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Original Article

Optimization of *in vitro* Propagation of Qare-Qat (*Vaccinium arctostaphylus*)

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Abstract

In order to optimize the micropropagation of Qare-Qat (*Vaccinium arctostaphylus* J.J.Sm), samples were collected from two regions of Iran (Asalem and Kelardasht). Anderson (AN) media containing different concentrations of zeatin (0, 0.5, 1, 2 and 4 mg L^{-1}) and different levels of pH (4.5, 5 and 5.5) were compared. The *in vitro* shoots were transferred on AN media supplemented with zeatin (1 and 2 mg L^{-1}) alone or in combination with Indole-3-butyric acid (IBA) (0.2 and 0.4 mg L^{-1}). To optimize the rooting stage, elongated shoots (1.5- 2cm) were cultured on half strength semi- solid media containing activated charcoal (0 and 7g L^{-1}), or half strength liquid AN medium supplemented with different concentrations of IBA (0, 0.5, 2 and 3 mg L^{-1}) and NAA (0 and 0.5 mg L^{-1}). The results indicated that at the establishment stage, AN media supplemented with 4 mg L^{-1} zeatin (pH= 5.5) and 1 mg L^{-1} zeatin (pH= 5) were the best treatment for Asalem and Kelardasht explants, respectively. For the Asalem explants, the highest number of proliferated shoots (4) was observed on AN media consisting of 2 mg L^{-1} zeatin and 0.4 mg L^{-1} IBA. Whereas for the Kelardash explants the highest number of proliferated shoots (3) was observed on AN media consisting of 2 mg L^{-1} zeatin and 0.2 mg L^{-1} IBA. The highest percent of root formation (86 and 66%) were observed in half strength AN medium containing 7 g L^{-1} activated charcoal and 3 mg L^{-1} IBA and 0.5 mg L^{-1} NAA in Asalem and Kelardasht explants, respectively. The plantlets were successfully transferred to soil and the survival rate was 85%.

Key words: Medicinal plants, Micropropagation, *In vitro*, Growth regulators, *Vaccinium arctostaphylus*

Introduction

Vaccinium arctostaphylus L. belongs to the Ericaceae family which grows in Turkey, Armenia, Azerbaijan, Russia, Bulgaria and northern mountain of Iran. The Persian name of this plant is Qare-Qat. Qare-Qat is a medicinal plant that has been used in Iranian folk medicine as an anti diabetic and anti hypertensive agent for centuries. The leaves and fruits of this plant have also strong antioxidant properties [1]. Iranian Qare-Qat has not been grown in a large scale and there is a potential for commercial production of its fruits in some parts of the country. Therefore, a protocol for rapid propagation of Qare-Qat would be advantageous to expand its cultivation.

Traditional vegetative propagation of *Vaccinium* species by cutting is not very effective due to their poor rooting ability. Sedaghathorr *et al.* (2006) reported that Qare-Qat seeds germinated only in the light/ darkness alternation after chilling expiring [2]. Thus, the seeds have positive photoblastic reaction. The seed germination has resulted in homogenous seedling production.

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Plant tissue culture techniques have recently been employed for the propagation of *Vaccinium* L. species. This technique is suitable for rapid mass propagation of elite genotypes and the production of virus free plants as well as independent from seasonal influences. For *in vitro* propagation of various *Vaccinium* species, different growing media, and plant growth regulators have been studied. [3-5]. Cytokinins such as N6-[2-isopentenyl] adenine (2iP), zeatin [6-(4-hydroxy-3-methylbuttrans-2-enylamino) purine], thidiazuron (TDZ) and auxins such as indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) have also been reported for shoot proliferation or rooting [5]. Zeatin was found to be more effective for shoot initiation in *Vaccinium* species [6] and for shoot proliferation of highbush blueberry (*V.* 124 *corymbosum* L.) [7] and lingonberry (*V. vitis-idaea* L.) [5]. Very high concentrations of zeatin (45-91 μM) were found most effective for shoot proliferation of highbush blueberry [7]. A low concentration of an auxin 5.7 μM (IAA) was beneficial when added to the induction medium [8]. However, the method for micropropagation of Iranian Qare-Qat has not been reported. As part of a project on expanding Qare-Qat fruits production in north region of Iran, samples from Asalem and Kelardasht were selected after a study on genetic variation of Qare-Qat from different regions of Iran [1, 9]. For future commercial propagation of *V. arctostaphylus*, in the present study, protocols for micropropagation of samples collected from Asalem and Kelardasht were optimized.

