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Effect of Medium pH on Shoot Regeneration of Three *Vaccinium* Species Naturally Growing in Turkish Flora

Türkiye Florasında Doğal Olarak Yetişen Üç Farklı *Vaccinium* Türünün Sürgün Çoğaltımı Üzerine Besi ortamı pH'sının Etkisi

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Abstract

Medium pH is one of the crucial factors affecting plant cell culture, and generally very little attention has been given to the control of it. The present study was designed to monitor the effects of pH changes on the course of shoot regeneration of *Vaccinium arctostaphylos* L., *Vaccinium myrtillus* L. and *Vaccinium uliginosum* L. Shoot regeneration was assessed by evaluating shoot numbers, shoot length, nod numbers, and callus formation. For this, McCown Woody Plant Medium (WPM) supplemented with 1.0/0.1 mg/L zeatin/indole-3- butyric acid (IBA) was employed as basal media being adjusted with initial pH at 4.0, 4.5, 5.0, 5.5 and 6.0 before autoclaving. When *in vitro* established nodal segments were employed, shoot regeneration was dramatically influenced depending on the species as well as the medium pH. The final pH changes were determined 3.66 and 5.42. The highest shoot number was obtained 5.03 (shoots per explant) at pH 5.5 in *V. arctostaphylos* whereas the highest shoot length (in average) was obtained (40.78 mm) at pH 4.5 pH in *V. myrtillus.* Both media having a pH of 6.0 gave the highest nodes number with 10.83 (nodes/explant) for *V. arctostaphylos* and *V. uliginosum*. In *V. arctostaphylos*, pH 5.5 was the most favorable for shoot number while pH 6.0 was found to be superior to the shoot length and nod number. In *V. myrtillus*, the highest values for all parameters were obtained at pH 4.5. When the pH is fluctuated tending an increase, the shoot multiplication rates significantly decreased in *V. uliginosum.*

Keywords: Micropropagation, pH, *Vaccinium arctostaphylos, V. myrtillus, V. uliginosum,* zeatin

Öz

Besi ortamlarının pH'sı bitki hücre kültürlerini etkileyen en önemli faktörlerden biridir ve genellikle bunun kontrolüne çok az önem verilmiştir. Bu çalışma *V. arctostaphylos*, *V. myrtillus* ve *V. uliginosum*'un sürgün çoğaltımında besi ortamı pH değişimlerinin etkilerini belirlemek amacıyla dizayn edilmiştir. Sürgün çoğaltımı, sürgün sayısı, sürgün boyu, nod sayısı ve kallus oluşum yüzdeleri açısından değerlendirilmiştir. Bunun için pH'sı otoklavdan önce 4.0, 4.5, 5.0, 5.5 ve 6.0'a ayarlanmış ve 1.0/0.1 mg/L zeatin/IBA ile desteklenmiş WPM, temel besi ortamı olarak kullanılmıştır. Nod segmentlerinin eksplant olarak kullanıldığı *in vitro* çalışmalarda sürgün çoğaltımı besi ortamı pH'sının yanı sıra türe bağlı olarak da büyük ölçüde etkilenmiştir. Besi ortamlarının son pH değişimleri 3.66 ve 5.42 olarak belirlenmiştir. En yüksek sürgün sayısı eksplant başına 5.03 ile 5.5 pH içeren besi ortamdaki *V. arctostaphylos*'tan, en yüksek sürgün boyu ise 40.78 mm ile 4.5 pH içeren besi ortamdaki *V. myrtillus*'tan elde edilmiştir. *V. arctostaphylos* ve *V. uliginosum* için en yüksek nod sayısını sürgün başına 10.83 ile pH'sı 6.0 olan ortam vermiştir. *V. arctostaphylos*'da en yüksek sürgün boyu ve en yüksek nod sayısı açısından 6.0 pH daha üstün bulunurken, en yüksek sürgün sayısı için 5.5 pH daha uygun olmuştur. *V. myrtillus*'da tüm parametrelerde en yüksek değerler 4.5 pH içeren ortamdan elde edilmiştir. pH değerleri bir artış eğiliminde olduğunda *V. uliginosum*'da sürgün çoğaltım oranları önemli bir şekilde azalmıştır.

