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In vitro production protocol of *Vaccinium uliginosum* L. (bog bilberry) growing in the Turkish flora

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Abstract: This novel approach was designed to increase the production capacity of *Vaccinium uliginosum* L. via direct organogenesis. Lateral buds containing one or two leaves were initially cultured in McCown woody plant medium (WPM), Anderson's rhododendron medium, and Murashige and Skoog basal media, each supplemented with zeatin/indole-3-butyric acid (IBA) and zeatin/naphthalene acetic acid (NAA) (1.0/0.1 mg L^{-1}). WPM containing the zeatin/IBA combination was the most effective as the basal medium. Various plant growth regulators (PGRs) were then investigated for the best shoot multiplication, and zeatin was found to be most favorable PGR in all cases. The highest shoot length and shoot number at 40.02 mm and 3.73-fold were obtained from the medium supplemented with 2.0/0.1/0.2 mg L^{-1} zeatin/IBA/gibberellic acid. Rooting capability was also studied by using WPM with IBA, indole-3-acetic acid, and NAA (0.25–1.0 mg L^{-1}) with or without activated charcoal (AC). It was found that 0.5/1.0 mg L^{-1} IBA/AC gave the highest rooting percentage at 18%. Rooted plantlets transplanted into peat:perlite (2:1) substrates were subsequently acclimatized under climate chamber conditions.

Key words: Indole-3-butyric acid, micropropagation, 2iP, *Vaccinium uliginosum*, sucrose, zeatin

1. Introduction

Horticultural plants and in particular wild ones are genetically more diverse and produce a range of products that are of considerable value to humankind. These include fresh and processed products from fruit, vegetables, and grapes, which provide essential food, minerals, phytochemicals, and vitamins that are critical to human nutrition (Benjak et al., 2005; Celik et al., 2007; Canan et al., 2016; Zorenc et al., 2016).

Vaccinium uliginosum L. (bog bilberry), belonging to the family Ericaceae, is a perennial deciduous shrub and an economically important wild plant growing in the boreal regions of Turkey, Europe, North America, and some parts of Asia (Jacquemart, 1996). The genus *Vaccinium* is represented by four species in the Turkish flora, namely *Vaccinium uliginosum* L. (bog bilberry), *Vaccinium arctostaphylos* L. (whortleberry), *Vaccinium myrtillus* L. (bilberry), and *Vaccinium vitis-idaea* L. (cowberry or lingonberry) (Davis, 1978), and their fruits are consumed by local people as dry or fresh fruit, marmalade, jam, compote, etc. *V. uliginosum* fruits contain large amounts of anthocyanin (glucosides, galactosides, and arabinosides of delphinidin, peonidin, petunidin, cyanidin, and malvidin), flavonols (galactosides of myricetin, quercetin, and syringetin; glucosides of quercetin and syringetin; and rhamnosides and arabinosides of quercetin), and flavonoids (Andersen, 1987; Määttä-Riihinen et al., 2004; Masuoka et al., 2007). Owing to the chemical constituents available, *Vaccinium* fruits find themselves in use for medicinal, nutritional, physiological, and genetic purposes (Jaakola et al., 2001), including use as antibacterials (Vučić et al., 2013), antioxidants (Kim et al., 2009), treatments for cardiovascular disease and cancer (Stoner et al., 2008), antiinflammatory and antiviral responses (Beattie et al., 2005), nutraceuticals (Liu et al., 2010), and the treatment of stomach disorders, rheumatic diseases, and bladder and kidney infections (Novelli, 2003). As a result of increasing demand, micropropagation of these species has gained momentum and led to the introduction of new approaches for commercial production (Cüce and Sökmen, 2015; Cappelletti et al., 2016). One of the main reasons for using such alternative production techniques is sexual production, which is disadvantageous for these plants as it causes high heterozygosity (Sedlák and Paprštein, 2009). Secondly, conventional production methods do not have any benefit in terms of shortening the growth cycle, effective

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clonal propagation, controlled environment, or production of disease-free plants as well as rapid mass production of high-quality planting material (Jaakola et al., 2002; Ostrolucká et al., 2004). Last but not least, these methods require intensive and excess labor, use much more time and space, and have deficiencies in root induction (Ostrolucká et al., 2004; Meiners et al. 2007; Ghosh, 2016). However, all aforesaid attempts showed great variations in terms of basal media as well as PGRs, growth conditions, explant types, sampling, physiological condition of the explants, etc. (Ružić et al., 2012). Only a few reports are available in terms of the micropropagation of *V. uliginosum* (Liu et al., 2008; Gu et al., 2009; Zong et al., 2012; Cüce et al., 2016). This present study can be envisaged as the first report on micropropagation of *V. uliginosum* growing in the Turkish flora. The aim of this study is to determine an efficient in vitro production protocol for *V. uliginosum* growing in the Turkish flora by finding out the best basal media and PGRs as well as comparing the micropropagation success that was previously accomplished for other *V. uliginosum* specimens.

