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ORIGINAL ARTICLE

Effects of nutrient media, different cytokinin types and their concentrations on in vitro multiplication of G × N15 (hybrid of almond × peach) vegetative rootstock

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KEYWORDS

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Thidiazuron

Abstract The objective of this research was to assess the effects of different media i.e. Murashige and Skoog (MS) and Quoirin and Lepoivre (QL), cytokinin type i.e. 6-Benzyladenin (BA) and 6-Benzylaminopurine (BAP) and cytokinin concentration on in vitro proliferation of the G × N15 rootstock. To evaluate the effects of different media and cytokinin type, two separate experiments were conducted as factorial based on completely randomized design, and single nodes were used as explants. The results showed that MS nutrient medium was found to be superior to QL nutrient medium. Regarding the interaction between media and growth regulators, the best interaction was found in MS medium supplemented with 1 mg l^{-1} BAP resulting in 8.5 new micro shoots/explant while 7.75 shoots were observed in MS medium containing 1.25 mg l^{-1} BA. The longest length of new micro-shoots (2.10 cm) was obtained in hormone-free MS medium. Findings of this study showed that there is a significant correlation between the hormone level and plantlet height and formed callus weight so that an increase in BAP and BA levels in both of MS and QL media resulted significantly in height decrease and callus weight increase. The results also suggest that the best and the worst plantlets in terms of quality were observed in hormone-free QL medium and MS medium supplemented with 1.25 mg l^{-1}, respectively. These results reflect the fact that the presence of high amounts of NH_4NO_3 and cytokinin especially BAP in culture medium triggered inhibitory effect on shoot growth.

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1. Introduction

Micro-propagation has been considered as an efficient tool for mass production of pathogen free and true to type fruit tree rootstocks in recent decades in Iran [31,21]. In order to

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establish commercial orchards, production of true to type vegetative rootstocks through tissue culture is a matter of high importance. In recent years, the use of stone fruit inter-specific hybrids as rootstock has received a proportionate attention in developed countries, which in turn solves many problems in stone fruit trees [36]. The rootstock G × N15 called "Garnem" was released in Spain by the Center of Investigation and Technology Agri Food of Aragon (CITA). G × N15 is a hybrid between almond (Garfi) and peach (Nemared) which has desirable features such as red leaves, good vigor, easy clonal propagation, resistance to root-knot nematodes, and graft compatibility with the whole range of peach and almond cultivars as well as some plum and apricot cultivars. The rootstock G × N15 is also suitable for replanting plots, which have been previously planted with peach. Furthermore, it shows very good performance with almond, both in irrigated and rain fed conditions [6].

In recent years, due to resistance of G × N15 rootstock to nematode and other desirable traits, a rising trend has been shown toward using G × N15 rootstocks in Iran. Mass production of G × N15 rootstock by conventional methods like hardwood and herbaceous cuttings is time-consuming and laborious. Micro-propagation techniques can enhance scale and speed of production of healthy plants [37].

Micro-propagation is affected by many factors such as genotype, culture medium [3], and plant growth regulators [9,11,12]. One of the most important factors in plant tissue culture especially in proliferation stage is cytokinin hormone. It is well known that cytokinins play multiple roles in the plant development such as promotion of cell division and cell expansion in plant protein synthesis stimulation and the activities of some enzymes. BAP as a synthetic cytokinin in combination with appropriate auxins has been used in micro-propagation of nut fruits [14,12]. BA and BAP are commonly used as cytokinins in prunus rootstock micropropagation. Moreover TDZ has been reported to be appropriate in in vitro proliferation step of some *prunus* spp. [23]. It is worth mentioning that some investigators have reported that TDZ and BA are the cytokinins used the most [12], while Arinaitwe et al. [17] and Góralski et al. [18] reported that kinetin and isopentenyl adenine (2iP) have been used less.

Recently, some researchers have suggested that regression analysis is one of the most efficient tools to compare the means of related quantitative treatments such as explant age, hormone concentration, and time course [20]. Biological processes are usually highly complex and difficult to describe accurately by simple statistical strategies based on analysis of variance (ANOVA). To identify the most appropriate model, as well as to describe the relationship between the treatment levels and also the response variable, regression analysis is commonly used. [20].

Micropropagation of fruit tree rootstocks plays a very important role in the commercialization of fruit orchards with desirable traits and disease-free plants. G × N15 is one of the vigorous vegetative rootstocks for both peach and almond around the world, and its propagation through tissue culture in order to establish industrial orchards is essential. Thus far, many researchers have been focusing on the influence of different cytokinins such as BAP, BA and TDZ in the proliferation phase of prunus rootstocks, the effect of medium culture has received much less attention. However few studies have reported about the micropropagation of G × N Series. This paper describes an effective protocol for in vitro proliferation of G × N15 via Single-node explants. Thus, the objectives of this study are to evaluate (a) effects of two media and (b) effects of two cytokinins (BA and BAP) in the culture medium during G × N15 growth and (c) a second-order polynomial to describe the relationship between the treatment levels and also the response variable.

