Determination of the optimized culture medium and growth conditions for micropropagation of Persian oak (*Quercus brantii* L.)

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Abstract

The aim of this investigation was micropropagation of *Quercus brantii* L. via culture of terminal and lateral buds for mass production of this plant. The plant materials were collected from Fars province Iran in spring and summer seasons. After surface sterilization and the removal of phenol materials from the explants, they were transferred into different culture media containing different concentrations of Indole-3-butyric acid and 6-benzyladenine as plant growth regulators. The results showed that 90 seconds sterilization with $HgCl_2$ (0.1% concentration) was suitable for contamination control. In order to remove phenol materials, floating explants in 100 mg ascorbic acid solution and 100 ml distilled water showed, the best result. The results also showed that the Woody Plant Medium (WPM) containing 0.1 mg/l 6-benzyladenine (BA) and 0.01 mg/l Indole-3-butyric acid (IBA) was suitable to establish this species with the highest survival rate. The highest number of proliferation was obtained in the Woody Plant Medium culture containing 0.7 mg/l 6-benzyladenine and 0.01 mg/l Indole-3-butyric acid. Also, the best rooting treatments were successfully achieved by using Woody Plant Medium containing 1 mg/l Indole-3-butyric acid. All experiments were carried out under growth chamber conditions with light intensity of 2500-3000 lux with 8 hours of dark period at 24-25 ºC. Totally, the results of this study showed an adequate potential of Persian oak for mass production by tissue culture in order to reconstruct Zagros oak forests and also for breeding tasks.

Key words: Adaptation, Proliferation, *Quercus brantii* L., Rooting, Tissue culture.

INTRODUCTION

Persian oak (*Quetcus brantii* L.) of Fagaceae family is a type of *Quercus* which contains 500 various species that grow in the form of tree and shrub in different parts of the world (Ozcan, 2006). Oak species are the most important trees in Zagros mountains that extend from northwest to south. The most abundant oak species in this region is called Persian oak (Naderi Shahab, 2013). The current method of propagation is the distribution of seeds (Koneshlu, 2001). Fire, grazing, collecting seeds for livestock, domestic and industrial consumptions, summer drought, pests and seed diseases are the most important factors of Zagros oak forests regeneration failure (Hosseini *et al*., 2008). Moreover, oak seed is related to recalcitrant seeds and due to the sensitivity to drought, holding it for a long time is impossible (Mohan Jainne and Haggman, 2007; Pasquini *et al*., 2011). In addition, the main problem of seed propagation is heterozygosity in progenies.

Using vegetative methods like micropropagation is one of the important solutions in resuscitation and reconstruction of oak forests. Micropropagation is a

valuable tool for clonal propagation of superior tree genotypes when integrated into conventional tree breeding programs (Li-Hua Zhu *et al*., 2010). In recent years micropropagation and tissue culture has become a powerful tool for propagation of many plant species. The mass production of plants with similar genetics is the most regular advantage of plant tissue culture.

Regarding *in vitro* culture of oak, few studies have been accomplished (Hernandes *et al*., 2003; Ostrolucka *et al*., 2007; Vieitez *et al*., 2009; Vengadesan and Pijut, 2009). Two cases have been reported in Iran; evaluating the *in vitro* propagation of *Quercus brantii* by Fayyaz *et al*. (2014) that stopped at rooting level and also the successful propagation of *Quercus Castaneifolia* by Zamani *et al*. (2012).

Due to limitation success in micropropagation of Persian oak in previous studies and the importance of this species in the resuscitation of Zagros forests, the results of this study can be helpful for mass production of Persian oak trees via tissue culture method. The purpose of this study was to develop and optimize Persian oak micropropagation. So in this research, the effects of different culture media and growth regulators were studied at three stages establishment, proliferation and rooting.

MATERIALS AND METHODS

Plant material, sterilization, elongation and shoot proliferation

Young apical shoots were collected in the growth season from Fars province, Iran. The lateral and apical buds were used as the source of the explant. The explants were surface sterilized with two different concentrations of $HgCl_2$ solution (0.1% and 0.05%) for 60, 90, 120, and 150 seconds (Table 1). Also, 2 drops of Tween 20 and 100 mg ascorbic acid were added to the sterilization solution. Then, plant materials were washed three times by sterile distilled water.