Material and Methods

Plant Material

The samples were collected from Asalem (Gilan: 37° 38´N, 48° 49´E, 1250 m) and Kelardasht (Mazandaran: 36° 32´N, 51° 07´E, 1704 m) districts in early spring. A botanical specimen was deposited in the Farabi Herbarium (FAR). The herbarium numbers were 31025 (Kelardasht) and 33899 (Asalem).

Establishment Stage

The plants were transferred to laboratory and the stem segments (3-5 cm) with apical and auxiliary buds were washed under running water for 1 h and then they were surface sterilized with 70% ethanol for 2 min. The segments were immersed in a solution of 3% sodium hypochlorite with 2 drops of Tween-20 (0.05%) for 10 min. Finally, the explants were rinsed 3 times for 15 min in sterile distilled water. This process was carried out under sterile conditions in a laminar air flow cabinet. The sterilized stem segments were cut into pieces (1- 2 cm) with single nodes. The explants were transferred to AN establishment media supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} Phyto agar, different concentrations of zeatin (0, 0.5, 1, 2 and 4 mg L^{-1}) (Fig. 1a and b). Different levels of pH (4.5, 5.0, and 5.5) compared. Media were autoclaved for 15 min at 121 ºC and 100 KPa pressure. Zeatin was sterilized through 0.25 µm filter sand added to the cooled media (40 ºC). The explants were cultured in 20 mL glass test tube (one shoot per flask), each with 5 mL of medium. All cultures were placed under high pressure metal halide lamps (PPFD 60 µmol m^{-2} s^{-1} at the plant surface at 25±1 ºC and 16-hourphotoperiod. The established shoots were sub-cultured onto fresh medium in 50 ml glass test tube after 4 weeks.

Shoot Proliferation Stage

The *in vitro* shoots were cut into single node segments and transferred to AN media supplemented with zeatin (1 and 2 mg L^{-1}) alone or in combination with Indole-3-butyric acid (IBA) (0.2 and 0.4 mg L^{-1}). The pH of media was adjusted to 5.5 and 5, before adding 7 g l^{-1} plant agar for Asalam and Kelardasht explants, respectively. Five explants were cultured in a 200 ml glass jar, containing 40 ml of culture medium. Number of shoots per explants, number of leaves per shoot and length of shoots (cm) were measured after 6 weeks for three subsequent sub-cultures. The cultures were kept at the same conditions described for establishment stage (Fig. 1c).

Rooting and Acclimatization Stages

To induce shoot elongation, *in vitro* shoots (1.5-2cm) from proliferation stage were cut individually and cultured on medium without any plant growth regulators for 2 weeks. The elongated shoots were transferred to rooting media (5 shoots per flask) (Fig. 1 d). The rooting media (AN) were either half strength semi- solid media containing activated charcoal (0 and 7g l^{-1}), or half strength liquid medium. All rooting media were supplemented with different concentration of IBA (0, 0.5, 2 and 3 mg L^{-1}) and Naphthalene acetic acid (NAA) (0 and 0.5 mg L^{-1}). Percent of rooting, length of roots (mm) and number of roots per plantlet were recorded 6 week later.

For acclimatization of the *in vitro* plantlets, the plantlets were taken out from the culture tubes. The roots were rinsed in water to remove remains of media and transplanted into pots containing sterile vermiculite equipped with plastic cover. The plantlets were kept at 24±2 ˚C, 95% humidity and 16-hours photoperiod. The plantlets were acclimated by gradually opening the cover in a 4 weeks period. The plants were placed in the greenhouse and the percent of survived plantlets was recorded after 4 weeks (Fig. 1 e and f).