2. Materials and methods

2.1. Source of explants

Actively growing young nodal segments were collected from an indigenous natural population of *V. uliginosum* plants from Demirkapı village-Çaykara, Trabzon (40°31′796″N, 40°23′475″E; 2760 m) between May and July. They were washed with tap water for 1 h and then surface-sterilized with 70% (v/v) ethanol for 1 min followed by 10, 15, 20, 25, and 30 min of incubation in 3% sodium hypochlorite (NaOCl) to determine the optimal sterilization time. Explants were washed with sterile deionized water three times for 15 min and cultured on approximately 50 mL of nutrient medium in 98.5 × 59 mm glass containers.

2.2. Experimental

McCown woody plant medium (WPM) (Lloyd and McCown, 1980), Anderson's rhododendron medium (AN) (Anderson, 1984), and Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), each containing zeatin/α-naphthalene acetic acid (NAA) and zeatin/indole-3-butyric acid (IBA) (1.0/0.1 mg L^{-1}), were used to determine the best initial basal medium for multiple shoot formation. For shoot multiplication, WPM basal medium containing 2% sucrose and 0.8% agar was supplemented with different combinations and concentrations of plant growth regulators, i.e. zeatin, N6-[2-isopentenyl] adenine (2iP), and thidiazuron (TDZ) with different concentrations (i.e. 0.5, 1.0, and 2.0 mg L^{-1} together with IBA (0.1 mg L^{-1})) and IBA and indole-3-acetic acid (IAA) with different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg L^{-1} together with zeatin (1.0 mg L^{-1})). Various concentrations of gibberellic acid (GA_3) (0.1, 0.2, 0.3, 0.4, and 0.5 mg L^{-1}) together with 1.0/0.1 mg L^{-1} zeatin/IBA were employed for investigation of their possible effects on shoot multiplication. Different sucrose concentrations (1%, 1.5%, 2%, 2.5%, and 3% g L^{-1}) were individually added to WPM medium supplemented with 1.0/0.1 mg L^{-1} zeatin/IBA to find the most effective sucrose percentage for shoot regeneration. WPM media supplemented with various concentrations (0.25, 0.5, and 1.0 mg L^{-1}) of IBA, IAA, and NAA with or without AC as well as medium without PGRs (control) were used for root induction. All plant growth regulators used in the study were sterilized with 0.22-μm filters and added to the cooled media after autoclaving. The medium pH was adjusted to 5.0 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}. A subculturing protocol was performed every 8 weeks. The regeneration ability of cultures was then evaluated on the basis of mean number of shoots per explant, length of shoots emerged from each explant, and mean number of nodes. Elongated shoots were then rooted and acclimatized in greenhouse conditions. Rooting percentage was also evaluated by number of rooted microshoots, root length, and number of root tips per explant by using the above-mentioned media. Each experiment was repeated in triplicate.

2.3. Statistical analysis

Each treatment included 6 Magenta B-caps (each containing 5 explants) and each was carried out in triplicate for shoot multiplication and root induction. All data were analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). The data collected for mean shoot length, mean number of shoots, mean number of nodes for shoot multiplication, mean number of roots, mean root length, and mean number of secondary roots were analyzed using analysis of variance (ANOVA) with Pearson's correlation. The Student t-test was used for assessing levels of statistical significance between binary comparisons ($P \leq 0.05$). Values are means ± standard deviation. 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 explants)/100.

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

3. Results

3.1. Shoot initiation

Sterilization procedure and agents employed as well as agents' duration are all important in terms of shoot initiation. As the most preferred agent, sodium hypochloride (NaOCl, 3%) was tested for its effectiveness

by using the duration of sterilization. The frequency of contamination varied from 6.6% to 41.11%. The most suitable duration was determined as 15 min with 58.89% survival percentage and 27.77% low contamination rate (Table 1). Determination of the suitable basal medium is a crucial factor for tissue culture studies with appropriate growth regulators. In particular, AN, WPM, and MS have great potential together with different concentrations and combinations of zeatin, 2iP, TDZ, IBA, IAA, and NAA for micropropagation of *Vaccinium* species. In all tested media, better initiation rates (percentage of explants growing new shoots) were obtained from WPM containing 1.0/0.1 mg L^{-1} zeatin/IBA with 63.33%, as well as higher shoot elongation with 13.97 ± 0.64 mm per explant. Compared to NAA, IBA was found to be superior when combined with zeatin (Table 1). AN and MS basal media containing 1.0/0.1 mg L^{-1} zeatin/NAA gave lower initiation rates of 47.77% and 43.33%, respectively. The aforesaid media supplemented with 1.0/0.1 mg L^{-1} zeatin/IBA led to higher necrosis and contamination when compared to WPM, where these parameters were low at 25.55 ± 1.92% and 14.44 ± 1.92%, respectively. As a result, the NAA combination with zeatin caused negative effects and WPM was found to be superior to AN and MS media in terms of shoot initiation (Table 1).