Anahtar Kelimeler: Mikroçoğaltım, pH, *Vaccinium arctostaphylos*, *V. myrtillus*, *V. uliginosum*, zeatin

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1. Introduction

Clonal propagation methods have gained a tremendous momentum in recent years (Henry et al. 1992; Gomez and Segura 1995) and modified microprogation approaches were introduced to the literature for rapid and efficient propagation for the economically important plant species as they serve as the controlled environment, effective clonal propagation, shortening the growth cycle and production of disease-free plants (Ostrolucká et al. 2004) *Vaccinium arctostaphylos* (whortleberry), *Vaccinium myrtillus* L. (bilberry), and *Vaccinium uliginosum* (bog bilberry or northern bilberry) are economically important wild berries in Turkish flora (Cüce et al. 2013). The fruits of these plants are used in food industry, cosmetic, and even in many fields including medicine because of the high anthocyanin content (Stark et al. 1978) and high antioxidant activities (Zheng and Wang 2003). There is a growing interest in *Vaccinium* species due to these biological profit and commercial production attracts the attention of many researchers in recent years (Ostrolucká et al. 2004; Meiners et al. 2007; Cüce et al. 2013; Cüce and Sökmen 2015).

Vaccinium species are typically found in locations with low pH (4.5 - 5.5) and moist soils containing high organic materials (Coville 1910; Harmer 1944; Cain 1952; Galletta 1975; Korcak et al. 1982). pH is the most significant factor for growing the these plants in natural environment as well as controlled culture conditions. For the latter case, it is well-known fact that the medium pH is one of the crucial factor affecting plant tissue and cell cultures, although little attention has been given to the control of it. If the medium pH is not suitable, some abnormalities occur in culture condition (Gürel and Gülsen 1998; Laukkanen et al. 2000), showing a decline in the shoot length, insufficient leaves growth and less shoot number yields. In terms of *in vitro* micropropagation of several *Vaccinium* species, different medium pH was adjusted and accordingly given in the literature. pH 5.0 was introduced to *Vaccinium vitis-idaea* (Debnath 2005; Ostrolucká 2010) tissue cultures, whilst pH 5.2 and 5.5 were applied to those of *Vaccinium corymbosum* (Liu et al. 2010), and *V. arctostaphylos* (Cüce et al. 2013) respectively. As mentioned above, no adequate and comprehensive study was conducted to show importance of pH range; here we present a model study to develop a protocol with suitable pH monitoring that could be valuable for the *in vitro* propagation of indigenous three *Vaccinium* species, selected as model plants.

2. Materials and Methods

2.1. Source of Explants

Nodal segments with buds collected from indigenous natural population of *V. arctostaphylos, V. myrtillus* and *V. uliginosum* plants were washed with top water for 1 h, then surface sterilized with 70% (v/v) ethanol for 1 min followed by 15 min incubation in 3% sodium hypochlorite (NaOCl). Lateral buds were washed with sterile distilled deionized water three times for 15 min, and cultured on approximately 50 ml nutrient media in 98.5 × 59 mm glass containers.

2.2. Experimental

For shoot multiplication, Woody plant medium (WPM**)** (Lloyd and McCown 1980) containing 0.1 g/L myo-inositol, 2% sucrose, 0.8% agar was supplemented with 1.0 mg/L zeatin and 0.1 mg/L IBA. 1N NaOH were used for preparing stock solution of both zeatin and IBA. 20 mg PGR was solved enough 1N NAOH and then completed to 20 mL with pure water. Plant growth regulators (PGRs) were filter-sterilized using 0.22 µm filters and added to the cooled media after autoclaving. The pH levels of media were adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 before autoclaving. After autoclaving, pH levels of media were measured again before IBA and zeatin addition respectively. Cultures were incubated in the growth chamber during eight weeks 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 multiplication ability of cultures was then evaluated on the basis of mean number of shoots per explant, length of shoots emerged from each explant, mean number of leaves, and callus formation at the end of the eight weeks. All pH values were measured again after the microshoots removed from the medium.

2.3. Statistical Analysis

Each experiment had three replicates of 30 plants for shoot multiplication (three explants in ten culture magentas). The data were statistically evaluated by using SPSS Statistics program. Analysis of variance (ANOVA) was used to calculate statistical significance, and the mean ± SE (standard error) differing significantly were determined using Duncan's multiple range test at $P < 0.05$ level. Correlation analyses were conducted using the mean values for shoot number, shoot length, and node number treatments (Pearson's correlation coefficient test).