2. Materials and methods

2.1. *Plant material* and explant preparation

The experiments were carried out in the Tissue Culture Laboratory, Department of Horticultural Sciences, University of Tarbiat Modares University (TMU) Tehran, Iran, during the period from 2012 to 2013. Plant materials were obtained from 2-year-old G × N15 vegetative rootstock grown in the greenhouse with normal daylight and a temperature range of 20–35 °C during night/day. Actively growing shoots with length of 15–20 cm of 'Garnem' were cut off and transferred to the fruit tree micro-propagation laboratory. Shoots were cut off to pieces of 1.5–2 cm length, each including one bud and then for surface disinfection, nodal cuttings without leaves were agitated in a solution containing water and 0.03% (v/v) Tween 20 (Merck, La Jolla, USA) for 5 min and finally explants were washed with running tap water for 1 h. For internal sterilization, explants were firstly agitated in alcohol 70% for 30 s and then explants were sterilized for 3.5 min in mercury chloride (0.01%). Later, they were washed twice with double distillated water containing citric acid 700 mg l^{-1} and finally, they were rinsed two times with distilled water.

2.2. Establishment step

After disinfection, sterile nodal cuttings from greenhouse grown G × N15 were placed on Murashige and Skoog [34] (MS) medium containing 0.5 mg l^{-1} of BAP (Sigma–Aldrich, Steinheim, Germany). MS medium was supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar (Duchefa). Nodal cuttings (15 mm) were vertically inoculated in test tubes (25 × 150 mm) containing 10 ml of experimental media. The pH of medium was adjusted to 5.8 with NaOH before autoclaving at 121C and 103.5 kPa for 20 min. The explants were maintained in a growth room with light intensity of 2500–3000 lux, 25 ± 1 °C and 16/8 h photoperiod for 4 weeks. After 4 weeks, the regenerated explants were transferred to MS medium supplemented with different concentrations of BAP (0.5, 1 and 1.5 mg l^{-1}).

2.3. Proliferation step

Two culture media were employed: The basic medium consisted of MS [34] and Quoirin and Lepoivre [27] (QL) macro and microelements (Duchefa). The media were supplemented with different cytokinins at various concentrations, sugar (30 g l^{-1}) and 7 g l^{-1} agar (Duchefa). The pH of medium was adjusted to 5.8 before autoclaving. After preparation of the media, plantlets (in second subculture) pre-cultured in hormone-free media, were transferred to experimental media. In vitro multiplication evaluation was performed on a weekly basis for 4–5 consecutive weeks. At the end of the fourth week, the parameters investigated in the experiments were the same, that is,

1. Shoot number/explant; number of new micro-shoots per explant: 0, 1, 2, 3, … .
2. Length of shoots (cm); Data included only new shoots longer than 0.75 cm.
3. Callus weight; callus derived from the base of stem explants.
4. Quality index; quality of plantlets was scored 1–5 based on growth parameters such as; vitrification, STN (shoot tip necrosis), yellowing, leaf area, and leaf quality and explants which exhibit normal growth were given the highest score (i.e. 5).

2.4. Statistical analyses

For the proliferation stage, experiments were conducted as factorial experiments based on Completely Randomized Design (CRD) with 5 replications and each replication included 4 explants in one glass baby food jar per treatment. Each experiment was repeated at least twice and the reported data are the means of two experiments. All data are presented as mean ± standard error. Data were analyzed with SAS software version 9.1. Differences were determined by analysis of variance, and significant ($P \leqslant 0.05$) differences among mean values were estimated using Duncan's new multiple range test. For each medium, the relationship between BAP and BA concentrations and shoot number, and shoot length was best described by a second-order polynomial.

3. Result

In the first experiment, the table of variance analysis indicated that different media (MS and QL) and various concentrations of BAP and BA had a significant effect on proliferation rate, height and callus weight. With respect to quality index, different media supplemented with various concentrations of BAP and not BA had significant effect on quality (Table 1). The highest number of new micro-shoot was obtained in MS medium containing 1 mg l^{-1}, in which the average number of new micro-shoot was 8.5 (Table 1). The maximum average of shoot length (2.10 cm) was observed in BAP-free MS medium which was longer compared to QL medium (Table 1). MS medium supplemented with 1.25 mg l^{-1} resulted in the production of the highest callus weight in comparison with other treatments. The highest quality of plantlets was due to BAP-free MS and QL media so that increasing BAP levels brought about a significant reduction in height and quality of plantlets as well as an increase in callus weight in both MS and QL media (Table 1). The regression analysis for shoot proliferation responses as affected by different BAP concentrations is summarized in Figs. 1 and 2. In MS medium culture with increasing concentrations of BAP to 1 mg l^{-1} proliferation was significantly increased and concentrations higher than 1 mg l^{-1} of BAP, not only did increase the rate of proliferation but also reduced the quality of plantlets (Fig. 1). Moreover regression analysis also showed that in the MS medium increasing the concentration of BAP to 1 mg l^{-1} reduced the height of micro-shoots but in 1.25 mg l^{-1} BAP the height of micro-shoots was increased. In QL medium culture, the increase in concentrations of BAP increased and decreased the proliferation rate and height of the Micro-shoots, respectively (Fig. 2). It may be explained by the reason that the high proliferation rate in MS medium containing 1 mg l^{-1} BAP compared to the low proliferation rate in MS medium containing 1.25 mg l^{-1} BAP caused a reduction in the height of micro-shoots.