3.2. Shoot multiplication

In terms of sucrose concentration, 2.0% sucrose in WPM significantly increased shoot multiplication with the highest shoot number as well as shoot length with 3.54 ± 0.62 per explant and 32.63 ± 2.10 mm per shoot, respectively (Table 1). Other tested sucrose concentrations were found ineffective for shoot multiplication. Determining the best growth regulator(s) and their combinations (if available) is a prerequisite for micropropagation studies as practical

Table 1. The effects of different basal media and different sterilization times on the initiation culture and different sucrose concentrations on shoot multiplication of *V. uliginosum*.

	1.0/0.1 mg L^{-1} zeatin/IBA			1.0/0.1 mg L^{-1} zeatin/NAA		
	WPM	AN	MS	WPM	AN	MS
Shoot length (mm)	13.97 ± 0.64 a	10.49 ± 0.34 b	9.43 ± 0.51 c	10.37 ± 0.86 b	9.22 ± 0.55 c	8.30 ± 0.43 d
Shooting (%)	63.33 ± 3.33 a	52.22 ± 1.92 bc	47.77 ± 3.85 cd	56.66 ± 3.34 b	47.77 ± 5.09 cd	43.33 ± 3.33 d
Necrosis (%)	25.55 ± 1.92 b	29.99 ± 3.33 ab	31.10 ± 5.09 ab	29.99 ± 3.33 ab	29.99 ± 3.33 ab	34.44 ± 1.92 a
Contamination rate (%)	14.44 ± 1.92 c	17.77 ± 1.93 b	21.11 ± 1.92 a	13.33 ± 0.00 c	21.11 ± 1.92 a	21.00 ± 1.73 a
WPM (1.0/0.1 zeatin/IBA)						
	10 min	15 min	20 min	25 min	30 min	
Shoot length (mm)	13.07 ± 0.40 b	13.90 ± 0.68 a	13.77 ± 0.82 a	13.37 ± 0.62 b	12.51 ± 0.59 c	
Shooting (%)	29.99 ± 3.33 d	58.89 ± 1.93 a	51.11 ± 1.92 b	41.11± 3.85 c	21.11 ± 1.92 e	
Necrosis (%)	28.89 ± 1.93 d	28.89 ± 1.93 d	38.89 ± 1.92 c	51.11 ± 5.09 b	51.11 ± 5.09 b	
Contamination rate (%)	41.11 ± 1.92 a	13.33 ± 3.33 b	9.99 ± 1.33 bc	7.77 ± 1.93 c	6.66 ± 1.34 c	
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 line are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.						
WPM (1.0/0.1 zeatin/IBA)						
Sucrose (%)	Shoot number/ explant	Shoot length (mm)	Node number/ explant			
1.0	2.68 ± 0.41 d	31.03 ± 1.20 c	8.86 ± 0.39 b			
1.5	2.93 b ± 0.48 c	31.94 ± 1.61 abc	10.00 ± 0.50 a			
2.0	3.54 ± 0.62 a	32.63 ± 2.10 a	10.06 ± 0.46 a			
2.5	3.20 ± 0.52 b	32.09 ± 1.97 ab	10.06 ± 0.60 a			
3.0	2.67 ± 0.38 d	31.51 ± 1.71 bc	10.10 ± 0.91 a			

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 $P \leq 0.05$.

and logical preferences reduce cost and labor and save time. Shoot multiplication was successfully achieved in all tested media with a frequency of 100% viability and shoot number except for cytokinin-free WPM and those with 0.5/0.1 mg L^{-1} TDZ/IBA (Table 2). WPM supplemented with zeatin (1.0 mg L^{-1}) and 0.1 mg L^{-1} IBA or zeatin (1.0 mg L^{-1}) and 0.2 mg L^{-1} GA_3 were found to be superior in terms of shoot number and shoot length, while zeatin (2.0 mg L^{-1}) with 0.1 mg L^{-1} IBA was the most favorable for obtaining node numbers (Figures 1a–1c). The highest shoot multiplication (3.73 shoots per explant), shoot length (40.02 mm), and node number (11.97 nodes per explant) were obtained from WPM supplemented with PGRs (Table 2). Although GA_3 augmentation significantly increased shoot multiplication, it caused the development of plantlets with thin, weak, and very long internode length, as well as small leaf diameter. A statistically significant difference was also observed between the GA_3-containing WPM and the others; media with GA_3 are not preferred because of these negative impacts ($P \leq 0.05$). WPM containing 2.0 mg L^{-1} zeatin and 0.2 mg L^{-1} IBA induced more vigorous and strong microshoots than the media with GA_3. Zeatin, 2iP, and TDZ gave shorter shoot length, nearly 22% less than that for zeatin-containing medium. On the other

Table 2. The effects of 3 different cytokinin combinations with different auxin and GA_3 concentrations on shoot proliferation of *V. uliginosum*.