3. Results

As mentioned elsewhere (Cüce et. al 2013; Cüce and Sökmen 2015), WPM medium supplemented with appropriate zeatin/IBA combination and concentration (1.0/0.1 mg/L) favors shoot proliferation three *Vaccinium* species studied here. Not only the medium as well as PGRs, but medium pH and plants selected for culturing also greatly effect micropropagation. It is noteworthy to emphasize that treatment of auxin treatment decreases the medium pH, zeatin acts as *vice versa* (Table 1). The measurements of pH showed a dramatic variation in medium shifting to high acidity after autoclaving. At initial medium pH 4, a decrease was recorded after autoclaving by 0.34 units, while at pH 6, the decline was sharper (0.87 units). Adding IBA into the autoclaved media caused another decrease medium pH except for pH 4.0. Afterwards, zeatin addition increased the pH and all media pHs measured became higher than the media after

autoclaving before adding IBA. At the end of the eight weeks, all medium pHs prepared for *V. arctostaphylos* and *V. myrtillus* were found to be more alkaline between 2.9 and 1.0 units higher when compared to those of initial medium pH. These two species made medium pH more alkaline whereas that of *V. uliginosum* was more acidic (Table 2).

Table 1. Changes in initial medium pH before and after autoclaving

Initial medium pH	Medium pH after autoclaving	Medium pH after PGR's	
	without zeatin and IBA	IBA addition	zeatin addition
4.0	3.66	3.66	3.74
4.5	4.07	3.96	4.10
5.0	4.35	4.21	4.41
5.5	4.58	4.48	4.75
6.0	5.13	4.96	5.19

Note: pH values before and after autoclaving are means of three measurements.

As far as survival percentages of the explants are concerned, pH adjustments confirmed a positive and significant effect on shoot regeneration of three different *Vaccinium* species. Changes in pH were dramatic after the completion of micropropagation experiments. The medium pH increased during micropropagation of *V. arctostaphylos* and *V. myrtillus* caused an increase whereas that of *V. uliginosum* exerted notable decrease in media pHs (Table 2). The highest shoot number per explant (5.03) was obtained at pH 5.5 in *V. arctostaphylos* case, whilst other pH adjustments produced lesser shoots (Table 3). Highest shoot length, node number and callus formation were 39.62 mm, 10.83 and 46.67%, respectively under the same condition and the same plant as well. The statistically significant difference was observed in terms of the shoot proliferation per explant ($P < 0.05$).

Table 2. Changes in medium pH after subculturing of 8 weeks.

Initial medium pH	Medium pH		
	V. arctostaphylos	*V. myrtillus*	*V. uliginosum*
4.0	6.72 ± 0.051	6.71 ± 0.066	4.82 ± 0.057
4.5	6.64 ± 0.027	6.50 ± 0.030	4.84 ± 0.075
5.0	5.95 ± 0.044	4.42 ± 0.197	4.93 ± 0.058
5.5	6.33 ± 0.034	6.69 ± 0.087	4.67 ± 0.042
6.0	6.27 ± 0.041	6.80 ± 0.088	4.75 ± 0.068

Note: pH values are means of three measurements after 8 weeks.

In contrast, pH 5.0 was superior to *V. myrtillus* in terms of shoot number (3.80 per explant), although no statistical difference was observed between pH 5.0 and 4.5 (Table 4). Highest shoot length (40.78 mm) was obtained from much lesser medium pH (e.g. pH 4.5), highest node number (10.83) was from the media possessing higher pH (6.0). The callus formation showed similarity in *V. arctostaphylos* case where the highest callus formation was 41.11% at pH 6.0. These results shows the significant differences for all parameters investigated.For *V. uliginosum*, pH 6.0 gave highest shoot number with 3.48. Only the statistical difference could be observed between pH 5.5 and other application in terms of shoot number. Otherwise the highest shoot length and node number were obtained at pH 4.0 with 34.94 mm and 10.76 respectively. This species did not produce any callus formation at the base of microshoots as happened in *V. arctostaphylos* and *V. uliginosum* cases. Shoot length and the node number decreased accordingly depending on increase in pH. Moreover, *V. uliginosum* microshoots were much weaker than those of *V. arctostaphylos* and *V. myrtillus* (Fig. 1). Shoot length and node number of *V. arctostaphylos* exhibited a strong positive correlation in terms of an increase in medium pH with r = + 0.631 and r = + 0.177 respectively. Correlation analyses of *V. myrtillus* were also similar to *V. arctostaphylos* when shoot length and node numbers were taken into account, exerting r = + 0.264 and r = + 0.402 while pH increases. Nevertheless, correlation analyses of *V. uliginosum* gave the significant negative correlation about shoot length (r = - 0.761) and