Table 1 Effect of different concentrations of BAP on Shoot Number, Shoot length, Callus rate and quality.

Effects	Proliferation	Height	Callus	Quality index
Medium				
MS	4.95 ± 0.42 a	1.75 ± 0.04 a	0.20 ± 0.02 a	3.60 ± 0.07 b
QL	4.47 ± 0.38 b	1.72 ± 0.03 a	0.12 ± 0.01 b	4.47 ± 0.06 a
Different concentrations of BAP				
0 mg l^{-1}	1.00 ± 0.00 d	2.06 ± 0.05 a	0.00 ± 0.00 d	4.53 ± 0.12 a
0.5 mg l^{-1}	3.25 ± 0.14 c	1.81 ± 0.02 b	0.13 ± 0.02 c	4.34 ± 0.13 a
0.75 mg l^{-1}	5.00 ± 0.20 b	1.71 ± 0.02 b	0.17 ± 0.01 bc	4.06 ± 0.12 b
1 mg l^{-1}	7.37 ± 0.35 a	1.56 ± 0.01 c	0.23 ± 0.03 ab	3.71 ± 0.12 c
1.25 mg l^{-1}	6.93 ± 0.26 a	1.57 ± 0.04 c	0.27 ± 0.03 a	3.51 ± 0.13 c
MS × BAP				
MS + 0 mg l^{-1}	1.00 ± 0.00 f	2.10 ± 0.09 a	0.00 ± 0.00	4.12 ± 0.08 cd
MS + 0. 5 mg l^{-1}	3.50 ± 0.19 e	1.83 ± 0.04 bc	0.15 ± 0.03	3.87 ± 0.08 de
MS + 0.75 mg l^{-1}	5.25 ± 0.25 cd	1.66 ± 0.03 cdef	0.19 ± 0.02	3.62 ± 0.08 ef
MS + 1 mg l^{-1}	8.50 ± 0.33 a	1.51 ± 0.03 ef	0.30 ± 0.04	3.31 ± 0.13 fg
MS + 1.25 mg l^{-1}	6.50 ± 0.26 b	1.68 ± 0.06 cd	0.36 ± 0.04	3.06 ± 0.06 g
QL + 0 mg l^{-1}	1.00 ± 0.00 f	2.01 ± 0.06 ab	0.00 ± 0.00	4.93 ± 0.06 a
QL + 0.5 mg l^{-1}	3.00 ± 0.19 e	1.78 ± 0.02 cd	0.11 ± 0.005	4.81 ± 0.09 ab
QL + 0.75 mg l^{-1}	4.75 ± 0.31 d	1.75 ± 0.03 cd	0.14 ± 0.009	4.50 ± 0.09 bc
QL + 1 mg l^{-1}	6.25 ± 0.25 bc	1.61 ± 0.01 def	0.16 ± 0.01	4.12 ± 0.08 cd
QL + 1.25 mg l^{-1}	7.37 ± 0.41 ab	1.45 ± 0.03 f	0.18 ± 0.01	4.00 ± 0.09 de
P-value				
Medium	0.004	0.215 n.s.	< 0.001	< 0.001
BAP	< 0.001	0.003	< 0.001	< 0.001
Medium × BAP	< 0.001	< 0.001	< 0.001	0.909 n.s.

Values in each column represent means ± SE. Different letters within columns indicate significant differences ($p < 0.05$).

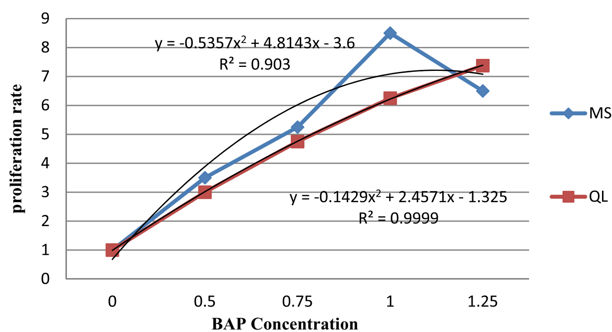

Figure 1 Interaction of different levels of BAP concentration and medium on in vitro proliferation of G × N15 micro-shoots.

