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Asian Journal of Plant Science and Research, 2018, 8(5):1-11

ISSN : 2249-7412
CODEN (USA): AJPSKY

Elaboration of Micropropagation Protocol for *Vaccinium corymbosum cv.* "Sunt Blue Giant"

Mohamed GRA*[1,2] Khusnetdinova LZ[1] and Timofeeva OA[1]

[1]*Department of Botany and Plant Physiology, Kazan Federal University, Kazan, Russian Federation*
[2]*Department of Plant Genetic Resources, Desert Research Center, Cairo, Egypt*

ABSTRACT

A reliable and reproducible protocol for micropropagation of Vaccinium corymbosum L. *cv "Sunt Blue Giant" was carried out from internodal segments isolated in late April and May from actively growing plants. Explants were established on three different types of basal media (MS, WPM and AN) without Plant Growth Regulators (PGRs). WPM showed the most promising basal medium for explant establishment in vitro. Zi (0.0, 0.5, 1.0 and 2.0 mg/L) and IBA (0.0, 0.1, 0.2 mg/L) and their combinations were tested for bud's induction. The maximum mean number of shoots formed per explant (3.9) with the highest mean length of axillary shoots formed on the internodal segments (3.2 cm) was obtained on WPM supplemented with 1.0 mg/L Zi 0.1 mg/L IBA. For shoot multiplication, the axillary shoots produced from the establishment stage were cultured on WPM containing 1.0 mg/L Zi in combination with 0.1 mg/L IBA through five successive subcultures and the results showed that, the fourth subculture gave the best results for multiplication. For rooting induction, WPM supplemented with different combinations and concentrations of IBA and IAA in addition to 0.1% AC were used. The highest mean number of roots formed per explant (5.9) were obtained on WPM fortified with 1.0 mg/L IBA after 10 weeks. In addition, the best mean length of adventitious roots/explant (1.05 cm) also obtained on the same medium. Successful acclimatization of rooted plantlets when transferred to soil (pH=3.5-4) was obtained and showed 100 % survival. For non-rooted plantlets, they required an acclimatization for ensure sufficient number of plants survive when transferred to soil. They should be dipped in different concentrations (1 and 2 g/L) of IBA solution for 5 and 10 min before they transferring to the soil (pH =3.5-4), results showed that, the number of survived plantlets (%) increased from 60% (control variant) to 80% (treated variants). Through this work, a reproducible protocol for micropropagation of V. corymbosum L. cv "Sunt Blue Giant" was developed and can help improve micropropagation systems for other Vaccinium species.*

Keywords: Blueberry, Internode segments, *Vaccinium corymbosum*, Sunt Blue Giant, Acclimatization, Non-rooted plantlets, Rooted plantlets

Abbreviations : AC: Activated Charcoal; AN: Anderson culture medium(1980); IAA: Indole-3-Acetic Acid; IBA: Indole-3-Butyric Acid; MS: Murashige and Skoog medium (1962); PGR(s): Plant Growth Regulator(s); WPM: Woody Plant Medium (1980); Zi: Zeatin.

INTRODUCTION

Highbush blueberry (*V. corymbosum* L.) is a member of family Ericaceae and considered to be one of a promising and economically important plants in Russia and many other countries [1,2]. Highbush blueberry is a soft fruit crop of large interest due to the high value of its edible fruits, and excellent source of health-promoting nutrients. The interest in large-scale production of *Vaccinium* species and their genetic improvement is growing constantly, due to the increasing commercial interest of these small fruits [3]. Blueberries has been hailed for their age-defying properties, fueling demand for the increasingly popular fruit. One of the main reasons for using alternative production techniques that is the sexual propagation, it is considered as disadvantageous for these plants due to the highest heterozygosity [4]. Also, traditional vegetative propagation of highbush blueberry by cuttings has not been successful due to poor rooting

ability, considerable demand for large amount of mother plants and their limited seasonal growth and relatively high price. Generative reproduction does not produce homogeneous progeny [5]. As a result of increasing demand, micro propagation of these species has gained momentum and led to the introduction of new approaches for commercial production [6]. Micro propagation is one of the best methods for the rapid propagation of elite plants and by the end of 1980 more than one million highbush blueberry plants were propagated annually worldwide [7]. Micro propagation can be used as a system for rapid mass production of high-quality plant material and give the opportunity to obtain homogeneous progeny [8]. However, all aforesaid attempts showed great variations in terms of basal media as well as Plant Growth Regulators (PGRs), growth conditions, explant types, sampling, physiological condition of the explants [9]. Cultivar under study "Sunt Blue Giant" is characterized size of fruit up to 30 mm in diameter the taste is sweet and its high constituent of vitamins and antioxidants yield from one plant up to 5 kg. This cultivar high frost resistance for this recommended cultivate it in the coldest regions because it can withstand upto -34°C without cover. The aim of this study is to determine an efficient micropropagation protocol for highbush blueberry (*V. corymbosum* L.) cv. "Sunt Blue Giant" by finding out the best basal medium and PGRs for shoot induction and to evaluate the effects of different auxins on *in vitro* rooting of shoots and improving of acclimatization plantlets *in vivo*.

