″N, 39°24′583″E; 1722 m) in May 2012 and used as source of explants for the present trial. Lateral buds were washed with tap water for 1 h, and then surface sterilized with 70% (v/v) ethanol for 1 min, followed by 15 min of incubation in 3% sodium hypochlorite (NaOCl). Finally, they were washed with sterile distilled deionized water 3 times for 15 min, and cultured on approximately 50 mL of nutrient media in 98.5 × 59 mm glass containers.

2.2. Experimental procedures

For the initial cultures, Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), Anderson's rhododendron medium (AN) (Anderson, 1980), and McCown's woody plant medium (WPM) (Lloyd and McCown, 1980) (Table 1), each supplemented with zeatin/indole-3-butyric acid (IBA) and zeatin/α-naphthalene acetic acid (NAA) (1.0/0.1 mg L^{-1}), were tested to determine the best basal medium for micropropagation.

For shoot regeneration, a WPM basal medium containing 0.1 g L^{-1} myo-inositol, 2% sucrose, and 0.8%

Table 1. Components of the culture media used in the present study.

		WPM	AN	MS
Macroelements (mg L^{-1})	$CaCl_2$	72.50	332.02	332.02
	KH_2PO_4	170.00	–	170.00
	$Ca(NO3)_2.4H_2O$	471.26	–	–
	$MgSO_4$	180.54	180.54	180.54
	NH_4NO_3	400.00	400.00	1650.00
	K_2SO_4	990.00	–	–
	KNO_3	–	480.00	1900.00
	NaH_2PO_4	–	330.60	–
Microelements (mg L^{-1})	$CoCl_2.6H_2O$	–	0.025	0.025
	$CuSO_4.5H_2O$	0.25	0.025	0.025
	FeNaEDTA	36.70	73.40	36.70
	H_3BO_3	6.20	6.20	6.20
	KI	–	0.30	0.83
	$MnSO_4.H_2O$	22.30	16.90	16.90
	$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
	$ZnSO_4.7H_2O$	8.60	8.60	8.60
Vitamins (mg L^{-1})	Glycine	2.00		2.00
	myo-Inositol	100.00	100.00	100.00
	Nicotinic acid	0.50	–	0.50
	Pyridoxine HCl	0.50	–	0.50
	Thiamine HCl	1.00	0.40	1.00
	Adenine sulfate	–	80.00	–

WPM = McCown's woody plant medium, AN = Anderson's rhododendron medium, and MS = Murashige and Skoog medium.

agar was supplemented with 3 PGRs, zeatin, N^6-[2-isopentenyl] adenine (2iP), and thidiazuron (TDZ) in different combinations and concentrations (0.5, 1.0, and 2.0 mg L^{-1}) together with IBA (0.1 mg L^{-1}). IBA with or without activated charcoal (AC) was employed for rooting in various concentrations (0.5, 1.0, and 2.0 mg L^{-1}). All PGRs used in the study were filter-sterilized with 0.22 μm filters and added to the cooled media after autoclaving. The medium pH was adjusted to 5.5 before autoclaving. Cultures were incubated in a growth chamber maintained at 24 ± 2 °C, under a 16/8 h photoperiod with a photosynthetic photon flux density of 50 μmol m^{-2} s^{-1}. The subculturing protocol was carried out every 6–8 weeks. The regeneration ability of the cultures was then evaluated on the basis of mean number of shoots per explant (multiple shoot formation), shoot elongation, and mean number of leaves on each shoot. Isolated microshoots (2–3 cm in length) were then transferred to the rooting medium containing the WPM basal medium supplemented with IBA (0.25, 0.5, and 1.0 mg L^{-1}). Finally, rooted plantlets were rinsed to remove any medium debris before being transferred to glass containers (80 mm in diameter). Rooting percentage was also evaluated via number of rooted microshoots, root length, and number of root tips per explant. Each experiment was performed in triplicate.