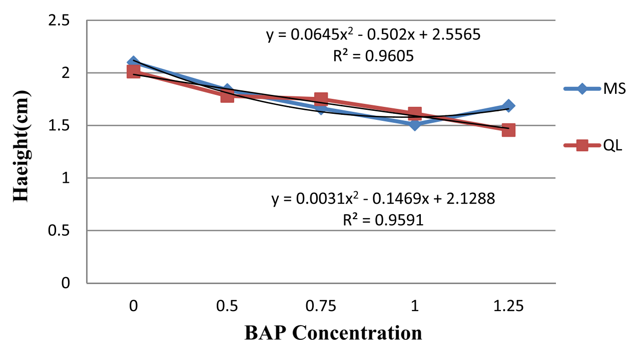

Figure 2 Interaction of different levels of BAP concentration and medium on Height of G × N15 micro-shoots.

In the second experiment, when MS and QL were used in a combination with different BA concentrations, the table of variance analysis showed that different culture media and various concentrations of BA and interaction between them led to a significant effect on new micro-shoot number and callus rate compared to the control. Different media and various concentrations of BA individually had a significant effect on height and quality index but interaction between them had no significant effects on quality index and height of G × N15 (Table 2). The best shoot proliferation with an average of 7.75 new micro-shoot was obtained in MS medium containing 1.25 mg l^{-1} BA (Table 2). With respect to height, an average length of 2.01 cm was produced in hormone-free MS medium (Table 2). The high amount of callus weight obtained in MS medium containing 1.25 mg l^{-1} and BA-free QL medium resulted in the production of the best plantlets in terms of quality (Table 2). Summary of the regression analysis for shoot proliferation responses as affected by different BAP concentrations is presented in Figs. 3 and 4. With increasing concentrations of BA in both MS and QL media culture proliferation rate was increased (Fig. 3). The regression analysis also showed that in the QL medium increasing the concentration of BA to 1 mg l^{-1} reduced the height of micro-shoots but in 1.25 mg l^{-1} BA the height of micro-shoots was increased (Fig. 4). In MS medium culture the height of micro-shoots reduced with increasing BA concentrations (Fig. 4). Furthermore, the results of the experiment suggested that callus weight was strongly influenced by our used cytokinin levels in both of QL and MS media. However, by increasing the hormone level, the height and quality of micro-shoots and callus weight were significantly decreased and increased, respectively. The results

Table 2 Effect of different concentrations of BA on Shoot Number, Shoot length, Callus rate and quality.

Effects	Proliferation	Height	Callus	Quality index
Medium				
MS	4.30 ± 0.39 a	1.80 ± 0.03 a	0.13 ± 0.01 a	3.9 ± 0.06 b
QL	3.35 ± 0.28 b	1.65 ± 0.03 b	0.08 ± 0.007 b	4.6 ± 0.05 a
Different concentration of BA				
0 mg l^{-1}	1.00 ± 0.00 e	1.89 ± 0.04 a	0.00 ± 0.00 e	4.69 ± 0.09 a
0.5 mg l^{-1}	2.50 ± 0.16 d	1.79 ± 0.03 ab	0.08 ± 0.008 d	4.47 ± 0.10 b
0.75 mg l^{-1}	3.81 ± 0.23 c	1.71 ± 0.03 bc	0.12 ± 0.009 c	4.25 ± 0.11 c
1 mg l^{-1}	5.06 ± 0.26 b	1.61 ± 0.04 bc	0.15 ± 0.01 b	4.03 ± 0.11 d
1.25 mg l^{-1}	6.75 ± 0.29 a	1.65 ± 0.07 c	0.17 ± 0.01 a	3.81 ± 0.08 e
M × BA				
MS + 0 mg l^{-1}	1.00 ± 0.00 f	2.01 ± 0.05 a	0.00 ± 0.00 g	4.37 ± 0.08 cd
MS + 0. 5 mg l^{-1}	2.75 ± 0.16 de	1.87 ± 0.04ab	0.11 ± 0.007 de	4.12 ± 0.08 de
MS + 0.75 mg l^{-1}	4.25 ± 0.31 c	1.80 ± 0.04ab	0.15 ± 0.01 bc	3.87 ± 0.08 ef
MS + 1 mg l^{-1}	5.75 ± 0.31 b	1.74 ± 0.03abc	0.18 ± 0.01 ab	3.62 ± 0.08 fg
MS + 1.25 mg l^{-1}	7.75 ± 0.25 a	1.61 ± 0.06bc	0.21 ± 0.01 a	3.50 ± 0.01 g
QL + 0 mg l^{-1}	1.00 ± 0.00 f	1.78 ± 0.02 ab	0.00 ± 0.00 g	5.00 ± 0.01 a
QL + 0.5 mg l^{-1}	2.25 ± 0.25 e	1.72 ± 0.03bc	0.05 ± 0.008 f	4.81 ± 0.09 ab
QL + 0.75 mg l^{-1}	3.34 ± 026 cd	1.61 ± 0.04bc	0.09 ± 0.004 e	4.62 ± 0.08 bc
QL + 1 mg l^{-1}	4.37 ± 026 c	1.48 ± 0.02 c	0.11 ± 0.002 de	4.44 ± 0.06 cd
QL + 1.25 mg l^{-1}	5.75 ± 0.16 b	1.68 ± 0.14bc	0.13 ± 0.003 cd	4.12 ± 0.08 de
P-value				
Medium	< 0.001	< 0.001	< 0.001	< 0.001
BA	< 0.001	< 0.001	< 0.001	< 0.001
Medium × BA	< 0.001	0.067 n.s.	< 0.001	0.641 n.s.