Cytokinin (mg L^{-1})									
Zeatin	2iP	TDZ	IBA	IAA	GA_3	Shoot number/ explant	Shoot length (mm)	Node number/ explant	Shoot-forming capacity (SFC)
0.0	0.0	0.0	0.0	0.0	0.0	1.81 ± 0.50 g	31.89 ± 1.75 i	9.88 ± 0.89 ghi	1.45
0.5			0.1			3.34 ± 0.55 b	33.45 ± 2.03 gh	10.86 ± 1.09 d	3.34
1.0			0.1			3.51 ± 0.59 ab	35.23 ± 1.79 ef	11.00 ± 0.99 cd	3.51
2.0			0.1			3.71 ± 0.43 a	35.75 ± 1.60 cde	11.97 ± 0.76a	3.71
	0.5		0.1			2.86 ±0.45 c	31.47 ± 1.89 i	10.82 ± 0.85 de	2.86
	1.0		0.1			2.39 ± 0.44 e	32.10 ± 2.18 i	10.11 ± 0.79 fg	2.39
	2.0		0.1			2.39 ± 0.37 e	32.44 ± 1.98 hi	10.58 ± 0.97 def	2.39
		0.5	0.1			2.10 ± 0.43 f	28.83 ± 2.35 j	9.49 ± 0.68 ij	2.03
		1.0	0.1			2.41 ± 0.34 e	27.44 ± 1.68 k	10.02 ± 0.74 gh	2.41
		2.0	0.1			3.01 ± 0.46 c	26.92 ± 1.94 kl	10.31 ± 0.82 efg	3.01
1.0			0.1			3.40 ± 0.47 b	35.48 ± 2.14 def	10.93 ± 1.04 cd	3.40
1.0			0.2			3.71 ± 0.45 a	36.98 ± 2.12 b	11.02 ± 1.31 cd	3.71
1.0			0.3			3.47 ± 0.46 ab	34.36 ± 3.29 fg	10.92 ± 1.17 cd	3.47
1.0			0.4			3.39 ± 0.38 b	34.74 ± 1.88 ef	10.68 ± 0.82 de	3.39
1.0			0.5			3.60 ± 0.40 ab	34.87 ± 2.49 ef	11.13 ± 0.85 cd	3.60
1.0				0.1		3.01 ± 0.45 c	26.59 ± 2.25 kl	8.64 ± 0.75 k	3.01
1.0				0.2		2.93 ± 0.41 c	26.08 ± 1.99 l	8.71 ± 0.72 k	2.93
1.0				0.3		2.77 ± 0.53 cd	24.79 ± 2.30 m	8.69 ± 0.70 k	2.77
1.0				0.4		2.82 ± 0.50 c	27.31 ± 2.44 k	9.59 ± 1.26 hi	2.82
1.0				0.5		2.57 ± 0.55 de	26.85 ± 2.40 kl	9.02 ± 0.64 jk	2.57
1.0			0.1		0.1	3.41 ± 0.44 b	36.86 ± 2.01 bc	11.44 ± 1.08 bc	3.41
1.0			0.1		0.2	3.73 ± 0.59 a	40.02 ± 2.14 a	11.82 ± 0.93 b	3.73
1.0			0.1		0.3	3.42 ± 0.59 b	37.33 ± 2.25 b	10.92 ± 0.87 cd	3.42
1.0			0.1		0.4	3.60 ± 0.54 ab	36.54 ± 2.16b cd	11.02 ± 1.01 cd	3.60
1.0			0.1		0.5	3.56 ± 0.41 ab	37.69 ± 1.73 b	11.47 ± 1.07 bc	3.56

Data were recorded 8 weeks after the culture and represent a total of 3 replicates of 30 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 $P \leq 0.05$.