Experimental Design and Statistical Analysis

The experiments were in a factorial based completely randomized design. Each experiment was repeated twice with fifteen replicate and five explants in each replicate. Analysis of variance was performed and comparisons of means were conducted using Duncan's Multiple Range Test. All analyses were regarded as significant at $P \leq 0.05$.

Results and Discussion

Establishment Stage

The analysis of variance (Table 1) showed that there was a significant difference between plant growth regulators, but there was no significant difference between the samples from different regions. There was not a significant difference between different pH levels (Table 1).The AN medium supplemented with 4 mg L^{-1} zeatin with pH= 5.5 and AN medium supplemented with 1 mg L^{-1} zeatin with pH= 5 were the best treatments for Asalem and Kelardasht explants, respectively (Fig. 2).

Recently Ostroluck *et al.* (2010) investigated the influence of initial pH on long-term axillary shoot proliferation in *Vaccinium vitis-idaea* L. cv. Koralle and Red Pearl [10]. They used AN media supplemented with 0.5 mg L^{-1}zeatin with initial pH of 4.0, 4.5, 5.0, 5.5 and 6.0 and concluded that some plants can tolerate a broader pH range, while in others pH tolerance was limited. They concluded that, it is necessary to determine optimal pH levels.

Borkowska (1996) also found that initial pH 5.0 to be suitable for culture of *Vaccinium corymbosum* L., but high medium acidity (pH= 3.0) inhibited shoot growth [11]. Debnath (2003, 2004 and 2005) and Jaakola (2001) determined that initial pH 4.8-5.0 was suitable for successful growth of lingonberry [12-15].

Proliferation Stage

Different concentrations of zeatin in combination with IBA were compared at the proliferation stage. The highest number of shoots per explants (4 and 3) was observed in AN media containing 2 mg L^{-1} zeatin in combination with 0.4 and 0.2 mg L^{-1} IBA for Asalem and Kelardasht, respectively (Table 2).

The maximum number of leaves was obtained in AN media supplemented with 0.4 mg L^{-1} IBA (7) alone and in combination with 1 mg L^{-1} zeatin (7.4) for Asalem ecotype. There was not a significant difference between different plant growth regulators in number of leaves for Kelardasht ecotype.

Table 1 Analysis of variance of different zeatin concentration, different pH levels of media and two ecotypes (Asalem and Kelardasht) on number of established explants of *V. arctostaphylus*.

Source	df	Mean square
		Number of established explants
Ecotype (E)	1	0.120 ns
Concentration of zeatin (C)	4	0.310 **
pH levels (P)	2	0.070 ns
E×C	4	0.156 **
E×P	2	0.153 *
C×P	8	0.177 **
E×C×P	8	0.377 **
Error	120	0.032