Values in each column represent means ± SE. Different letters within columns indicate significant differences ($p < 0.05$).

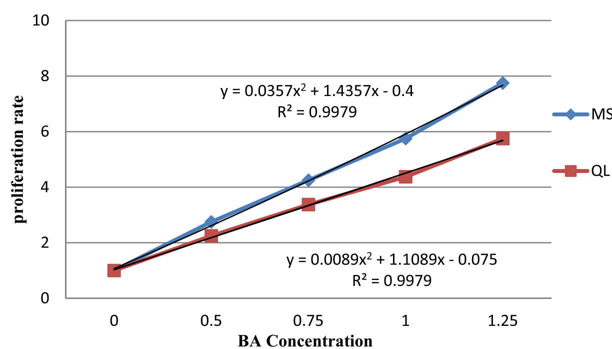

Figure 3 Interaction of different levels of BA and culture media on in vitro proliferation of G × N15 micro-shoots.

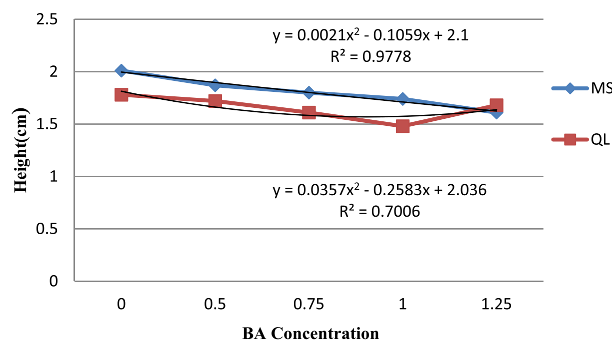

Figure 4 Interaction of different levels of BA concentration and medium on height of G × N15 micro-shoots.

also showed that the quality of plantlet in QL medium was better than MS medium and an increase in BAP and BA concentrations brought about a negative effect on quality index. MS medium compared with the QL medium was more effective on the proliferation phase; and a very low proliferation rate and longer length occurred in hormone-free medium. The findings of this study indicate that BAP had a stronger effect than BA on formation of new micro-shoot in proliferation step (Tables 1 and 2).

4. Discussion

One of the basic steps in the establishment of industrial orchards is choosing appropriate rootstock. G × N15 is known as one of the best vegetative rootstocks for peach and almond trees all over the world. This rootstock has many desirable horticultural traits such as resistance to common root-knot nematodes, good vigor, easy clonal propagation and graft compatibility with the whole range of peach and almond cultivars [6]. In order to establish high density orchard, propagation of G × N15 through tissue culture is essential but few studies have been conducted on the micro-propagation of this rootstock.

Variation in proliferation rate among prunus cultivars and rootstocks has been previously reported [30,13]. This variation may have resulted from the different requirements of each cultivar or rootstock for growth regulators. Moreover, differences in stimulation of shoot proliferation may be related to differences in macronutrients [29,19,7,2,5,16,12]. Culture medium and used cytokinins displayed a strong effect on proliferation rate of micropropagation process. Many studies have been conducted to identify the optimal medium culture and the type of cytokinin, for in vitro proliferation in *prunus* spp. [30,12]. Media compositions have a key role in morphogenesis. MS was the most commonly used basal medium for *prunus* micropropagation, but QL and WPM showed to be useful in some cultivars [13]. The inclusion of cytokinins in the media is generally considered necessary for in vitro proliferation [15,22]. Differences in internal growth regulator contents of each genotype have been suggested to be the reason for different in vitro responses of several plant species [35], and variability between *prunus* cultivars in micropropagation may be attributed to that difference. MS, QL, and WPM medium culture as well as BA, BAP, Kinetin, and TDZ have been the most commonly used media and plant growth regulators in prunus micropropagation [19,2,5,16,12]. To describe the relationship between treatment levels and response variable, regression analyses are commonly used [20]. The present investigation compared the effects of two nutrient media and two types of cytokinins (BAP and BA), which have been used by many researchers in micropropagation of prunus rootstocks using analysis of variance (ANOVA) and regression analyses.