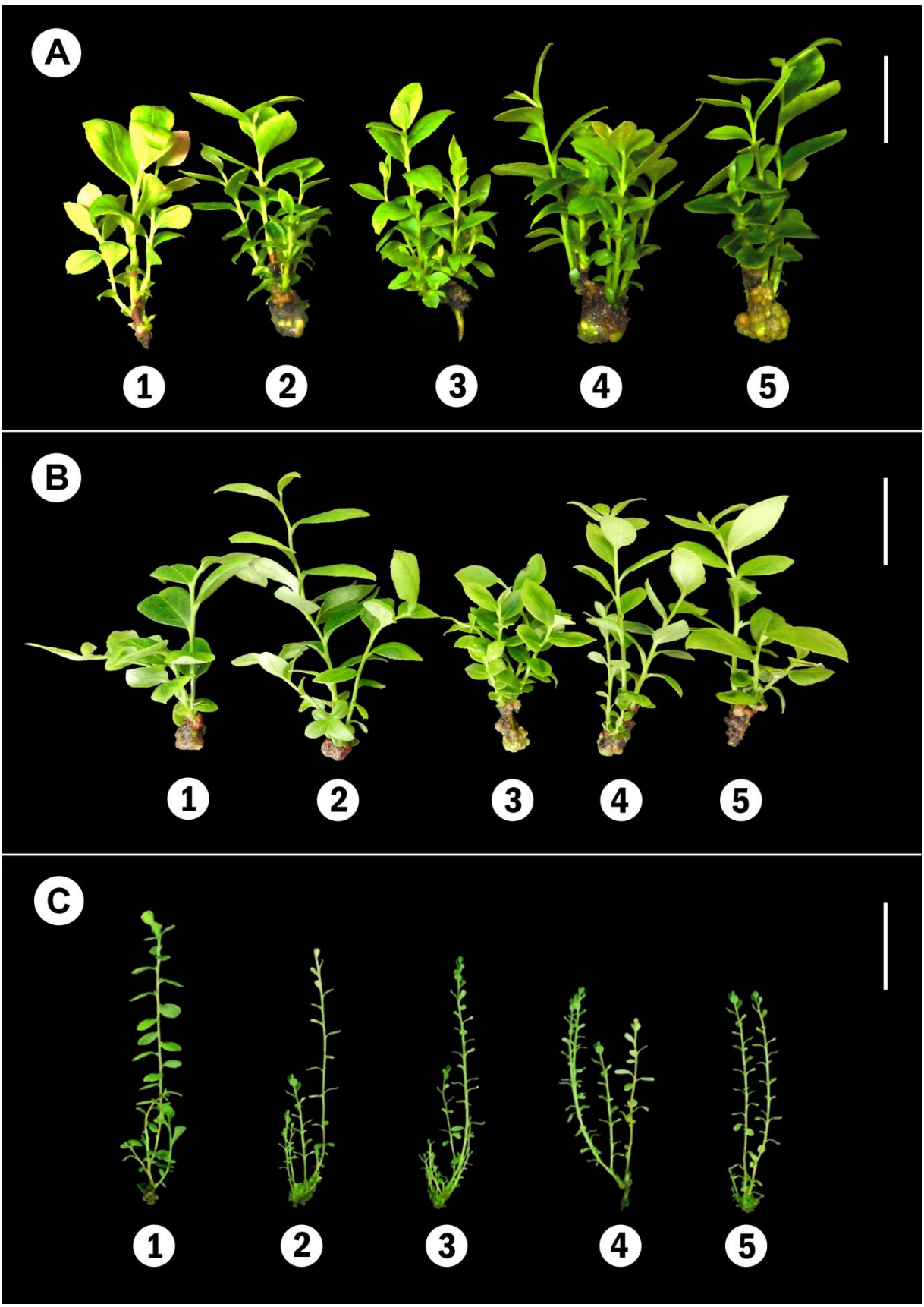

Figure 1. Effect of WPM basal media supplemented with 1.0/0.1 mg/L Zeatin/IBA combination, on shoot multiplication of **A)** *V. arctostaphylos*, **B)** *V. myrtillus*, **C)** *V. uliginosum.* 1 = 4.0 pH, 2 = 4.5 pH, 3 = 5.0 pH, 4 = 5.5 pH and 5 = 6.0 pH. Shoot proliferation and callus formation at the base of the shoot after eight weeks on culture medium at 4.0, 4.5, 5.0, 5.5 and 6.0 pH, respectively. *Bars,* A= 11.32 mm, B = 12.34 mm, C = 9.5 mm.

node number (r = - 0.889, Table 6). As a result, fluctuations in media pHs exert statistically significant impacts on shoots regeneration and responses from three *Vaccinium* species varies greatly depending on the pH.