**: Significant at 1% Probability level; *: Significant at 5% Probability level, ns: no

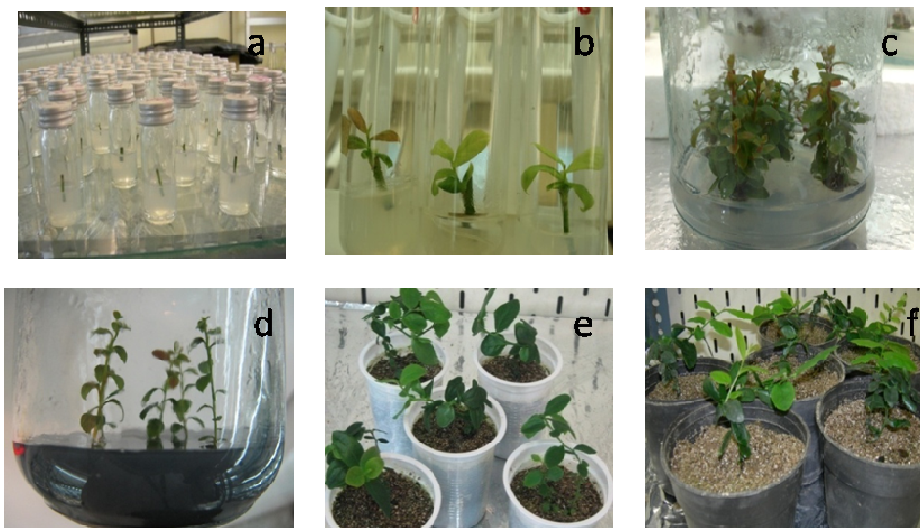

Fig. 1 Shoot and root formation from explants of *V. arctostaphylus* (a), (b) Establishment Stage. (c) Shoot Multiplication stage, (d) Rooting stage and (e) Acclimatization. (Color only on-line)

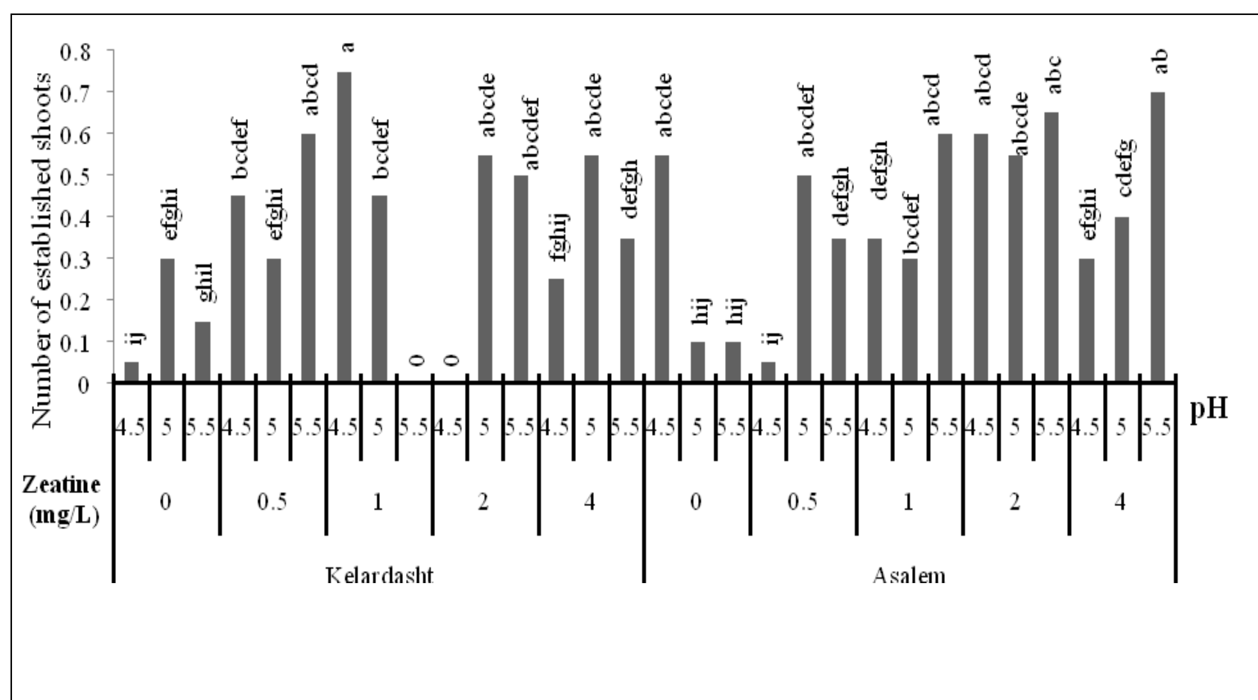

Fig. 2 The effect of interaction between different concentrations of zeatin (0, 0.5, 1, 2 and 4 mg L^{-1}) with different pH levels (4.5, 5 and 5.5) on number of established shoots of *V. arctostaphylus* (Kelardasht and Asalem)