The results revealed that MS medium was superior for in vitro proliferation of G × N15 rootstock in comparison with QL medium. Our results are in agreement with those of Unek et al. [10] on G × N15 (Garnem) that MS medium containing BAP was the best medium for proliferation of garnem. In contrast to our results, earlier, for G × N9, WPM and QL were found to have a better effect on shoot proliferation rate than either MS or ½ MS medium and the possible explanation given for this was the reduced nitrogen content in WPM [13] Although Nazary Moghaddam Aghaye et al. [32] and Molassiotis et al. [3] reported that for GF677, use of MS resulted in the best shoot proliferation rates. Dejampour et al. [21] found that, the use of DKW resulted in the best proliferation rate. Different results clearly indicated that requirement of medium compositions for micropropagation of various prunus species is genotype dependent. The findings of the present study indicated that MS medium was more effective compared to QL medium which may be explained by the fact that MS full strength medium contains very high doses of macro elements, specially nitrogen (KNO_3 and NH_4NO_3). On the other hand, the differences between MS and QL media cannot be explained solely on the basis of total ionic strength due to inhibitory role of high levels of elements in culture medium on in vitro growth of woody plants [8,33] (see Fig. 5).

There is a significant cluster of evidence indicating that one of the most important aspects of successful proliferation of prunus rootstocks is to determine an appropriate type and concentration of plant growth regulators [12]. It is well known that cell division, shoot multiplication and axillary bud formation can be promoted by the cytokinin. And also high concentrations of cytokinins of adenine type are often necessary for growth and differentiation in prunus *spp.* [15]. Cytokinin stimulates the initiation and activity of axillary meristems, which result in shoot induction and formation [22]. The influence of hormones is conflicting on shoot regeneration and some investigators reported that TDZ is more effective than BAP [29,7,2,5,16]. However, some investigators have reported that BAP is more effective than TDZ [19,12]. These conflicting

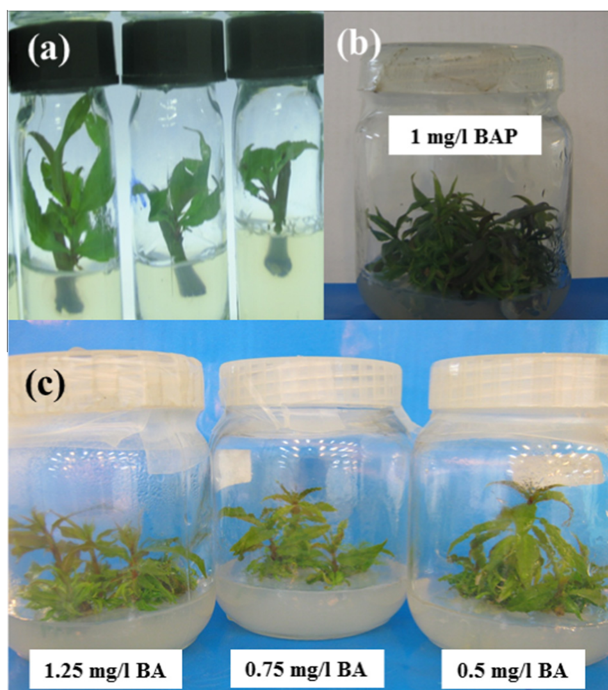

Figure 5 In vitro shoot regeneration from nodal cuttings. (a) Shoot development from nodal segment explants of G × N15 vegetative rootstock after 25 days of culture on MS medium containing 0.5 mg l^{-1} BAP + 0.05 mg l^{-1} IBA, (b) Development of multiple shoots after 30 days of culture on the MS medium containing 1 mg l^{-1} BAP, (c) in vitro multiplication of G × N15 micro-shoot after 30 days of culture on the MS medium containing 0.5, 0.75, and 1.25 mg l^{-1} BA.