Table 3. Shoot multiplication per explant of *V. arctostaphylos* at different pH of culture medium supplemented with zeatin and IBA

pH	Shoot Number	Shoot Length (mm)	Node Number	Callus (%)
4.0	$3.70^{c} \pm 0.71$	$31.99^{d} \pm 2.94$	$9.73^{b} \pm 0.98$	$32.22^{b} \pm 1.92$
4.5	$4.90^{ab} \pm 0.99$	$34.01^{c} \pm 2.73$	$10.53^{a} \pm 1.07$	$42.22^{a} \pm 5.92$
5.0	$4.53^{b} \pm 0.78$	$35.94^{b} \pm 3.44$	$10.83^{a} \pm 0.99$	$41.11^{a} \pm 1.92$
5.5	$5.03^{a} \pm 0.93$	$35.25^{bc} \pm 1.49$	$9.77^{b} \pm 0.93$	$41.11^{a} \pm 3.85$
6.0	$3.90^{c} \pm 0.61$	$39.62^{a} \pm 2.81$	$10.83^{a} \pm 1.26$	$46.67^{a} \pm 3.33$

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

4. Discussion

Vaccinium species has a great diversity as they grow in different habitats, so some species would require further study to optimize micropropagation protocols in terms of suitable pH values. These species has a narrow range of pH (4.5-5.5) for optimum growth and pH at varying levels as being emphasized by various researchers for different *Vaccinium* species (Debnath 2005; Ostrolucká et al. 2010; Cüce and Sökmen 2015). Many authors have studied the influence of medium pH on micropropagation of different plant species (Gürel and Gülşen 1998; Andersone and Ievinsh 2008; Huda et al. 2009). But, as far as literature survey can ascertain, there is no report was available concerning pH application of indigenous *Vaccinium* species.

Table 4. Shoot multiplication per explant of *V. myrtillus* at different pH of culture medium supplemented with zeatin and IBA

pH	Shoot Number	Shoot Length (mm)	Node Number	Callus (%)
4.0	$2.53^{b} \pm 0.63$	$35.96^{b} \pm 2.39$	$8.57^{c} \pm 0.94$	$24.44^{b} \pm 1.93$
4.5	$3.63^{a} \pm 0.76$	$40.78^{a} \pm 3.79$	$10.27^{ab} \pm 0.98$	$38.89^{a} \pm 3.94$
5.0	$3.80^{a} \pm 0.96$	$36.03^{b} \pm 2.23$	$10.37^{ab} \pm 1.03$	$37.78^{a} \pm 3.85$
5.5	$2.60^{b} \pm 0.67$	$39.93^{a} \pm 2.40$	$9.77^{b} \pm 1.01$	$34.45^{ab} \pm 3.85$
6.0	$2.93^{b} \pm 0.69$	$39.61^{a} \pm 2.96$	$10.83^{a} \pm 1.84$	$41.11^{a} \pm 4.39$

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

With this protocol, we tried to find the optimal medium pH being suitable for *in vitro* shoot multiplication of the whortleberry bilberry, and bog bilberry. According to the preliminary studies, WPM supplemented with zeatin/IBA-containing medium including 4.5-5.5 pH was very effective for shoot induction and multiplication for *Vaccinium* species (Zhao et al. 2011; Clapa et al. 2012; Cüce and Sökmen 2015; Hung et al. 2016). In this study, stable zeatin/IBA (1.0/0.1 mg/L) combination and different pH value was used in three different indigenous *Vaccinium* species. Adjustment of medium pH showed variety after autoclaving and adding PGRs. These changes were most probably due to the nature of WPM medium, IBA and zeatin added to the culture medium, as well as the NaOH used in preparing the zeatin storage. According to the Ostrolucká et al. (2010), medium pH shows variety between 0.24 and 1.63 units depending on the basal media and PGRs employed. A reported before, heat sterilization might significantly shift medium pH through denaturing of proteins, hydrolysis of carbohydrates and dissolution of salts the medium pH. Selby et al. (1989) also reported that changes in medium pH after autoclaving. Low initial medium pH decreased by 0.1-0.2 units while the higher initial medium pH acidified by 0.8–0.9 units depending on initial medium pH. Many researchers have paid attention to fluctuations in medium pH caused by changes in medium components (Owen et al. 1991; Druart and De Wulf 1993). All reports are in accordance with the results presented here. When the shoot multiplication rates of *V. arctostaphylos*, *V. myrtillus,* and *V. uliginosum* were compared, some notable differences can be seen in terms of shoot numbers, shoot length, node numbers and callus formation. In addition to having shorter shoot length, *V. uliginosum* microshoots did not produce any callus at all. Many researchers were used different pH value for shoot proliferation of whortleberry (Cüce et al. 2013), bilberry (Jaakola et al. 2001; Cüce and Sökmen 2015), lingonberry (Debnath and McRae 2001b), highbush blueberry (Eccher and Noe 1989; Hung et al. 2016), lowbush blueberry (Kaldmäe et al. 2006), and although Ostrolucká et al. (2010) reported that 5.5 and 4.0 pH significantly enhanced shoot number in Koralle and