2.3. Statistical analysis

Each treatment had 3 replicates of 15 plants for shoot organogenesis and in vitro rooting (3 explants in 5 culture jars). The data collected were shoot length, shoot number, leaf number, root number, and root length. These data were subjected to tests of analysis of variance (ANOVA) with Spearman's correlation. After rejecting the null hypothesis of equal means using the ANOVA F-test, Duncan's multiple range test was used for comparing treatment group means at $P < 0.05$. The statistical software used was SPSS 21.0. Values were means ± standard deviation.

At the end of the 6 weeks, shoot-forming capacity (SFC) (Lambardi et al., 1993) and root-forming capacity (RFC) (Koç et al., 2014) were also calculated as follows:

SFC index = (average number of shoots per regenerating explant) × (% of regenerating explant)/100.

RFC index = (average number of roots per shoot) × (% of multiplying root)/100.

3. Results

According to our preliminary observations, multiplication (shoot proliferation intensity) depends not only on the presence or absence of the growth regulators but the basal medium also has a great impact on micropropagation. Therefore, 3 different basal media, WPM, AN, and MS, each having a potential use in the micropropagation of *Vaccinium* species, were individually tested. In terms of survival percentages of the explants, WPM was superior to the other media tested (MS and AN), at 86.6% versus 76.6% and 80%, respectively. Furthermore, the multiple shoot formation percentage obtained from WPM was significantly higher than those of MS and AN, being 70%, 53.33%, and 60%, respectively, in the presence of zeatin/IBA (1.0/0.1 mg L^{-1}), and 50%, 43.33%, and 46.66%, respectively, in the presence of zeatin/NAA combinations (1.0/0.1 mg L^{-1}) ($P < 0.05$) (Table 2). Based on these findings, WPM can be considered the most effective basal medium for the initial micropropagation steps for *V. myrtillus*.

3.1. The effects of zeatin/IBA and zeatin/NAA concentrations on the initiation of shoot multiplication

The highest shoot elongation (13.01 mm) was determined when the WPM plus the 1.0/0.1 mg L^{-1} zeatin/IBA combination was employed. In case of the AN and MS media containing 1.0 mg L^{-1} zeatin and 0.1 mg L^{-1} IBA, individually, 60% and 53.33% of the explants developed multiple shoots with sizes of 9.77 mm and 7.75 mm, respectively. In the zeatin/NAA application, however, the shoots were much smaller than those of the zeatin/IBA applications. For the WPM, AN, and MS media supplemented with 1.0 mg L^{-1} zeatin and 0.1 mg L^{-1} NAA, the percentages of explants which developed multiple shoots were 50%, 43.3%, and 46%, respectively. The type

Table 2. The effects of different basal media supported with the same cytokinin concentration and 2 different auxin concentrations on the initiation culture of *V. myrtillus* explants.

	WPM		MS		AN	
	Rate of shoots (%)	Shoot length (mm)	Rate of shoots (%)	Shoot length (mm)	Rate of shoots (%)	Shoot length (mm)
1.0/0.1 mg L^{-1} Zeatin/IBA	70 ± 3.33	13.01 a	53.3 ± 5.77	7.75 e	60 ± 3.33	9.77 b
1.0/0.1 mg L^{-1} Zeatin/NAA	50 ± 3.33	9.34 c	43.3 ± 3.84	6.86 f	46.7 ± 3.33	8.03 d

Data were recorded 8 weeks after the culture with a total of 3 replicates of 30 explants per treatment. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

of auxin also influenced the length of shoots produced per explant (Table 2); the length was significantly less ($P < 0.001$) with NAA than with IBA in all media.