The highest length of proliferated shoots (8 and 9 cm) were observed in media consisting of 0.4 mg L^{-1} IBA for Kelardasht and 0.2 mg L^{-1} IBA with 1 mg L^{-1} zeatin for Asalem (Table 2). Qu *et al.* (2000) reported a negative effect of NAA (0.1 mM) on shoot induction cranberry [16]. Meiners *et al.* (2007) demonstrated a positive effect of NAA on the shoot formation rate in *V. vitis-idaea* and *V corymbosum* [3]. Our results showed that with increasing the concentration of IBA, number of shoots in kelardasht explants did not increase significantly; moreover, IBA inhibited the formation of new shoots in Asalem explants. The effect of cytokines in combination with auxinson multiplication of some *Vaccinium* speices, has been reported by Marcotrigiano and McGlew (1991) [4]. They showed that the medium containing10 µM TDZ and 1 µM NAA was most appropriate medium for shoot induction in cranberry. Meiners *et al.* (2007) reported that zeatin (9.1 µM) in combination with IAA (5.7 µM) was most suitable for multiplication in *Vaccinium vitis-*

idaea L. cv.'Red Pearl' [3]. Ruzic *et al.* (2012) studied the effect of three medium (MS, AN and MAN) supplemented with zeatin (0.5 mg L^{-1}) alone or in combinaion with IBA (0.1, 1 and 5 mg L^{-1}) [17]. They found that low concentration of IBA (≤1 mg L^{-1}) added in zeatin- supplemented AN medium, increased shoot multiplication in V. *corymbosum*. However, other authors have showed that zeatin alone was effective for shoot proliferation of blueberry (*V. corymbosum*) (Eccher and Noe 1989) and lingonberry (*V. vitis-idaea* L.) (Debnath and McRae 2001)[7,18]. Eccher and Noe(1989) reported that very high concentrations of zeatin (45-91 μM) were most effective for shoot proliferation of blueberry [7].The most interesting finding was that on all media, any abnormality in morphology or callus initiation were not observed at shoot proliferation stage.

Rooting and Acclimatization

The results of rooting stage are shown in Table 3. Induction of roots from shoots produced *in vitro* is a critical step [19].

Table 2 The effect of different concentrations of zeatin and IBA alone or in combination on mean number of shoots per explants, mean number of leaves per explants and mean length of shoots at proliferation stage of *V. arctostaphylus*.

Plant growth regulators (mg L^{-1})		Mean number of shoot (±SE)		Mean number of leaves (±SE)		Mean length of shoot (cm)(±SE)	
		Asalam	Kelardast	Asalam	Kelardasht	Asalam	Kelardasht
Control	0	0.4 bc ±o.54	0 c±0	5.8 ab±1.6	4 cd ±1.140	5.8 abc±2.7	5.5 ab±1.14
Zeatin	1	1 abc±0.7	1 bc±o.o7	5 abc±1.22	5.4 a ±2.07	6 abc±1	3.8 b±1.9
	2	1.6 ab±1.14	0.20 cd±1.41	4.6 bc±2.19	5.4 a ±0.54	6.2 abc±1.3	5.2 ab±2.8
IBA	0. 2	0 c±0	0 c±0	2.6 c±0.54	5 ab ±1	2.4 d±0.54	4.6 ab±1.5
	0. 4	0.4 bc ± 0.54	0 c ± 0	7 ab ± 1.58	5.8 a ± 1.4	8 ab ± 2.5	8 a ± 2.8
Zeatin+IBA	1+0. 2	0.4 bc ± 0.54	1.8 b±0.83	6 ab ±1	4 cd ± 0.70	9 a ± 3.5	4.8 ab ± 0.83
	2+0. 2	0 c ± 0	3 a±0.4	4 bc ±1.5	5 ab ± 0.83	5 bcd ± 2.2	3.2 b ± 0.44
	1+0.4	1.2 abc ± 2.1	0.4 c±0.8	7.4 a ± 2.9	4.4 c ± 3.6	6.4 abc ± 2.8	5.8 ab ± 4.8
	2+0. 4	4 a ± 1.58	0 c±0	6.2 ab ± 2.16	4.8 c ±1.3	4.6 cd ± 1.8	4.4 b ±1.6

The letters in each column show significant differences according to Duncan's Multiple Range Test.