results could be attributed to the use of different species as well as the possible effects of different genotypes. Our results also showed that the presence of a cytokinin like BAP or BA in the proliferation phase is essential. Goudarzi [38], in proliferation of colt and F12.1 rootstocks, showed that BA is the most effective cytokinin. Besides, Daneshvar-Hosseini et al. [1] and Nazary Moghaddam Aghaye et al. [31] stated that BAP is more effective on proliferation on Gisela 6 rootstock. These findings confirmed our results. The influence of cytokinins on micro-propagation can be varied based on the kind of culture medium, the variety of plant and the age of explants [35]. Analysis of the effect of various cytokinins on proliferation of G × N15 showed that BAP was more effective than BA. The regression analyses showed that there is a positive correlation between concentrations of BAP and number of new micro-shoots up to a certain concentration of BAP. In the current study, with increasing concentrations of BAP to 1 mg l^{-1} in MS medium number of micro-shoot per explants increased significantly, and the best proliferation was obtained in 1 mg l^{-1}, but BAP concentration higher than 1 mg l^{-1} resulted in the reduction of new micro-shoot per explant. Our results are in agreement with those of Thorpe et al. [35] that reported BAP is required at low concentrations ranging from 0.5 to 2.5 mg l^{-1} and higher concentrations of BAP had an adverse effect on proliferation rate. The current study indicated that the number of shoots was increased as the concentration of BAP increased. Our results are supported with those of Ruzic [12] for sweet cherry cv. Lapins (*P. avium* L.) and Shekafandeh [4] for Prunus dulcis Mill, in which the best proliferation was reported in medium containing BAP. In our both first and second experiments, regression analyses showed that MS medium containing 1 and 1.25 mg l^{-1} BAP resulted in the highest number of new micro-shoot per explant. Also regression analyses indicated that higher concentrations of BAP had an inhibitory effect on proliferation. Our results are in agreement with those of Akbas et al. [39] on *Amygdalus communis* L. that the decrease of proliferation rate, length of micro-shoot and an increase of callus end of micro-shoot are due to the strong concentrations of BAP which inhibit them.

Comparison of cytokinin type shows that the applied BAP can be more effective than BA on G × N15 shootlet formation, micro-shoot number, and shoot length in both MS and QL media. Low proliferation rate on BA-containing medium compared to those of medium with BAP may be consistent with its low cytokinin activity. Previous research indicated that high concentrations of cytokinins of adenine type are often necessary for in vitro proliferation of tissue culture previously and BAP could be used successfully to induce new micro-shoot multiplication in *Prunus* spp. [24].

In both experiments, the maximum length of micro-shoot was observed in hormone-free medium. Shoot length was not affected significantly by the type of medium but concentration of hormone significantly affected the length of shoot in both experiments so that hormone level increase triggered a significant decrease in micro shoot length. The findings of the present study showed that hormone-free media resulted in maximum length of micro-shoot which may be explained by the fact that cytokinin containing medium produced more micro-shoot, nutrients are used by more sinks and so there may be lower nutrient sources for shoot elongation.

These results are conformed with the results reported by Nazary Moghaddam Aghaye et al. [32] in GF677 micropropagation, which suggested that BAP is more suitable for shoot multiplication of GF677. Our results showed that callus was significantly affected by medium and cytokinin type so that increasing concentrations of BAP and BA in culture medium brought about a significant enhancement in the callus weight in both experiments. These findings are in agreement with that of Gisela 6 semi-dwarf rootstock [31]. It shows that when concentration of BAP was in excessive amount, it resulted in an increase of callus weight and reduction in micro-shoot length. One of the possible explanations given for that may be consistent with its high cytokinin activity. Apparently a certain amount of BAP is required to result in the best effect. Although reduction of BAP and BA concentration in both MS and QL media resulted in quality improvement of plantlets, plantlets with the best quality are produced in QL medium due to its lower amount of macroelements specially NH_4NO_3. Our results are in agreement with those of Ivanova and Van staden in *Aloe polyphylla* [25,26], and Shirdel et al. in dog rose [28] which suggest that high amount of NH_4NO_3 and cytokinin in medium increase the hyperhydricity and decrease quality of plantlets.

The present investigation indicated that regression analysis is an appropriate method to compare means of related quantitative treatments (e.g., doses of cytokinin). In our study, straight line functions and high *R*2 coefficient (70–99%) demonstrated that regression contributed to clarity of our biological results.

All these results clearly showed that determination of appropriate type and concentration of cytokinin for having successful proliferation phase in various prunus rootstocks depends on genotype. These varied results on the effectiveness of various cytokinins may be due to the effect of different factors such as plant genotype, tissue type and age of explant. Overall, the results of the current study indicate that MS medium and BAP were more efficient than QL medium and BA for in vitro proliferation of G × N15 rootstock. In terms of proliferation rate, the best results were obtained in MS medium containing 1 mg l^{-1} concentration of BAP.