MATERIAL AND METHOD

Plant Material and Explant Preparation

All experiments carried out *in vitro* under controlled conditions. Seedlings of *V. corymbosum* L. cv "Sunt Blue Giant" were used as plant material. They were obtained from Bekker company in Kazakhstan. Internodal segments used as explants they have been taken from young, soft, and actively growing shoots. Explants washed five times with antioxidant solution (150 mg/L citric acid in combination with 100 mg/L ascorbic acid) to inhibit browning of the tissues, after that all explants were rinsed with flow of tap water 2 h. Explants were surface-sterilized with three different successive concentrations (10, 20 and 30%) of commercial Clorox containing 5.25% sodium hypochlorite (NaOCl) with rinsing with sterile distilled water each time. The sterilization time 10 min with each concentration of commercial Clorox, containing three drops of tween 80. Then, they rinsed six times with sterile distilled water to remove all traces of the disinfectant.

Culture Media and Conditions

The sterilized explants were cultured on (MS) [9,10], (WPM) [11] and (AN) [12] even alone or supplemented with different concentrations and combinations of plant growth regulators for different micro propagation stages. All mediums were supplemented with 2.5% sucrose, 0.1 g/L myo- inositol and was gelled with 2.7 g/L phytagel, finally pH was adjusted to 5.0 ± 0.2 prior to addition of gelling agent [3,8]. Media were autoclaved for 20 min at 121°C under 1.1 kg/cm^2 pressure. All cultures were kept in the growth room at 25±2°C under 16 h photoperiod under cool white fluorescent tubes (F 140 t ad/38, Toshiba).

Establishment stage

Sterilized explants were cultured on three different medium types (MS, WPM and AN) free of plant growth regulators to determine the best basal medium for establishment of lateral buds *in vitro*. For establishment of explant and axillary shoots induction on the internodal segments, WPM has been used supplemented with various concentrations of Zi (0.0, 0.5, 1.0 and 2.0 mg/L) and IBA (0.0, 0.1, 0.2 mg/L) and their combinations. After four weeks of culture, the mean number of axillary shoots on the internodal segments and mean length of axillary shoots (cm)/explant were recorded.

Multiplication stage

Uniform axillary shoots obtained from the establishment stage about 2-3 cm long were cultured on WPM fortified with 1.0 mg/L Zi in combination with 0.1 mg/L IBA. Formed shoots subcultured every 8 weeks on the same medium for five times. After eight weeks of culture, the mean number of adventitious shoots and mean length of adventitious shoots (cm)/explant recorded.

Rooting stage

Adventitious shoots with a suitable length (2.5-3 cm) were introduced to WPM augmented with 0.1% activated charcoal and supplemented with IBA (0.0, 0.5, 1 and 2 mg/L) and IAA (0.0, 0.5 and 1 mg/L) and their combinations for effective root formation. After ten weeks of culture the percentage of roots induction, mean number of formed roots and mean length of adventitious roots (cm)/propagule recorded.

Acclimatization

In the spring, plantlets were taken out from the culture vessels, then completely washed with distilled water to remove any medium residual.

1. Rooted plantlets were immediately transferred to pots (8 cm) containing a sterile soil (BONA FORTE pH=3.5-4).

2. Non-rooted plantlets were soaked in different concentrations (1 and 2 g/L) of IBA solution for 0, 5, and 10 min, then transferred to pots (8 cm) containing sterile soil (BONA FORTE pH=3.5-4). With both approaches plantlets covered with white plastic bottles for maintain high humidity, then the plantlets transferred to green house 25±1°C and 80-90% relative humidity and they irrigated regularly two times a week by adding a solution of (¼ strength of WPM salts with the addition of (50 mg/L citric acid and 15 mg/L ascorbic acid, then the pH of the solution was adjusted to 3-3.5). After 30 days, the plastic bottle that covered the plantlets opened gradually from a few minutes a day until normal greenhouse conditions could be maintained without desiccation of the plantlets. Plantlets replanted into bigger containers (12 cm in diameter) and placed in open-air conditions. The acclimatization period was 60-90 days, depending on external climatic conditions. The percentages of survived plantlets were recorded 90 days after potting.

Statistical analysis

Variance analysis of data were done using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using [13]. New multiple range tests as described by [14].