Table 1. Sterilization treatments on Q. *brantii* explants.

$HgCl_2$ (%)	Time (second)			
	60	90	120	150
0.05	T1	T2	T3	T4
0.1	T5	T6	T7	T8

Immediately after explant preparation, they were immersed in the antioxidant solutions (Torres, 2012). Ascorbic and citric acids were used as antioxidants. So 0, 25, 50, 75, 100, 125 and 150 mg/l ascorbic acid in 100 ml distilled water were used for one hour.

After eliminating contamination and phenolic compounds, to determine the effect of different culture media and plant growth regulators on *in vitro* growth and development of *Q. brantii* the explants were cultured on the MS (Murashige and Skoog, 1962) and WPM (Woody Plant Medium) media without plant growth regulators and 0.1 mg/l BA in each culture. In separate experiments, WPM culture medium was supplemented with 12 different combinations of auxins and cytokinins (Table 2). After 21 days, leaf number, shoot length and shoot number per explants were recorded.

Table 2. Proliferation treatments of Q. *brantii* explants.

IBA (mg/l)	BA (mg/l)					
	0	0.1	0.3	0.5	0.7	0.9
0	A1	A2	A3	A4	A5	A6
0.01	A7	A8	A9	A10	A11	A12

Each culture medium was supplemented with 3% (w/v) sucrose. The pH of all the culture media was adjusted to 5.7, before autoclaving. Culture media were solidified with 0.5% (w/v) agar (Sigma-Aldrich, USA) and sterilized under 121.5 ºC and 1.2 kg/cm^2 pressure for 30 minutes. Cultures were incubated in a growth room at 25 ºC under a 16-h photoperiod (5000 lux).

Rooting and adaptation

Elongated micro shoots (1.5-2.5 cm in length), were cultured on WPM with 0 and 1 mg/l IBA and ½ MS medium with 1 mg/l IBA. Culture media contained 0.4% (w/v) agar (Difco Bacto Agar) and 2% (w/v) sucrose. pH of all media was adjusted at 5.7 before autoclaving. They were placed in the dark at 25 ºC for 7 days and then transferred to a 16-h photoperiod. After 30 days, root number and root length per explant were recorded.

Rooted plantlets were washed under tap water. Then they were transferred into pots containing peat and perlite at 2:1 ratio. Established plants were transferred to the greenhouse.

Statistical analysis

Data were subjected to the analysis of variance using a completely randomized design after reviewing the data distribution (the review of normality with Kolmogorov Smirnov test).

A completely randomized design with factorial arrangement with three replications and 10 explants in each replication were used to analyze the effect of $HgCl_2$

concentrations and time on explants survival.

A completely randomized design with three replications and 10 explants in each replication were used to analyze the effect of ascorbic acid concentrations on explants survival.

Also, a completely randomized design with factorial arrangement with three replications and 10 explants in each replication were used to analyze the effect of the culture medium type and BA concentration on survival.

Moreover, a completely randomized design with factorial arrangement with 10 replications was used. Since some of the samples did not survive during the test, the design was unbalanced and therefore, analyzed using variance analysis for unbalanced designs to analyze the effect of BA and IBA levels on leaf number and shoot length.

A completely randomized design with 10 replications was used to analyze the data of rooting stage.

The means of treatments were compared with Duncan's multiple range test (DMRT) to distinguish differences between treatment means. However, shoot number did not show the normal distribution in proliferation and was analyzed by the Kruskal-Wallis and Manvitny tests. Data analysis was performed using the SAS software.

RESULTS

Sterilization

The results showed that $HgCl_2$ is a suitable solution for the control of fungal and bacterial contaminations. $HgCl_2$ significantly affected the survival of cultured buds. Moreover, the time level significantly affected the buds survival. The interaction between $HgCl_2$ concentration and time was significant as well (Table 3). The highest survival was obtained in 0.1% $HgCl_2$ in 90 seconds (Figure 1).

Table 3. Analysis of variance of sterilization effect with $HgCl_2$ in the presence of Tween 20 and ascorbic acid on survival of Q. *brantii* cultured explants.