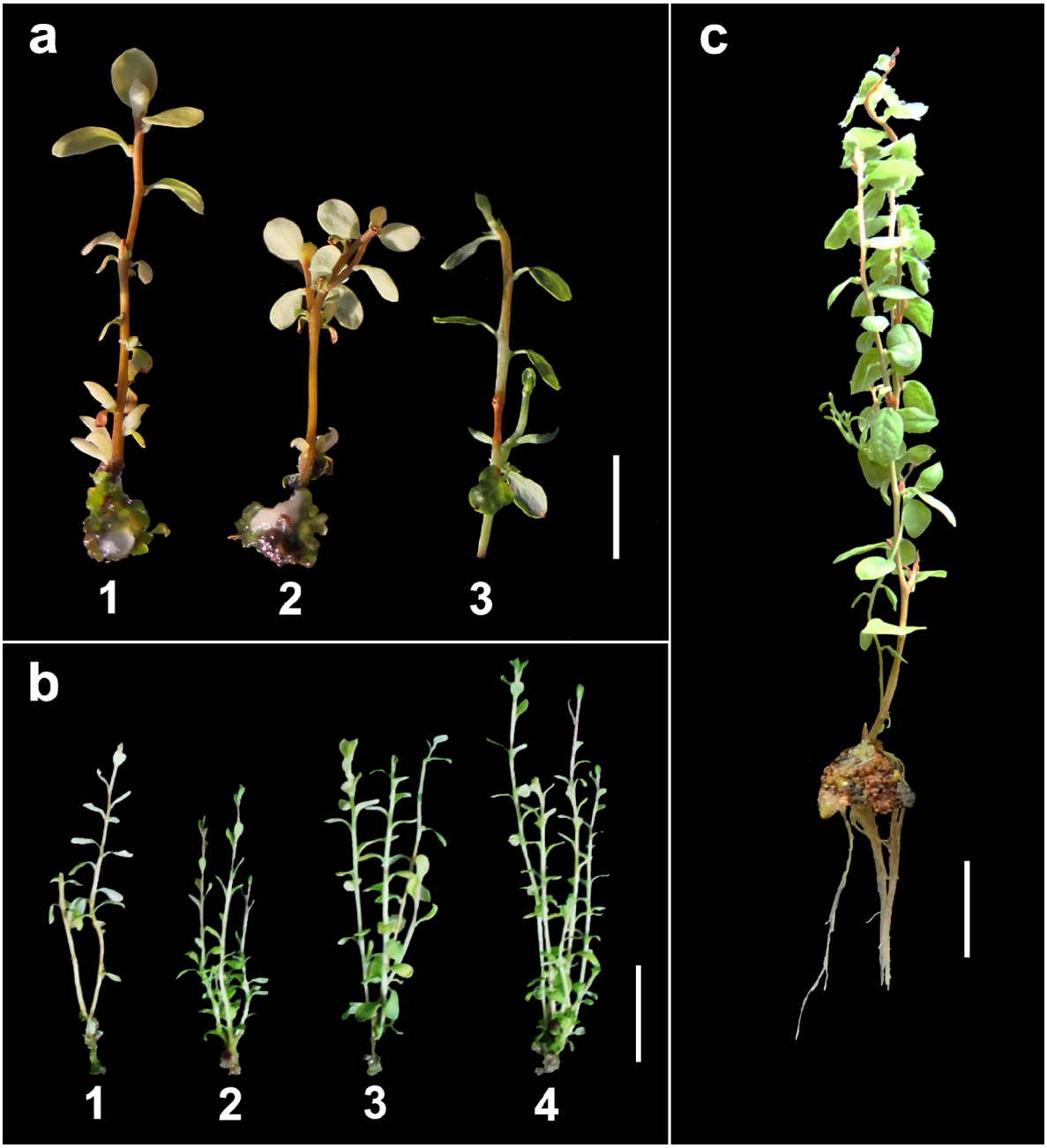

Figure 1. Micropropagation protocol from lateral bud explants on *V. uliginosum*. a) Effect of different basal media supplemented with 1.0/0.1 mg/L zeatin/IBA combination on shoot initiation of *V. uliginosum*. 1 = WPM, 2 = AN, and 3 = MS. b) The effect of WPM on shoot regeneration of *V. uliginosum* from lateral buds explants. 1 = Control, 2 = 2.0/0.1 mg/L TDZ/IBA, 3 = 2.0/0.1 mg/L 2iP/IBA, and 4 = 2.0/0.1 mg/L zeatin/IBA. c) The effect of 0.5 mg L^{-1} IBA and 1.0 g/L AC on rooting of bog bilberry from shoot-bud culture-derived seedlings. Bars: a = 2.7 mm, b = 8.08 mm, c = 5.45 mm.

hand, the SFC index made it possible to calculate the average number of shoots per explant and revealed general information about the multiplication potential and percent regeneration of the explants easily. The highest SFC index was calculated from WPM with zeatin (1.0 mg L^{-1}), 0.1 mg L^{-1} IBA, and 0.2 mg L^{-1} GA_3 at 3.73, while PGR-free free media gave the lowest value of 1.45 (Table 2). A significant positive correlation can be seen between shoot length and node number (r = 0.389) in the presence of zeatin. Furthermore, a remarkable positive correlation was also determined between increasing concentration of zeatin and shoot number (r = 0.283) as well as shoot

length (r = 0.462). On the other hand, TDZ gave rise to a significant negative correlation in terms of shoot length (r = –0.366). Increasing 2iP applications also caused negative effects on shoot number (r = –0.405) (Table 3). As far as the node numbers are concerned, zeatin (2.0 mg L^{-1}) was found to be very effective with 11.97 nodes per explant, exerting a significant statistical difference in comparison to other applications ($P \leq 0.05$). Correlation results show that increasing zeatin and TDZ concentrations elevated the node number at a significant level (Table 3, $P \leq 0.01$). When different auxin concentrations were combined with zeatin (1.0 mg L^{-1}), IBA and IAA treatments exerted negative effects by decreasing the shoot length (r = –0.191) and shoot number (r = –0.279) depending on the increase in concentration, respectively (Table 4). As a result, zeatin in combination with IBA and GA_3 were the most favorable PGRs for shoot number and shoot length, and zeatin with IBA should be preferred as strong and healthy plantlets are required for multiplication and rooting processes.