RESULTS AND DISCUSSION

Establishment stage

In vitro culture of woody plants has always been difficult due to the difficulty of determination of nutritional medium requirements [15]. Also, application of *in vitro* plant micropropagation depends on the correct combination of exogenous hormones (auxin and cytokinin) which added to the medium [16]. Data recorded in Table 1 and Figures 1 & 2 represented the effect of different mediums (MS, WPM and AN) without of plant growth regulators on parameters of establishment stage for explants after four weeks of culture. Results showed that, the mean number of axillary shoots on the internodal segments explants were significantly affected by all medium types under investigations as well as mean length of axillary shoots (cm) per explant and it was obvious that WPM and AN mediums are greatly affected the mean number of axillary shoots on the internodal segments (2.00 shoot/explant) in comparing with MS media (1.1 shoot/explant). Also, it was clearly that WPM medium was more promotive for producing high length of axillary shoots per explant (2.75 cm) than MS and AN medium (1.47, 1.89 cm) respectively Table 1 and Figures 1 & 2 these results showed that WPM medium is the most effective for shoot establishment of *V. corymbosum* L. cv "Sunt Blue Giant" *in vitro*.

Table 1: Effect of different media (MS, WPM and AN) without of plant growth regulators on establishment of lateral buds of *V. corymbosum* L. cv "Sunt Blue Giant".

Parameters	MS	WPM	AN
Mean number of axillary shoots on the internodal segments	1.10 b	2.00 a	2.00 a
Mean length of axillary shoots (cm)/explant	1.47 b	2.75 a	1.89 c

Data recorded after four weeks of culture with five replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

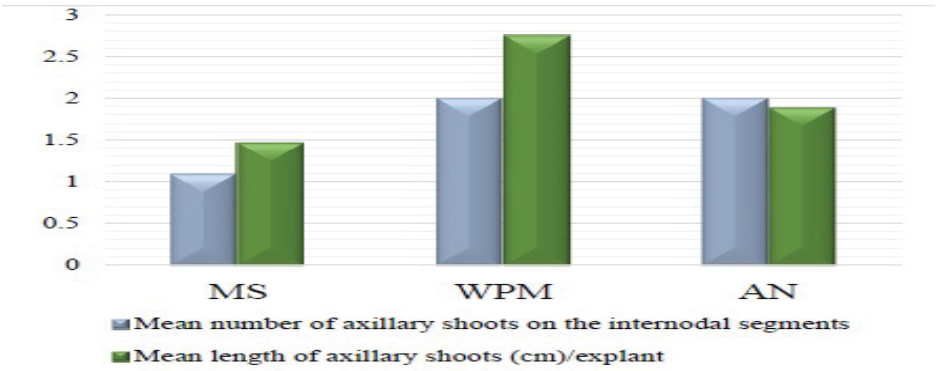

Figure 1: Effect of different media (MS, WPM and AN) without PGR(s) on establishment of lateral buds of *V. corymbosum* L. cv "Sunt Blue Giant"

Figure 2: Effect of different media (MS, WPM and AN) without without PGR(s) on establishment of lateral buds of *V. corymbosum* L. cv "Sunt Blue Giant" A – MS medium, B – WPM medium, C – AN medium

Data recorded in the following Table 2 and Figure 3 studied the effect of WPM either alone or supplemented with different concentrations and combinations of Zi and IBA on the different parameters of establishment stage for the *in vitro* cultivated explants after eight weeks of culture. With respect to the percentage of explant forming growth the heights value (100%) obtained on WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA and the lowest value of survival percentage (80%) obtained on WPM free of plant growth regulators. But it was statistically insignificantly different by both variables under the study and their interaction. On the other hand, the mean number of shoots formed per explant, and mean length of axillary shoots (cm)/explant affected significantly. The highest mean number of formed shoots per explant (3.9 shoot/explant) obtained on WPM fortified with 1.0 and 2.0 mg/L Zi in combination with 0.1 mg/L IBA. While the highest values for mean length of axillary shoots per explant (3.22 cm) were obtained with WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA (Table 2).

Generally, the optimum medium for shoot proliferation of *V. corymbosum* L. cv "Sunt Blue Giant" was WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA. Therefore, this medium was chosen for further shoot multiplication [6,9,17,18]. The results of the present study are in agreement with the work of other authors, who found that the use of Zi strongly stimulates shoot growth and considerably increases multiplication rate in the highbush blueberry [19]. Combinations of cytokinin and auxin have shown that effective results for better shoot proliferation than when both growth regulators are used singly [20]. However, application of Zi along with IBA in WPM promoted shoot proliferation of *V. corymbosum* L. cv "Sunt Blue Giant" also Zi in the lower concentrations showed some effective results for shoot proliferation of *V. corymbosum* L. cv "Sunt Blue Giant", which is an important factor for reducing the micro propagation cost, as well as minimizing undesirable somaclonal variation as it was confirmed by Ostrolucka *et al*. [3]. In addition, the use of high concentrations of cytokinin in the medium increase the hyperhydricity and decreases quality of plantlets [21]. Nevertheless, this is in contrary with Debnath and Mcrae [22] and Meiners *et al*. [23] obtained the used higher Zi concentrations (4.0 mg/L and more).