Source of variation	df	Sum of square	Mean of square	F value	P
Time	3	12416.667	4138.889	90.30	<0.0001
$HgCl_2$	1	600.000	600.000	13.09	0.0023
Time×$HgCl_2$	3	8433.333	2811.111	61.33	<0.0001
Error	16	733.333	45.833		

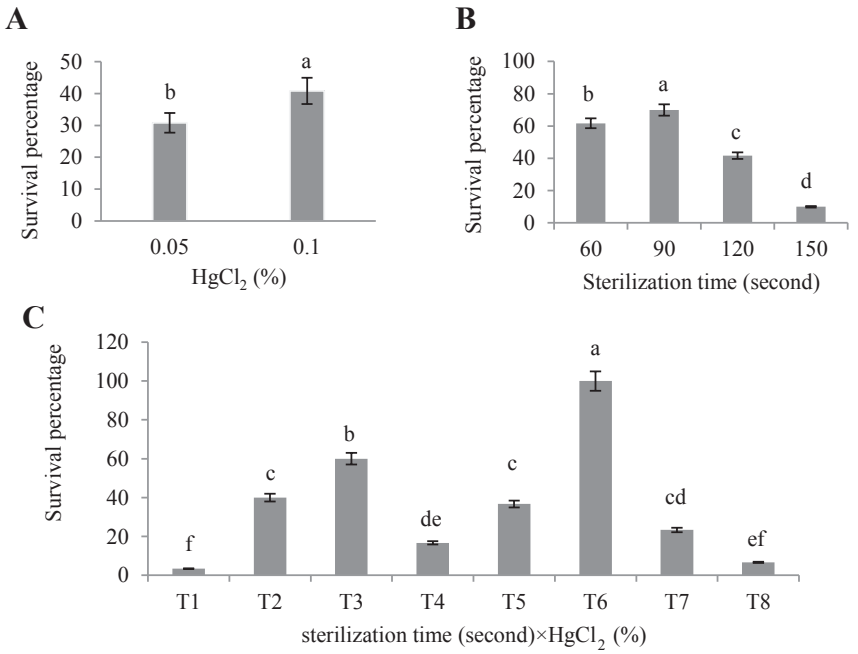

Figure 1. Effect of **A:** $HgCl_2$ concentration, **B:** sterilization time and **C:** sterilization time×$HgCl_2$ concentration on survival of Q. *brantii* explants.

The concentration of ascorbic acid significantly affected the buds survival (Table 4). The highest rate of buds survival was obtained in 100 mg/l ascorbic acid (Figure 2).

Establishment, elongation and proliferation

Medium type significantly affected the survival of cultured buds. Moreover, BA level significantly affected the survival. But the survival was not significantly affected by the interaction of medium with BA level (Table 5). The highest survival was obtained in the WPM culture medium containing 0.1 mg/l BA. It was found that the WPM culture medium containing 0.1 mg/l BA was suitable for the establishment stage (Figure 3).

BA level significantly affected the leaf number but it was not significantly affected by IBA level as well as the interaction between BA and IBA levels (Table 6). The highest leaf numbers were obtained in 0.7 and 0.9 mg/l BA concentrations (Figure 4).

BA level significantly affected the shoot length but it was not significantly affected by IBA level. The interaction between BA and IBA levels significantly affected the shoot length (Table 7). The longest shoot was obtained in 0.7 mg/l BA and 0.01 IBA (Figure 5).

The results of nonparametric test showed that shoot number was significantly affected by BA level. The highest shoot number was obtained in the culture medium with 0.7 mg/l BA (Figure 6). Shoot proliferation is shown Figure 8-A.

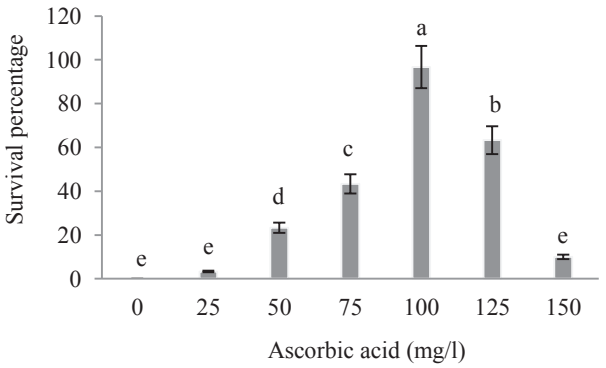

Figure 2. Effect of ascorbic acid concentration on survival of Q. *brantii* buds.

Table 4. Analysis of variance of ascorbic acid concentration effect on survival of Q. *brantii* buds.