3.3. Rooting

Various concentrations of IBA, IAA, and NAA (0.25, 0.5, and 1.0 mg L^{-1}), each combined with AC (1.0 g/L) or without AC, were employed for in vitro rooting experiments. Healthy and sufficiently elongated multiple shoots (>3 cm in length) were liberated from each other and subcultured for 2 months, and then each was transferred to WPM media supplemented with different concentrations of IBA, IAA, and NAA with or without AC for in vitro rooting experiments. NAA supplementation did not show a positive effect on rooting in vitro irrespective of the presence or absence of AC (Table 5). IBA (0.5 mg L^{-1}) with 1.0 g L^{-1} AC gave the highest rooting percentage and root length at 18.89% and 12.27 mm (Figures 1a–1c). AC augmentation increased rooting by 29.4%. IBA applications were significantly different from IAA and NAA. The highest root number was obtained from 1.0 mg L^{-1} IBA treatments, whereas the highest secondary root number (3.50 per root) was determined from WPM containing IAA (0.25 mg L^{-1}) and 1.0 g L^{-1} AC. As the most effective auxin, IBA could be preferred at high concentrations (1000 and 2000 mg L^{-1} in particular) for ex vitro rooting. Sufficiently long shoots were dipped in IBA solution at different high concentrations and planted in a 2:1 peat:perlite (v/v) acidic substrate for ex vitro rooting. Higher concentrations of IBA gave statistically meaningful values. Rooting percentage and root length demonstrated similarities between 1000 and 2000 mg L^{-1} IBA treatments, whereas a high root number was obtained from 2000 mg L^{-1} IBA treatments with 2.54 per microshoot. Rooting ability was calculated by means of the RFC index and the highest value of 0.31 was obtained from ex vitro rooting (Table 5).

3.4. Acclimatization

Rooted plantlets were transferred to plastic containers (71 × 71 mm) containing the 2:1 peat:perlite (v/v) acidic substrate under climate room conditions and irrigation on a regular basis for 7 days. At the beginning of the acclimatization period, plastic pots were covered by other

Table 3. Pearson correlation coefficients between different cytokinin sources and concentrations calculated for all shoot multiplication parameters of *V. uliginosum*.

mg L^{-1}			Zeatin			2iP			TDZ		
			SN	SL	NN	SN	SL	NN	SN	SL	NN
	Zeatin	0.283*	0.462*	0.426*							
	2iP				–0.405*	0.196	–0.110				
	TDZ							0.672**	–0.366**	–0.366**	0.413**
0.5	SN	1	0.185	0.136	1	–0.329	0.051	1	–0.047	–0.047	0.050
	SL	0.185	1	0.389*	–0.329	1	0.369*	–0.047	1	1	–0.076
	NN	0.136	0.389*	1	0.051	0.369*	1	0.050	–0.076	–0.076	1
1.0	SN	1	0.215	0.207	1	0.016	–0.007	1	–0.183	–0.183	0.085
	SL	0.215	1	0.433*	0.016	1	0.260	–0.183	1	1	0.098
	NN	0.207	0.433*	1	–0.007	0.260	1	0.085	0.098	0.098	1
2.0	SN	1	0.191	0.017	1	0.095	0.046	1	–0.210	–0.210	0.031
	SL	0.191	1	0.421*	0.095	1	0.083	–0.210	1	1	0.038
	NN	0.017	0.421*	1	0.046	0.083	1	0.031	0.038	0.038	1

Significant differences are given. * = $P < 0.05$. ** = $P < 0.01$. SN = Shoot number. SL = Shoot length. NN = Leaf number.

Table 4. Pearson correlation coefficient between different cytokinin and auxin sources and concentrations calculated for all shoot multiplication parameters of *V. uliginosum*.

mg L^{-1}		IBA			IAA		
		SN	SL	NN	SN	SL	NN
	IBA	0.025	–0.191*	0.007			
	IAA				–0.279**	0.102	0.256**
0.1	SN	1	0.467**	0.451*	1	0.119	0.160
	SL	0.467**	1	0.202	0.119	1	0.036
	NN	0.451*	0.202	1	0.160	0.036	1
0.2	SN	1	0.151	0.069	1	–0.086	–0.226
	SL	0.151	1	0.653**	–0.086	1	0.563**
	NN	0.069	0.653**	1	–0.226	.0563**	1
0.3	SN	1	0.001	0.061	1	0.205	–0.109
	SL	0.001	1	0.730**	0.205	1	–0.227
	NN	0.061	0.730**	1	–0.109	–0.227	1
0.4	SN	1	0.312	–0.086	1	–0.013	–0.029
	SL	0.312	1	0.149	–0.013	1	–0.296
	NN	–0.086	0.149	1	–0.029	–0.296	1
0.5	SN	1	0.262	0.254	1	–0.265	0.310
	SL	0.262	1	0.529**	–0.265	1	0.053
	NN	0.254	0.529**	1	0.310	0.053	1