From the previous data it can be concluded that, WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA was the most effective for shoot proliferation of highbush blueberry (*V. corymbosum* L.) cv "Sunt Blue Giant" *in vitro* after 8 weeks.

Table 2: *In vitro* establishment of *V. corymbosum* L. cv "Sunt Blue Giant" cultured on WPM supplemented with different combinations of PGRs.

Parameters	IBA conc. (mg/L)	Zi conc. (mg/L)			
		0.0	0.5	1.0	2.0
% of explants forming growth	0.0	80 a	90 a	90 a	90 a
	0.1	90 a	90 a	100 a	90 a
	0.2	90 a	90 a	90 a	90 a
Mean number of axillary shoots on the internodal segments	0.0	1.30 c	1.40 c	3.00 b	3.30 ab
	0.1	1.30 c	3.30 ab	3.90 a	3.90 a
	0.2	1.20 c	3.30 ab	3.20 ab	3.10 ab
Mean length of axillary shoots (cm)/ explant	0.0	1.63 d	1.72 d	2.96 ab	2.86 ab
	0.1	2.20 cd	2.70 abc	3.22 a	1.76 d
	0.2	2.40 bc	2.94 ab	2.40 bc	2.98 ab

Data recorded after eight weeks of culture with five replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

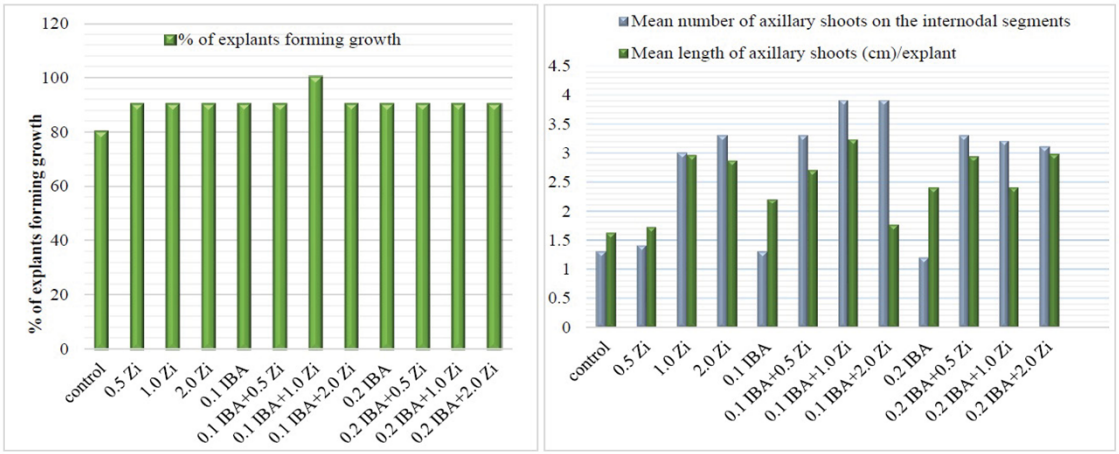

Figure 3: Effect of different levels of Zi, IBA and their combinations on different parameters of establishment stage of *V. corymbosum* L. cv "Sunt Blue Giant".

Multiplication stage

Data in Table 3 and Figure 4 illustrate the effect of WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA on the enhancement of the multiplication rate (Mean number of adventitious shoots and mean length of adventitious shoots (cm)/explant) of *V. corymbosum* L. cv "Sunt Blue Giant" *in vitro* during five successive subcultures. Axillary shoots, which obtained from the establishment stage excised and cut into 1-2 bud segments and subcultured on fresh media WPM containing 1.0 mg/L Zi in combination with 0.1 mg/L IBA through five successive subcultures every eight weeks. The mean number of adventitious shoots per explant gradually increased with each subculture. Meanwhile, shoot length decreased with subcultures up to the fifth subculture. But from the fifth subculture microshoots began to show hyperhydricity. Fourth subculture gives the maximum multiplication rate with healthy adventitious shoots of 5.5 shoot/explant with mean length of 3.33 cm/explant (Figure 5). Previous reports showed that, subculturing help to achieve continuous production of healthy adventitious shoots through a number of subculture cycles until the third subculture of the highbush blueberry and lingonberry cultivars [24,25]. In addition, it is reviewed that the first subculture on the same medium resulted in an increased number of shoots formed per explant in several *Vaccinium* species [26,27]. In the present study, the reason of hyperhydric adventitious shoots generated in the fifth subculture may be the change in growth requirements of adventitious shoots after the fourth subculture. Another explanation may be a deficiency of microelements, since WPM contains neither cobalt nor iodine [28]. This opens the way for further research to overcome this problem.