Table 3 Interactive affect of different auxin type concentration on percent of *in vitro* root formation and the root length of *V. arctostaphylus* in ½ AN medium.

Plant growth regulator (mg L^{-1})		Rootformation [%] ± SE		Length of root [mm] ± SE	
		Asalem	Kelardasht	Asalem	Kelardasht
NAA	IBA				
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	0.5	0 ± 0	5 f ±0	0 ± 0	1 e ± 0
	2	13.3 e ± 0.1	8 e ± 0.1	2.3 d ± 0.8	1 e ± 0.1
	3	33.3 d ± 0.1	19 c ± 0.1	3.3 c ± 0.6	1.5 d ± 0.1
0.5	0	33.3 d ± 0.08	18.3 cd ± 0.08	4.3 b ± 0.09	2 c ± 0.08
	0.5	40 c ± 0.06	20b c ± 0.06	3.5 c ± 0.1	2 c ± 0.06
	2	53.3 b ± 0.1	25 b ± 0.1	8.5 a ± 0.2	3 b ± 0.1
	3	86.6 a ± 0.04	66.6 a ± 0.04	9 a ± 0.17	4 a ± 0.04

The letters in each column show significant differences according to Duncan's Multiple Range Test.

The first roots were observed after 2 weeks. The results illustrated that interaction effect of the three media and different IBA concentrations on percent of rooting and number of root per explants were not significant (Table 3).The highest percent of rooting (86.6 and 66.6) and the highest root lengths (9 mm and 4 mm) were achieved in the medium containing 3 mg L^{-1} IBA with 0.5 mg L^{-1} NAA for both Asalem and Kelardasht, respectively (Table 3).

Meiners *et al.* (2007) used various concentration of NAA and IBA for *in vitro* rooting of *V. corymbosum* and *V. vitis-idaea* and reported that in medium containing NAA, the roots often originated from callus or leaves touching the medium surface [3]. Therefore, they suggested that NAA was not suitable for root induction.

Ostrolucka *et al.* (2009 and 2004) suggested the AN medium with 8 g L^{-1} activated charcoal and 0.8 mg L^{-1} IBA for *in vitro* rooting of *V. corymbosum* L. and *V. vitis-idaea* L [20,21]. Ruzic *et al.* (2012) reported that *in vitro* rooting of three cultivars of highbush blueberries was obtained in the medium supplemented with 0.8 mg L^{-1} IBA and 4 g L^{-1}activated charcoal [17]. Gaung-jie *et al.* (2008) examined *in vitro* rooting of *V. cyanococcus* on WPM media with various concentrations of IBA and suggested that the medium containing 10 µM IBA was suitable for rooting stage [22].

The rooted plantlets were transplanted into vermiculite substrate, maintained in a humidity chamber and acclimatized by gradually lowering the humidity during a 4 weeks period (temperature 24±2, 95% RH at 16 hour photoperiod and 55 µmol m^{-2} s^{-1} PPF). After one month the highest percent of survived plantlets was observed in plantlets that had higher length of roots. Plantlets in length 50- 80 mm (after 5 months) were replanted into bigger plastic containers containing peatmoss: cocopeat: perilite (2:2:1) substrate and were maintained in the greenhouse (temperature 20 ± 2 °C, 85% RH, maximum PPF = 90 µmol m^{-2} s^{-1}, 16 h photoperiod).85% of the plants survived which showed normal growth and morphological characteristics.

In conclusion, this paper has given an efficient method for *in vitro* propagation of Iranian *V. arctostaphylus* which could be used in its commercial production in order to expand the cultivation of this important medicinal plant.

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