Source of variation	df	Sum of square	Mean of square	F value	P
Ascorbic acid	6	22980.952	3830.16	100.542	<0.0001
Error	14	533.333	38.095		

Table 5. Analysis of variance on the culture medium and BA concentration on survival of Q. *brantii* buds.

Source of variation	df	Sum of square	Mean of square	F value	P
Culture media	1	2453.357	2453.357	25.88	<0.0001
BA	1	1988.595	1988.595	20.98	0.001
Culture media×BA	1	77.357	77.357	0.82	0.387
Error	8	947.833	94.783		

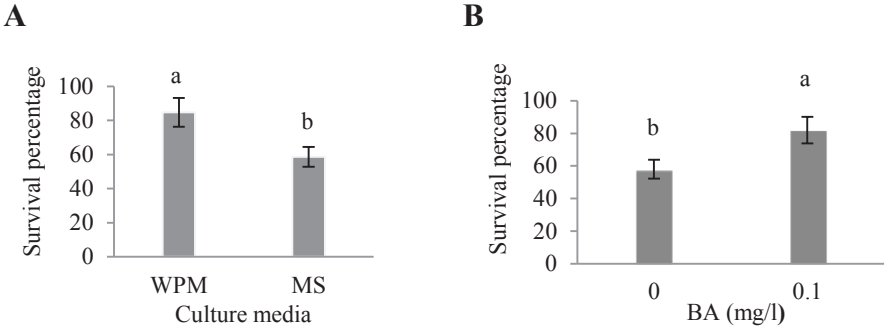

Figure 3. Effect of **A:** culture media and **B:** BA concentration on survival of Q. *brantii* explants.

Table 6. Analysis of variance on BA and IBA concentration effects on leaf number of Q. *brantii* explants.

Source of variation	df	Sum of square	Mean of square	F value	P
BA	5	585.451	117.090	10.83	<0.0001
IBA	1	34.776	34.776	3.22	0.077
BA×IBA	5	70.319	14.064	1.30	0.275
Error	68	670.081	10.808		

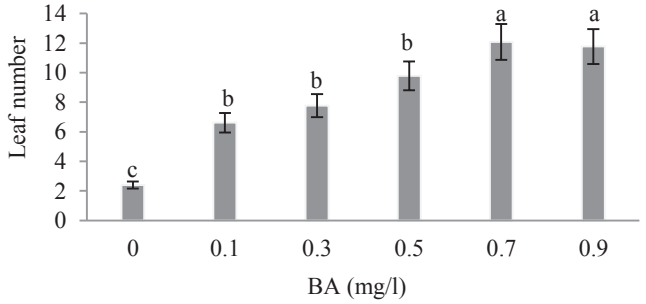

Figure 4. Effect of BA concentration on leaf number of Q. *brantii* explants.

Table 7. Analysis of variance on BA and IBA concentrations on shoot length of Q. *brantii* buds.

Source of variation	df	Sum of square	Mean of square	F value	P
BA	5	4.800	0. 960	3.46	0.008
IBA	1	0.294	0.294	1.06	0.307
BA×IBA	5	3.558	0.711	2.57	0.036
Error	62	17.194	0.277		

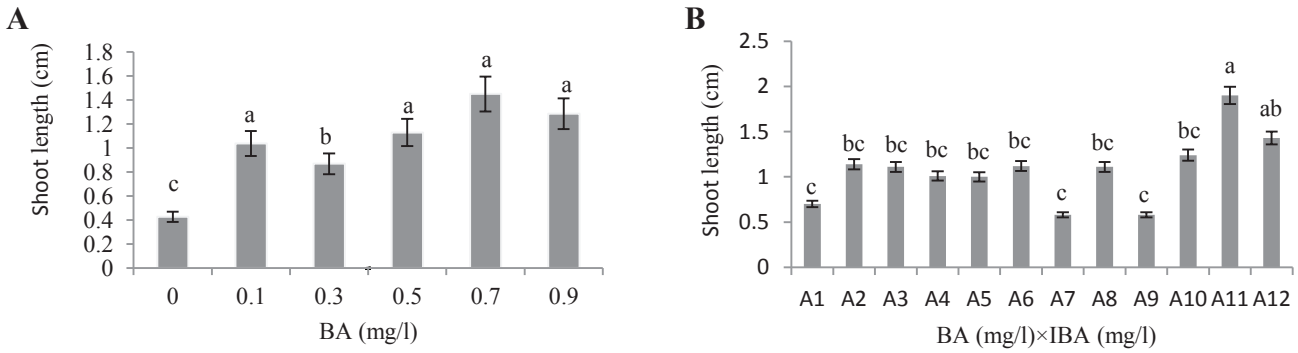

Figure 5. Effect of **A:** BA concentration and **B:** BA×IBA concentration on shoots length of Q. *brantii* buds.