Table 3: Effect of WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA on multiplication of *V. corymbosum* L. cv "Sunt Blue Giant" through five successive subcultures.

Parameters	1st Subculture	2nd Subculture	3rd Subculture	4th Subculture	5th Subculture
Mean number of adventitious shoots	3.9 c	4.2 c	5 b	5.5 ab	5.8 a
Mean length of adventitious shoots (cm)/explant	3.22 a	3.36 a	3.37 a	3.33 a	2.57 b

Data recorded after eight weeks of culture with five replicates of 10 explants per treatment. Means having the sameletter within columns are not significantly different at 0.05 level of probability.

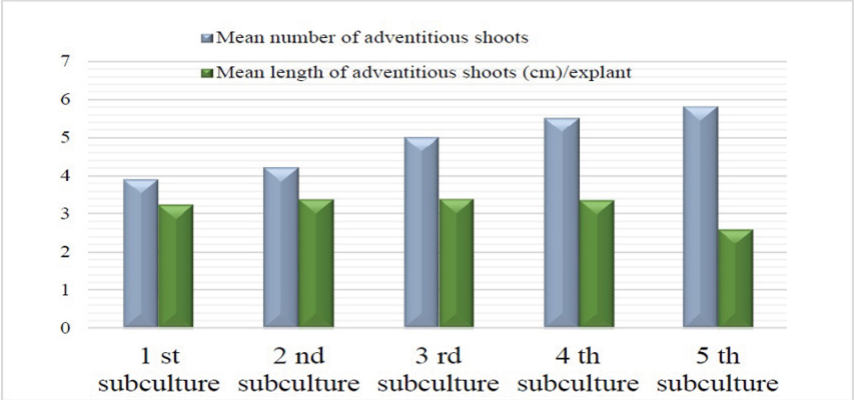

Figure 4: Effect of WPM supplemented with 1.0 mg/L Zi in combination with 0.1 mg/L IBA on multiplication of *V. corymbosum* L. cv "Sunt Blue Giant" through five successive subcultures

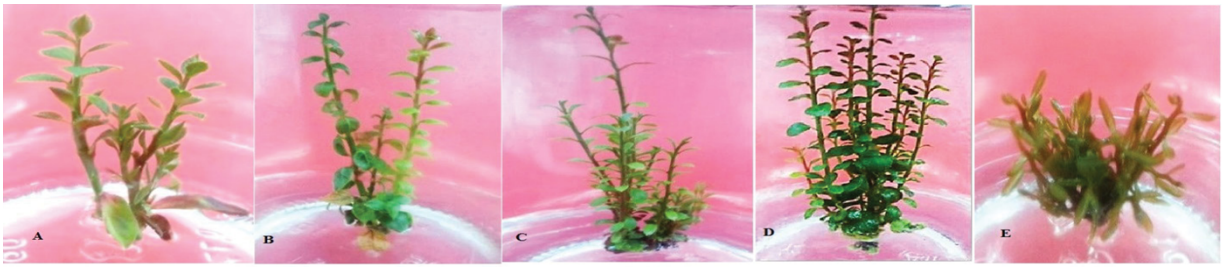

Figure 5: Multiplication of *V. corymbosum* L. cv "Sunt Blue Giant". On WPM supplemented with 1.0 mg/L Zi with 0.1 mg/L IBA through five successive subcultures. A – The first subculture, B – The second subculture, C – The third subculture, D – The fourth subculture, E – The fifth subculture