3.2. The effect of zeatin, TDZ, and 2iP concentrations on shoot multiplication

Shoot regeneration was successfully established in all media tested with a frequency of 90% viability and number of shoots. Shoot lengths and leaf numbers per explants were determined at the end of 6 weeks of culturing. After the fourth subculture, zeatin (2.0 mg L^{-1}) was found to be more effective than TDZ, 2iP, and the cytokinin-free medium in terms of the aforesaid parameters. The highest shoot multiplication (5.73 shoots per explant) was achieved in the presence of zeatin (2.0 mg L^{-1}). However, the same concentration was less effective than the other cytokinins or the cytokinin-free medium that still produces new shoots (Table 3). A statistically significant difference was observed between the cytokinin-free medium, zeatin, TDZ, and 2iP when shoot multiplication per explant was taken into account ($P \leq 0.05$). WPM containing 2.0 mg L^{-1} zeatin was the best medium for shoot multiplication (Table 3). Furthermore, the SFC index facilitated the average number of shoots per explant and gave general information about the proliferation potential and percent regeneration of the explants. The highest SFC index of 5.73 was for 2.0 mg L^{-1} zeatin and the lowest of 0.68 was for 1.0 and 2.0 mg L^{-1} 2iP (Table 3).

A significant correlation was also established between shoot number and shoot length ($r = +0.534$) in the presence of zeatin. Moreover, remarkable positive correlations can be established between an increasing concentration of zeatin and shoot number ($r = +0.921$) as well as shoot length ($r = +0.585$). On the other hand, the effects of the TDZ applications were opposite, having a significant negative correlation with shoot length ($r = -0.738$) and leaf number ($r = -0.570$). Furthermore, the 2iP applications were negatively correlated with shoot length depending on increasing concentrations ($r = -0.533$) (Table 4). Although zeatin (2.0 mg L^{-1}) gave the best leaf number (25.52%), it was not significantly different from the cytokinin-free medium (24.28%) (Figure 1a).

As a result, it can be said that zeatin is the most effective PGR in terms of shoot multiplication, leaf number, and shoot length (Figure 1a1, 1a2, and 1a3). Although TDZ was less effective than zeatin (Figure 1b, 1b1, 1b2, and 1b3), it exerted a better response than the 2iP applications (Figure 1c, 1c1, 1c2, and 1c3).

3.3. The effects of the combination of IBA and AC on rooting

Multiple shoots were separated and the shoots (2–3 cm in length) were excised individually during subculture. Then each was transferred to the rooting media supplemented with different concentrations of IBA. Rooting ability was successfully progressed concomitantly by increasing IBA concentrations ranging from 0.25 to 1.0 mg L^{-1}. Adding AC to the rooting medium also inhibited callus formation and increased the percentage of root formation. Root initiation did not occur at the shoot base, but often originated from

Table 3. The effects of 3 different cytokinin concentrations on shoot proliferation, shoot length, and leaf numbers.

PGR	PGR conc. (mg L^{-1})	Starting material			Cultured after 6 weeks			
		Shoots/ explant (number)	Shoot length (mm)	Leaves/ shoot (number)	Shoots/ explant (number)	Shoot length (mm)	Leaves/ shoot (number)	Shoot forming capacity (SFC)
Control	–	1.00 a	40.00 b	10.46 c	1.26 e	49.79 bc	13.00 ab	0.32
Zeatin/ IBA	0.5/0.1	1.00 a	39.79 b	10.60 c	3.40 c	49.11 cd	12.60 abc	3.40
	1.0/0.1	1.00 a	39.83 b	10.26 c	4.00 b	50.80 b	12.26 bc	4.00
	2.0/0.1	1.00 a	40.01 b	10.46 c	5.73 a	53.95 a	13.13 a	5.73
TDZ/ IBA	0.5/0.1	1.00 a	39.54 b	10.33 c	3.00 c	47.81 de	12.40 abc	2.8
	1.0/0.1	1.00 a	39.88 b	10.60 c	2.20 d	45.86 fg	12.53 abc	2.05
	2.0/0.1	1.00 a	40.02 b	10.13 c	3.13 c	44.59 g	11.00 d	3.13
2iP/IBA	0.5/0.1	1.00 a	40.75 b	10.60 c	1.73 e	46.93 ef	12.00 c	1.26
	1.0/0.1	1.00 a	40.42 b	10.13 c	1.46 e	45.76 fg	12.00 c	0.68
	2.0/0.1	1.00 a	40.16 b	10.60 c	1.46 e	45.52 fg	12.60 abc	0.68