Red Pearl of lingonberry cultivars. In this study, *V. arctostaphylos*, *V. myrtillus* and *V. uliginosum* gave the highest shoot number values at pH 5.5, 5.0 and 4.0, respectively.

V. arctostaphylos showed the significant positive correlation between increase in pH and the shoot length, reaching the highest value (39.62 mm) at pH 6.0. However, *V. myrtillus* and *V. uliginosum* gave the highest shoot length at 4.5 and 4.0 lower pH value, respectively. In *V. uliginosum* case, node numbers also increased in accordance with shoot length and increase in pH, whereas *V. arctostaphylos* and *V. myrtillus* gave the highest node numbers at higher pH with 10.83. At the end of the eight weeks, all studied *Vaccinium* species demonstrated significant difference in terms of callus formation.

Table 5. Shoot multiplication per explant of *V. uliginosum* at different pH of culture medium supplemented with zeatin and IBA

pH	Shoot Number	Shoot Length (mm)	Node Number
4.0	$3.47^{a} \pm 0.54$	$34.94^{a} \pm 2.89$	$10.76^{a} \pm 0.82$
4.5	$3.47^{a} \pm 0.59$	$33.38^{b} \pm 2.66$	$10.29^{b} \pm 0.71$
5.0	$3.34^{a} \pm 0.53$	$31.44^{c} \pm 2.48$	$8.53^{c} \pm 0.99$
5.5	$3.07^{b} \pm 0.51$	$28.70^{d} \pm 1.41$	$7.68^{d} \pm 0.78$
6.0	$3.48^{a} \pm 0.54$	$27.89^{d} \pm 1.51$	$6.44^{e} \pm 0.66$

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

Callus formation is undesirable phenomenon in micropropagation studies as it creates problems in rooting. As a result, pH fluctuations did not cause callus formation in *V. uliginosum*, but the others, *V. arctostaphylos* in particular, produced more callus.

As far as literature survey can ascertain, there is a little information concerning *in vitro* propagation of indigenous *Vaccinium* species and there is no reports about micropropagation of *V. uliginosum* at all (Cüce et al. 2013; Cüce and Sökmen 2015).

These researchers studied to determine micropropagation spring explants of *V. arctostaphylos* and *V. myrtillus* and obtained different shoot proliferation depending on the plant species employed. These differences are obtained in the shoot proliferation may also originate from the plant species, physiological condition of explants, collection times of explants, frequency of subculture, and habitats of the plants. All these results showed that the most suitable pH value can change according to the all these features as we mentioned above.

Table 6. Pearson's correlation coefficient between plant growth regulators calculated for all parameters of *V. arctostaphylos*, *V. myrtillus* and *V. uliginosum*

	V. arctostaphylos			*V. myrtillus*			*V. uliginosum*		
	SN	SL	NN	SN	SL	NN	SN	SL	NN
V. arctostaphylos	0.079	0.631**	0.177*	-	-	-	-	-	-
V. myrtillus	-	-	-	-0.036	0.264**	0.402**	-	-	-
V. uliginosum	-	-	-	-	-	-	-0.096	-0.761**	-0.889**
SN	1	-0.013	-0.013	1	-0.079	0.184*	1	0.077	0.094
SL	-0.013	1	0.254**	-0.079	1	0.394**	0.077	1	0.674**
NN	-0.013	0.254**	1	0.184*	0.394**	1	0.094	0.674**	1

The significant differences are given. * = $P < 0.05$, ** = $P < 0.01$, SN = Shoot Number, SL = Shoot Length, and NN = Node Number.

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