***In vitro* rooting stage**

Data recorded every eight weeks of culture with 5 replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability. For *Vaccinium* species L. IBA and IAA are frequently used for *in vitro* root initiation and increasing root number and length even alone or in combination with each other [18]. IBA was the most suitable auxin for rooting of *Vaccinium* species. This result is in accordance with our rooting studies [23,24]. In our study, adventitious shoots which obtained from multiplication stage with suitable length 2.5-3 cm were transferred to WPM fortified with various concentrations of IAA (0.0, 0.5 and 1.0 mg/L) in combination with IBA (0.0, 0.5, 1.0 and 2.0 mg/L) and 1.0 g/L activated charcoal to study their effect on root formation of *V. corymbosum* L. cv "Sunt Blue Giant". Data represented in Table 4 and Figures 6 & 7 showed the significant effect of auxins on the studied parameters of *V. corymbosum* L. cv "Sunt Blue Giant". Concerning percentage of roots induction, the highest value was recorded on WPM supplemented with 1.0 mg/L IBA (90 %). On the other hand, WPM free of plant growth regulators did not show any root formation. The highest mean value (5.9 roots/propagule) of formed roots per propagule obtained on WPM supplemented with 1.0 mg/L IBA after 10 weeks with the best mean length of adventitious roots/ propagule (1.05 cm). These results are in agreements with Sedlak *et al*. who reported that, the highest rooting percentage (70%) was obtained with WPM containing 1.0 mg/L IBA and 0.1% AC with some highbush blueberry cultivars, while in the same article very low rooting success was achieved with anther cultivars the percent of rooting of only 9% [29]. Also, the results agree with Sedlak *et al*. who reported a similar effect of this auxin on direct root induction [4]. Cuce *et al*. showed that rooting ability was successfully progressed concomitantly by increasing IBA [6]. On the other hand, Ostrolucka *et al*. [3,30] used AN medium supplemented with 0.8 mg/L IBA and 0.8 g/L charcoal and Ružić *et al*. [9] used AN medium supplemented with 0.8 mg/L IBA and 4 g/L charcoal for getting the highest rooting percentage, Sedlak *et al*. [24] said that for *in vitro* rooting microcuttings were cultured on half-strength Anderson medium supplemented with 0.49 µM IBA. Sedlak *et al*. [31] founded that NAA was better than IBA and IAA. The results show that auxins (IBA, IAA) controlled root development of *V. corymbosum* L. cv "Sunt Blue Giant" where the cell differentiation process to roots depends on cellular auxin gradients. During this process the auxin-driven cell elongation of root hairs is supported by auxin import into non-root forming epidermal cells [32]. The cellular levels of auxin, in turn contribute to the regulation of gene expression that defines cell fate and pharmacological or genetic disruptions of auxin movement dramatically impacts root patterning [33,34].

One of the most important steps in tissue culture is the acclimatization of plantlets to *in vivo* conditions. A successful tissue culture method of propagation must result in re-establishment of high frequency of the tissue culture derived Data recorded after 10 weeks of culture with 5 replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

Table 4: Effect of different levels of IAA, IBA and their combinations on *in vitro* rooting of *V. corymbosum* L. cv "Sunt Blue Giant".

Parameters	IAA conc. (mg/L)	IBA conc. (mg/L)			
		0.0	**0.5**	**1.0**	**2.0**
% of root induction	0.0	0 f	80 ab	90 a	80 ab
	0.5	80 ab	70 abc	60 abcd	40 cde
	1.0	60 abcd	50 bcde	30 def	20 ef
Mean number of adventitious roots/ propagule	0.0	0.00 f	4.30 abc	5.90 a	4.40 ab
	0.5	4.30 abc	4.40 ab	3.60 bcd	2.50 cd
	1.0	3.60 bcd	3.70 bcd	2.20 de	0.60 ef
Mean number of adventitious roots (cm)/propagule	0.0	0.00 b	1.04 a	1.05 a	0.92 a
	0.5	1.04 a	0.92 a	0.74 a	0.64 a
	1.0	0.74 a	0.75 a	0.61 a	0.7 a

Data recorded after 10 weeks of culture with 5 replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