Data were recorded 6 weeks after the culture with a total of 3 replicates of 15 plants per treatment for shoot regeneration. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Table 4. Spearman's rank correlation coefficient between plant growth regulators calculated for all parameters of *V. myrtillus.*

	Zeatin			TDZ			2iP		
	SN	SL	LN	SN	SL	LN	SN	SL	LN
Zeatin	0.921**	0.585**	–0.001						
TDZ				0.558**	–0.738**	–0.570**			
2iP							0.075	–0.533**	–0.131
SN	1.000	0.534**	0.147	1.000	–0.354**	–0.238	1.000	0.044	–0.139
SL	0.534**	1.000	0.012	–0.354**	1.000	0.503**	0.044	1.000	0.276*
LN	0.147	0.012	1.000	–0.238	0.503**	1.000	–0.139	0.276*	1.000

The significant differences are given. * = $P < 0.05$, ** = $P < 0.01$, SN = shoot number, SL = shoot length, and LN = leaf number.

callus or leaves touching the medium surface. At the end of the 8 weeks, the highest rooting percentage was obtained (60%) in the presence of 0.5/1.0 mg L^{-1} IBA/AC and the highest RFC index of 1.59 was calculated in the same auxin/AC concentration (Figure 1d) (Table 5).

3.4. Acclimatization

Rooted plantlets were transferred to glass containers containing a 2:1 substrate ratio of peat to perlite (v/v) and irrigated on a regular basis for 15 days. At the beginning (4 weeks), the containers were covered with lids and later the lids were opened. After 3 months, the plantlets were transplanted into bigger containers (120 mm in diameter) due to the elongation of roots (65 mm, Figure 1e) and were placed under open-air conditions. Then the survival percentage of the plantlets was estimated as 90%.

4. Discussion

Because of the great variability within the genus *Vaccinium*, some species require further study to optimize micropropagation protocols. The micropropagation studies of *V. myrtillus* are inadequate in the literature. A wide range of basal media and PGRs at varying concentrations have been used by various researchers for micropropagation of different genotypes in *Vaccinium* species. Although a high concentration of cytokinins (e.g., 9.8–19.9 mg L^{-1} zeatin) was found to be the most effective for shoot proliferation (Eccher and Noe, 1989), a low concentration of auxin (0.8 mg L^{-1} IBA) was found to be the most effective for rooting of *Vaccinium* species (Ostrolucká et al., 2004).

In this protocol, in vitro shoot initiation and proliferation of the bilberry were performed on WPM supplemented with different concentrations of cytokinin and in a cytokinin-free medium. The zeatin-containing medium was very effective for shoot induction and multiplication. Cüce et al. (2013) studied *V. arctostaphylos* and reported that WPM supplemented with 1.0 mg L^{-1} zeatin in combination with 0.1 mg L^{-1} IBA significantly enhanced shoot multiplication (74%). When the percentages of shoot formation between *V. arctostaphylos* and *V. myrtillus* were compared, no dramatic difference was observed with the exception that the shoots of the former were significantly longer (Cüce et al., 2013) than the latter (presented here). Although zeatin was also found to be effective for shoot proliferation of lowbush blueberry (Kaldmäe et al., 2006), lingonberry (Debnath and McRae, 2001b), and highbush blueberry (Chandler and Draper, 1986; Eccher and Noe, 1989; Tetsumura et al., 2008), 2iP supplemented with IBA significantly enhanced axillary shoot elongation in highbush blueberry (Litwińczuk and Wadas, 2008). In agreement with this, the highest shoot elongation of the present study (13.01 mm) was observed when WPM plus 1.0/0.1 mg L^{-1} zeatin/IBA combination was employed.