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References

[1] A. Daneshvar-Hossini, E. GanjiMoghadam, S. Anahid, Ann. Biol. Res. 1 (2010) 135–141.
[2] A. Matt, J.A. Jehle, Plant Cell Rep. 24 (2005) 468–476.
[3] A. Molassiotis, K. Dimassi, I. Therios, G. Diamantidis, Biol. Plantarum. 47 (2003) 141–144.
[4] A. Shekafandeh, World Appl. Sci. J. 8 (2010) 1322–1326.
[5] A.C. Espinosa, P.M. Pijut, C.H. Michler, HortScience 41 (2006) 193–201.
[6] A.J. Felipe, HortScience 44 (2009) 196–197.
[7] B. Bhagwat, D. Lane, Plant Cell, Tissue Organ Cult. 78 (2004) 173–181.
[8] B. McCown, J. Sellmer, General media and vessels suitable for woody plant culture, Forestry Sci. (1987).
[9] B. Nowak, K. Miczyński, L. Hudy, Plant Cell, Tissue Organ Cult. 76 (2004) 255–260.
[10] C. Unek, E. Tanriver, A.B. Kude, Acta Hortic. 923 (2010) 195–197.
[11] D. Ruzic, M. Saric, R. Cerovic, L. Culafic, Biol. Plant. 47 (2003) 463–465.
[12] Dj.V. Ruzic, T.I. Vujovic, Hort Sci. 3 (2008) 12–21.
[13] E.B. Radmann, V.J. Bianch, J.C. Fachinello, R.P. Oliveira, Sci. Agrar. 10 (2009) 95–101.
[14] E.F. George, M.A. Hall, G.J. De Klerk, Plant Tissue Culture Procedure – Background. Plant Propagation by Tissue Culture, Springer, 2008.
[15] E.G. Sutter, in: R.N. Trigiano, Gray (Eds), Plant Tissue Culture Concepts and Laboratory EXERCISES, CRC Press, New York, 1996, pp. 11–25.
[16] F.A. Canli, L. Tian, Sci. Hortic. 116 (2008) 34–40.
[17] G. Arinaitwe, P. Rubaihayo, M. Magambo, Sci. Hortic. 86 (2000) 13–21.
[18] G. Góralski, M. Popielarska, H. Ślesak, D. Siwińska, M. Batycka, Acta Biol. Cracov. Bot. 47 (2005) 121–128.
[19] H. Tang, Z. Ren, G. Reustle, G. Krczal, Sci. Hortic. 93 (2002) 235–244.
[20] J.C. Lorenzo, M. Garcia-Borroto, In Vitro Cell Dev. Biol. Plant 44 (2008) 229–232.
[21] J. Dejampour, V. Grigorian, E. Majidi, N. AliAsgarzadeh, Iran. J. Hortic. Sci. Technol. 8 (2007) 43–54.
[22] J. Dobránszki, J.A. Teixeira da Silva, Biotechnol. Adv. 28 (2010) 462–488.
[23] J. Durkovic, A. Misalova, Funct. Plant Sci. Biotechnol. 2 (2008) 1–19.
[24] K. Pruski, T. Lewis, T. Astatkie, J. Nowak, Plant Cell, Tissue Organ Cult. 63 (2000) 93–100.
[25] M. Ivanova, J. Van Staden, Plant Cell, Tissue Organ Cult. 104 (2011) 13–21.
[26] M. Ivanova, J. Van Staden, Plant Cell, Tissue Organ Cult. 99 (2009) 167–174.
[27] M. Quoirin, P. Lepoivre, Acta Hortic. (1977).
[28] M. Shirdel, A. Motallebi-Azar, S. Masiha, N. Mortazavi, M. Matloobi, Y. Sharafi, J. Med. Plants Res. 5 (2011) 4605–4609.
[29] O. Perez-Tornero, J. Egea, A. Vanoostende, L. Burgos, Plant Sci. 158 (2000) 61–70.
[30] P. Andreu, J.A. Marín, Sci. Hortic. 106 (2005) 258–267.
[31] R. Nazary Moghaddam Aghaye, A. Yadollahi, A. Moeini, S. Sepahvand, J. Biol. Environ. Sci. 7 (2013) 57–64.
[32] R. Nazary Moghaddam Aghaye, A. Yadollahi, J. Agric. Sci. 4 (2012) 1916–9752.
[33] R.L. Bell, C. Srinivasan, D. Lomberk, In Vitro Cell Dev. Biol. Plant 45 (2009) 708–714.
[34] T. Murashige, F. Skoog, Physiol. Plantarum. 15 (1962) 473–497.
[35] T. Thorpe, C. Stasolla, E.C. Yeung, G.J. de Klerk, A. Roberts, E.F. George, in: E.F. George, M.A. Hall, G.J. De Klerk (Eds.), Plant Propagation by Tissue Culture, Springer, 2008, pp. 115–173.
[36] T.G. Beckman, G.A. Lang, Acta Hortic. 622 (2002) 23–25.
[37] Y. Aka-Kacar, C. Akpinar, A. Agar, Y. Yalcin-Mendi, S. Serce, I. Ortas, Rom Biotech. Lett. 15 (2010) 5246–5252.
[38] Goudarzi. R, MSc Thesis, Tarbiat modares university, Tehran, Iran (1995).
[39] F. Akbas, S. Namlı, C. Isikalan, B.E. Ak, Afr. J. Biotechnol. 22 (2009) 6168–6174.