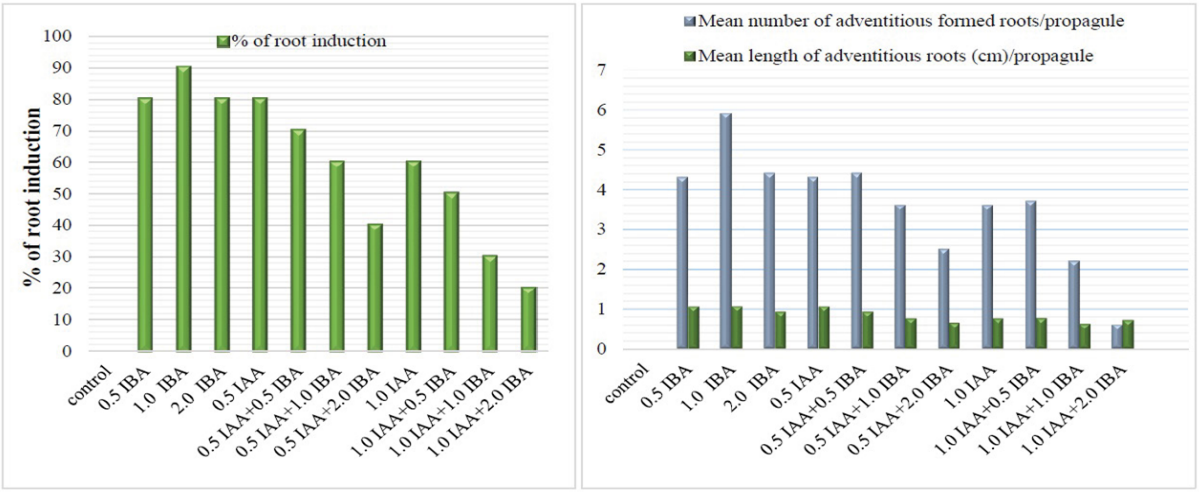

Figure 6: Effect of different levels of IAA, IBA and their combinations on different parameters of rooting stage of *V. corymbosum* L. cv "Sunt Blue Giant".

Figure 7: *In vitro* rooted plantlets of *V. corymbosum* L. cv "Sunt Blue Giant" after 10 weeks of culture. A- Plantlets on WPM without PGRs, B-Plantlets on WPM supplemented with 1.0 mg/L IBA.

Acclimatization stage

Plantlets in soil. Data in Table 5 and Figures 8 &10 clarify survival percentage of rooted and non-rooting plantlets. It reached from 60-100% after transfer of rooted plants to *ex vitro* conditions. These plantlets showed normal growth and developmental characteristics. Represented data showed that, there were no significant differences between the different treatments. Therefore, root quality closely related to the internal physiological activities of plantlets. Strong and healthy roots have led to increase the plantlets acclimation potential. Plantlet acclimatization depends on the properties of the rooting medium [35]. A similar response was previously obtained by Meiners *et al*. [23] who found that shoots of *Vaccinium* spp. were either allowed to rooted *in vitro* on medium containing IBA or *ex vitro* under fog tunnel. These results were similar with regard to acclimatization of rooted shoots, which was the highest in'Goldtraube' (91.8%), and the lowest in 'Berkeley' (66.7%).

Table 5: Effect of different concentrations of auxins in rooting media on survival percentage of rooted plantlets of *V. corymbosum* L. cv "Sunt Blue Giant". in acclimatization stage.

Parameters	IAA conc. (mg/L)	IBA conc. (mg/L)			
		0.0	0.5	1.0	2.0
% of survived plantlets	0.0	60 a	100 a	100 a	80 a
	0.5	100 a	100 a	100 a	80 a
	1.0	100 a	80 a	60 a	60 a

Data were recorded after 12 weeks of culture with 5 replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

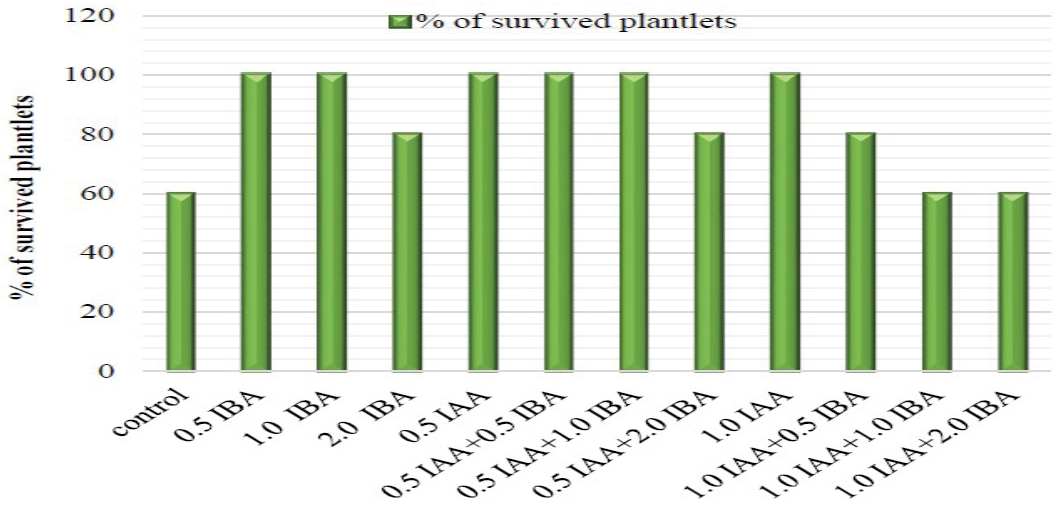

Figure 8: Effect of different concentrations of auxins in rooting media on survival percentage of rooted plantlets of *V. corymbosum* L. cv "Sunt Blue Giant". in acclimatization stage.