Jaakola et al. (2001) studied the influence of increasing 2iP concentration on spring and autumn explants of *V. myrtillus* and *V. vitis-idaea.* They obtained high initiation of the growth rate for bilberry in a modified MS medium supplemented with 10 mg L^{-1} 2iP during the 5 weeks, but in subsequent culture experiments they observed brownish shoots. Therefore, they used 5 mg L^{-1} 2iP and obtained 44% shoot proliferation. In our study, 3 different cytokinins in various concentrations, each supplemented with 0.1 mg L^{-1} IBA, were used and their influence over shoot multiplication and growth was compared. Cüce et al. (2013) reported on WPM supplemented with various concentrations of zeatin. WPM was found to be the best basal medium for shoot proliferation of *V. arctostaphylos*. Similarly, for the survival percentages of the explants, WPM was superior to the other media tested (MS and AN) at a rate of 86.6% versus 76.6% and 80%, respectively. Our results are also in accordance with a previous study where zeatin was proven to be the most suitable compared to other cytokinins in terms of shoot multiplication (Cüce et al., 2013).

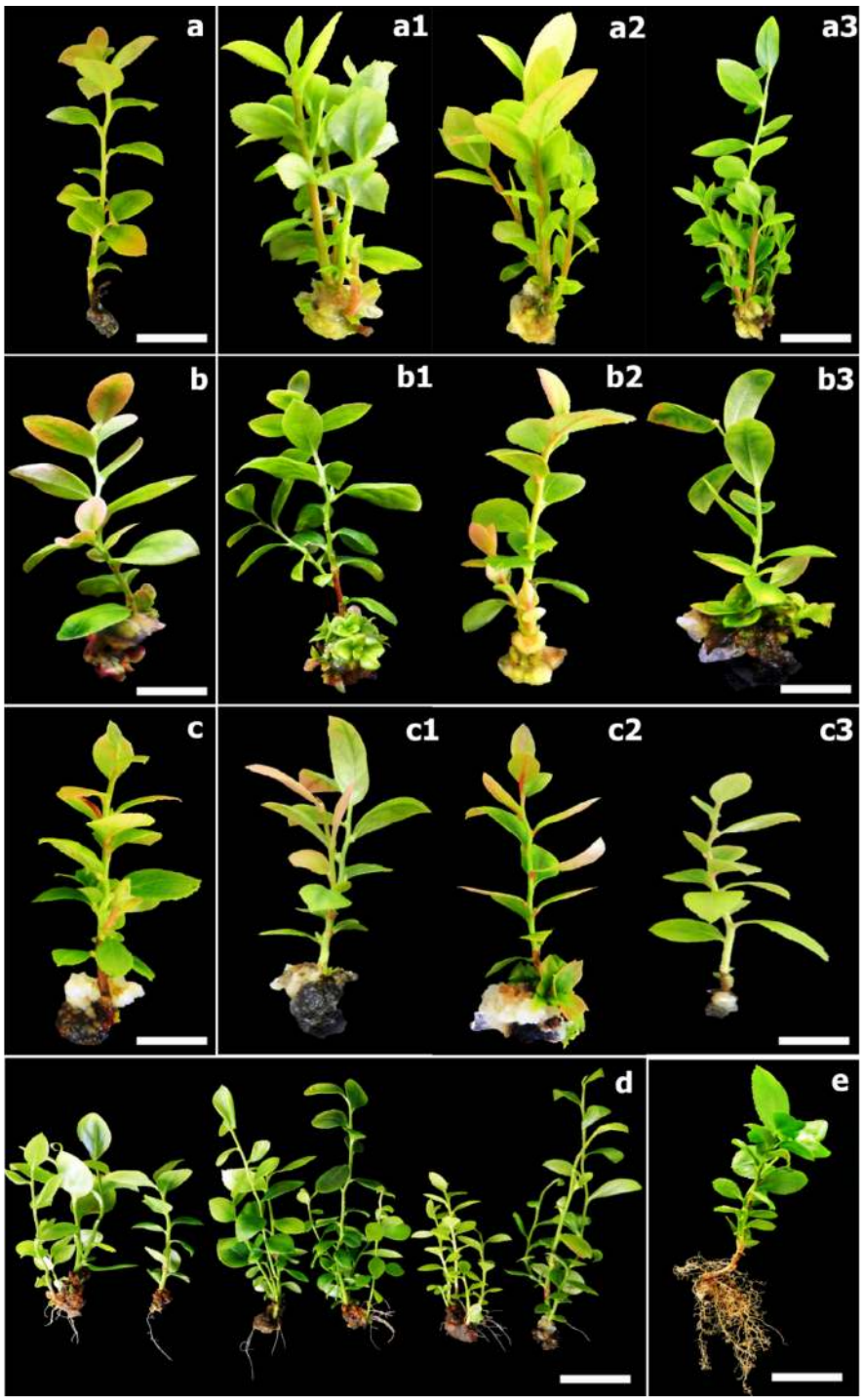

Figure 1. The effect of WPM supplemented with different concentrations of zeatin, TDZ, and 2iP on shoot regeneration from lateral buds explants. Shoot and root formation on lateral bud explants of bilberry, *V. myrtillus.* a, b, and c: Control group of *V. myrtillus*. a1, a2, and a3: Shoot proliferation and callus formation at the base of the shoot after 6 weeks on culture medium supplemented with 0.5, 1.0, and 2.0 mg L^{-1} ^{z}eatin, respectively. b1, b2, and b3: Shoot proliferation and callus formation at the base of the shoot after 6 weeks on culture medium supplemented with 0.5, 1.0, and 2.0 mg L^{-1} ^{TDZ,} respectively. c1, c2, and c3: Shoot proliferation and callus formation at the base of the shoot after 6 weeks on