Significant differences are given. * = $P < 0.05$. ** = $P < 0.01$. SN = Shoot number. SL = Shoot length. NN = Leaf number. IBA = Indole-3-butyric acid. IAA = Indole-3-acetic acid.

transparent plastic pots and were progressively opened until the end of the 8th week. After 3 months, plantlets were transplanted into bigger containers (120 mm in diameter) due to the elongation of roots and placed under open-air conditions. Transfer of plantlets from in vitro to ex vitro conditions and their acclimatization was successful, as almost 60%–70% of transferred plants survived

4. Discussion

Selection of an appropriate methodology is a crucial aspect for the rapid multiplication of species that are difficult to propagate. Shoot multiplication of *V. uliginosum* growing in the Chinese flora was reviewed by Zong et al. (2012), Liu et al. (2008), and Gu et al. (2009). Shoot multiplication capacity may change depending on the original position of the explant from which nodal segments were excised for micropropagation (George, 1993; Debnath and McRae, 2001b). Explants excised from different parts of the plant can show very different multiplication success rates because of the differences between the physiological state of the buds on different regions of the stem, as reported by Debnath and McRae (2001a), as well as the natural growth environmental factors and collection time of the explants. The genus *Vaccinium* has greater variability than other Ericaceae members, and therefore they could need special optimization protocols for micropropagation (Cüce and Sökmen, 2015). As far as our literature survey could ascertain, no comprehensive report is available concerning the comprehensive micropropagation possibilities of *V. uliginosum* growing in the Turkish flora. The findings presented here can be envisaged as the first report in this sense by using explants that were excised from the middle section of young, soft, and actively growing *V. uliginosum* rootstocks. The physiological conditions of the initial culture materials may affect the surface sterilization processes together with the duration allocated for sterilization. NaOCl applications at different concentrations have been reported to be effective on surface sterilization of explants excised from *Vaccinium* plants (Debnath, 2009a; Liu et al., 2010). Although the sterilization agent and time coincided with previous studies (Debnath, 2009a; Han et al., 2013), shorter sterilization time (15 min) was found to be more effective and efficient for *V. uliginosum*. Half- or full-strength WPM, AN, and MS media each individually supplemented with varying concentrations of PGRs have been used for the

Table 5. The effects of different auxin concentrations and activated charcoal on in vitro and ex vitro rooting of *V. uliginosum* microshoots.

	Auxin (mg L^{-1})			Activated charcoal (g L^{-1})			WPM		
	IBA	IAA	NAA	AC	Rooting rate (%)	Root number/ explant	Root length (mm)	Secondary root number/explant	Root-forming capacity (SFC)
In vitro	0.0	0.0	0.0	0.0	-	-	-	-	-
	0.25				11.11 ± 1.92 bc	1.70 ± .07ab	6.09 ± 0.20g	1.88 ± 0.12h	0.19
	0.5				13.33 ± 3.33 b	1.53 ± 0.17c	9.31 ± 0.27d	2.47 ± 0.19e	0.20
	1.0				12.22 ± 1.92 bc	1.75 ± 0.10a	9.55 ± 0.32c	2.17 ± 0.11g	0.21
		0.25			12.22 ± 3.85 bc	1.33 ± 0.15d	8.32 ± 0.45e	3.00 ± 0.32b	0.16
		0.5			-	-	-	-	-
		1.0			-	-	-	-	-
			0.25		-	-	-	-	-
			0.5		-	-	-	-	-
			1.0		-	-	-	-	-
	0.0	0.0	0.0	1.0	-	-	-	-	-
	0.25			1.0	15.55 ± 1.93 ab	1.40 ± 0.10d	5.65 ± 0.12h	2.27 ± 0.16f	0.22
	0.5			1.0	18.89 ± 1.92 a	1.53 ± 0.17c	12.27 ± 0.55a	2.58 ± 0.18d	0.29
	1.0			1.0	7.78 ± 1.92 c	1.67 ± 0.09b	6.68 ± 0.15f	2.89 ± 0.05c	0.13
		0.25		1.0	11.11 ± 1.92 bc	1.58 ± 0.16c	10.63 ± 0.28b	3.50 ± 0.26a	0.18
		0.5		1.0	-	-	-	-	-
		1.0		1.0	-	-	-	-	-
			0.25	1.0	-	-	-	-	-
			0.5	1.0	-	-	-	-	-
			1.0	1.0	-	-	-	-	-
							Peat/perlite		
Ex vitro	0.0	0.0	0.0	0.0	-	-	-	-	-
	500				-	-	-	-	-
	1000				12.22 ± 1.83	1.79 ± 0.08	15.41 ± 0.25	3.54 ± 0.05	0.22
	2000				12.11 ± 1.92	2.54* ± 0.10	15.42 ± 0.13	4.21* ± 0.08	0.31