Data represented in Table 6 and Figures 9 & 10 illustrated that survival percentage of non-rooted shoots taken from rooting stage (60-80%) was obtained after dipping in different concentrations (1.0 and 2.0 g/L) of IBA solution for 0, 5, and 10 min, followed by direct planting in *ex vitro* conditions. Also, no significant difference between the different treatments was observed.

It was noticed that shoots cultivated on medium containing IBA and IAA remained green and most of them formed new leaves and shoots although they did not have roots. After transferring of non-rooted shoots to *ex vitro* conditions, they did not show any detectable variation in morphology or growth characteristics, but they were growing slowly compared with rooted plantlets. The best results (80% survival) were observed after dipping in different concentrations (1.0 and 2.0 g/L) of IBA solution for 5 and 10 min. While the lowest percentage of survived plantlets (60%) was observed without, dipping of non-rooted shoots in IBA solutions.

A similar response was previously obtained by Zhang *et al*. [36] who found that, good *ex vitro* rooting percentages were achieved after dipping briefly in IBA (or NAA) at 1000-2000 mg/L with the survival rate more than 90%. Also, Jiang *et al*. [37] worked on non-rooted shoots of 2-3 cm and induced rooting in a perlite-peat mixture (1:1 v/v) under intermittent mist after dipping in 2000 mg/L IBA solution.

Microshoots of *V. corymbosum* and *V. vitis-idaea*. 'Red Pearl' and 'Koralle' are directly rooting in peat substrate after dipping into 0.5 to 0.8 mg/L IBA solution under *ex vitro* conditions (80-90-95%), depending on the cultivar [8]. And Fan *et al*.[24] showed that by dipping microcuttings into 4.92 µM IBA for 10 s and rooting *ex vitro* in a substrate composed of 90% peat and 10% perlite, the rooting percentages could be improved to 89% for 'Bluejay' and 97% for 'Pink Lemonade'.But this in contrary with Ruzic *et al*. [9] who obtained acclimatization of non-rooted shoots with a considerably lower acclimatization rate. Anyway, with both approaches, the acclimatization rates depend on external climatic conditions and physiological conditions of the explant. This study is a complete protocol for micro propagation of highbush blueberry (*Vaccinium corymbosum* L.) cultivar "Sunt Blue Giant" and can help to improve micro propagation systems for other *Vaccinium* species.

Table 6: Effect of different concentrations of IBA and duration of dipping non-rooted shoots in IBA solutions and their combinations on survival percentage of *V. corymbosum* L. cv "Sunt Blue Giant" in acclimatization stage.

Parameters	IBA conc. (g/L)	Duration(min)			
		0.0	5.0	10	2.0
% of survived plantlets	1.0	60 a	80 a	80 a	80 a
	2.0	60 a	80 a	80 a	80 a

Data were recorded after 12 weeks of culture with 5 replicates of 10 explants per treatment. Means having the same letter within columns are not significantly different at 0.05 level of probability.

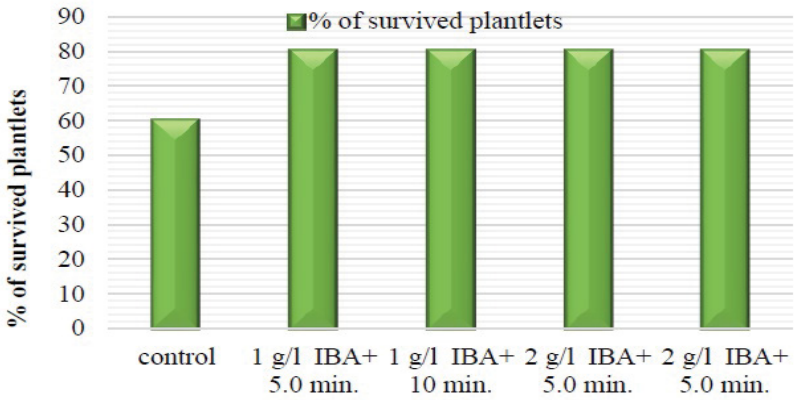

Figure 9: Effect of different concentrations of IBA and duration of dipping non-rooted shoots in IBA solutions and their combinations on survival percentage of *V. corymbosum* L. cv "Sunt Blue Giant" in acclimatization stage.

Figure 10: Acclimatized rooted and nonrooted plantlets of *V. corymbosum* L. cv "Sunt Blue Giant" after 12 weeks from culture transfer to the greenhouse.

CONCLUSION

It could be stated that an efficient cloning protocol was developed for *Vaccinium corymbosum* L. cv "Sunt Blue Giant" which enables large-scale propagation of high quality, true to type plants. The results of the present study prevailingly confirmed that *in vitro* regeneration ability is highly genotype depending, therefore small protocol modifications may be necessary for different cultivars.

ACKNOWLEDGMENTS

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

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