culture medium supplemented with 0.5, 1.0, and 2.0 mg L^{-1} ^{TDZ,} respectively. d: The effect of IBA on rooting of bilberry from shoot-bud culture-derived seedlings. The roots were developed in vitro from nodal segments after 8 weeks on 0.25, 0.5, and 1.0 IBA and 0.25/1.0, 0.5/1.0, and 1.0/1.0 IBA/AC mg L^{-1}. e: Rooted shoots grown in the climate chamber for 3 months. Bar lengths: a = 9 mm; a1, a2, and a3 = 9.5 mm; b = 9 mm; b1, b2, and b3 = 10 mm; c = 9.5 mm; c1, c2, and c3 = 12.77 mm; d = 13.7 mm; and e = 32.5 mm.

Table 5. The effects of IBA concentrations and activated charcoal on in vitro rooting of *Vaccinium myrtillus* shoots.

PGR	PGR Conc.	Rooting rate (%)	Root length (mm)	Root number (no/plant)	Root forming capacity (RFC)	Callus formation (%)
IBA (mg L^{-1})	0.25	33.3 ± 6.7	6.08 d	2.27 b	0.755	53.3 ± 13.3
	0.5	33.3 ± 13.3	9.69 c	1.54 e	0.512	33.3 ± 6.8
	1.0	26.7 ± 6.7	11.95 b	1.77 d	0.472	40 ± 13.3
IBA/AC (mg L^{-1})	0.25	40 ± 6.7	6.43 d	2.15 c	0.86	15.5 ± 10.2
	0.5	60 ± 13.3	14.95 a	2.65 a	1.59	27 ± 6.7
	1.0	13.3 ± 6.8	6.61 d	1.54 e	0.204	–

Data were recorded 8 weeks after the culture and represents a total of 3 replicates of 15 plants per treatment on WPM. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

A few reports are also available in the literature concerning in vitro rooting of *Vaccinium* species. For instance, Ostrolucká et al. (2009) used AN medium supplemented with 0.8 mg L^{-1} IBA and 0.8 mg L^{-1} charcoal for in vitro rooting of *V. corymbosum* and *V. vitis-idaea* shoots and the rooted plantlets were then transferred to a peat substrate, followed by acclimatization under greenhouse conditions. These researchers obtained the highest rooting percentage at 95% and 60% for *V. corymbosum* and *V. vitis-idaea*, respectively, while it reached 60% for the microshoots of *V. myrtillus* in our experiment presented here.

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