Data were recorded 8 weeks after the culture and represent a total of 3 replicates of 30 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 $P \leq 0.05$. The Student t-test was used for assessing levels of statistical significance between binary comparisons for ex vitro rooting - = Not detected.

micropropagation of different genotypes of *Vaccinium* species by several researchers (Cappelletti et al., 2016; Scalzo et al., 2016). For the shoot initiation, the aforesaid media with full strength were found to be appropriate and all gave successful results. Since the highest values were obtained from the WPM medium in shoot initiation studies, WPM containing various concentrations of cytokinins, auxins, and GA_3 and PGR-free medium were employed in the shoot multiplication studies for bog bilberry. In several reports, researchers suggested WPM medium for high shoot multiplication success (Liu et al., 2008; Cüce and Sökmen, 2015), while MS was suggested as a very effective medium for several *Vaccinium* species (Gu et al. 2009; Cappelletti et al., 2016). In this report, zeatin-supplemented WPM in combination with IBA was found to be most favorable for shoot induction with 63.33% success and the result was similar to our previous report (Cüce and Sökmen, 2013). In the latter case, the shoot induction rate was found as 74% for *V. myrtillus* when the aforesaid medium was used. Several carbohydrates have been used as carbon and energy sources in plant tissue culture experiments, e.g., sucrose, glucose, fructose, galactose, sorbitol, maltose, and lactose (Lemos and Blake, 1996; Kunitake et al., 1997; Debnath, 2005a; Litwińczuk

and Wadas, 2008), but nonreducible disaccharide, sucrose, is often preferred for micropropagation studies of *Vaccinium* species (Liu et al., 2010; Debnath, 2011b). To the best of our knowledge, the effect of sucrose concentrations on the micropropagation of *V. uliginosum* has not been studied before and therefore determining the precise sucrose concentration is very crucial. WPM containing 2% sucrose has been found operative in many *Vaccinium* species for both shoot formation and propagation. However, our findings presented here are not in agreement with a previous report that suggested that a lower sucrose concentration (1.5%) would be beneficial for higher shoot multiplication in *V. corymbosum* varieties (Cao et al., 2003). This report also recommended that optimum sucrose levels for propagation are genotype-specifics. When compared, no difference was observed between zeatin/BA and zeatin/IBA/GA_3 applications in terms of multiple shoot production, while taller, thin, and weak shoots were produced in the latter case. Therefore, GA_3 applications were concluded as inappropriate for shoot multiplication. This finding is also consistent with a previous report dealing with *V. macrocarpon* (Marcotrigiano and McGlew, 1991). Different plant parts have been used as explants in many studies and in most cases zeatin was more effective in terms of shoot multiplication of bilberry (Cüce and Sökmen, 2015), lowbush blueberry (Kaldmäe et al., 2006), whortleberry (Cüce et al., 2013), and highbush blueberry (Tetsumura et al., 2008). Contrarily, the 2iP/IBA combination was effective for shoot elongation in highbush blueberry (Litwińczuk and Wadas, 2008). Researchers have also reported the effectiveness of low zeatin or high TDZ and 2iP for *V. uliginosum* (Liu et al., 2008; Gu et al., 2009; Zong et al., 2012). They obtained high shoot multiplication success with WPM containing 15 mg L^{-1} 2iP and 0.2 mg L^{-1} NAA or modified MS containing 0.5 mg L^{-1} zeatin and 0.2 mg L^{-1} IBA. Their results are completely different from our findings, implying that variability in results depends on the plant species, explant type and collection time, and the physiological condition of the explants (Ružić et al., 2012). As far as the rooting process was concerned, IBA and IAA concentrations increased the root formation, whereas auxin-free medium and NAA treatments did not give a positive response. Meiners et al. (2007) previously obtained a similar response with NAA treatments for different *Vaccinium* species. Addition of 1.0 g L^{-1} AC also increased the percentage of root formation and 0.5 mg L^{-1} IBA/1.0 g L^{-1} AC gave the highest in vitro rooting success with 18.89%. Ostrolucká et al. (2009) used AN medium with 0.8 mg L^{-1} IBA and 0.8 g L^{-1} AC for in vitro rooting of *V. corymbosum* and *V. vitis-idaea* microshoots and obtained the highest rooting success at 95%, 90%, and 85% for the former case and 60% and 40% for the latter. Similarly, Sedlák and Paprštein (2009) achieved very low rooting success (9%) with a different *V. corymbosum* cultivar. These results suggest that rooting percentage may vary depending on the plant species, growth conditions of the mother plant, and physiological conditions of the explant. Finally, in ex vitro rooting, microshoots of *V. uliginosum* were dipped in high concentrations of IBA and obtained a 12.22% rooting percentage. The final-phase rooted plantlets were transferred to 2:1 peat:perlite substrate and were acclimatized under climate chamber conditions. The results of this study have potential to help optimize shoot propagation systems for other *Vaccinium* species.

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