Rooting

Culture medium and IBA had significant effects on root number but not on root length (Table 8). The highest root number was obtained in WPM with 1 mg/l IBA (Figure 7). The longest root was obtained in WPM culture medium containing 1 mg/l IBA too (Figure 7). Rooting and adaptation in the greenhouse is shown in Figures 8-B and C.

DISCUSSION

The results showed that using 0.1% $HgCl_2$ in the sterilization of explants was an appropriate treatment for eliminating contamination, but using more concentrations of $Hgcl_2$ caused explants browning and finally explants death. The method used for oak was different with previous investigation. For example,

Zamani *et al.* (2012) in a research on *Quercus castaneifolia* species showed that using 0.1% $HgCl_2$ for 2 minutes, was least lethal to the explants. Based on the result of micropropagation of *Q. brantii* and *Q. ruber* (Chalupa, 1984, Chalupa, 1993), the 0.1%-0.3% $HgCl_2$ solution for 20 to 40 minutes were suitable.

In the reported studies, $HgCl_2$ effectively had sterilized surface of lateral shoots in *Sequoiadendron giganteum* (Bon *et al*., 1988) and growing shoots of various hardwood species (Chalupa, 1987). $HgCl_2$ was more effective in eliminating contamination of *Populus* than calcium hypocholorite or H_2O_2 (Naujoks *et al*., 1987). 0.1% $HgCl_2$ in the sterilization of *Eucalyptus occidentials* explants, was suitable as well (Abravesh *et al*., 2013). Using 2-3 drops of tween 20 for *Eucalyptus globules* leads to the permeability of more sterilization solution (Emam and Asareh, 2010).

One method of preventing browning is to remove the phenolic compounds produced during several subcultures of explants, especially in the establishment stage (Torres, 2012). Due to the fact that phenol distribution starts from the very beginning hours, this method (Torres, 2012) of making a culture is not only time-consuming, but also more expensive. Hence, the reduction of oxidation potential with revival materials or antioxidants can reduce browning.

Cutting starts the production of polyphenol oxide that leads to tissue browning (Marks and Simpson, 1990). Often, to prevent browning, the explants are exposed to antioxidants after cutting (Compton and Preece, 1986). Establishment of *Brahylaena huillensis* in a culture medium containing 200 mg/l ascorbic acid, helped the survival of the explants up to 77% (Ndakidemi *et al*., 2014). In this study, putting the explants for an hour in a solution containing 100 mg/l ascorbic acid decreased phenoloxidase and increased survival. Based on the results of this study, it was obvious that due to the survival of explants there is a significant difference between MS and WPM cultures, and WPM cultures with low concentration of BA is suitable for primary establishment which is in agreement with other studies on oak, such as Vieteza

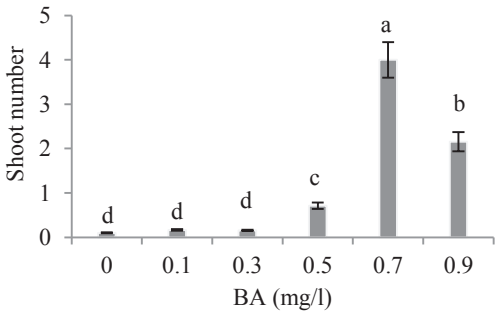

Figure 6. Effect of BA concentration on shoot number of Q. *brantii* cultured buds.

Table 8. Analysis of variance on modified culture media and IBA.

Character	Source of variation	df	Sum of square	Mean of square	F value	P
Root number	Culture medium and IBA	2	42.47	21.23	3.96	0.031
	Error	27	144.90	5.37		
Root length	Culture medium and IBA	2	23.89	11.94	2.75	0.082
	Error	27	117.24	4.34		

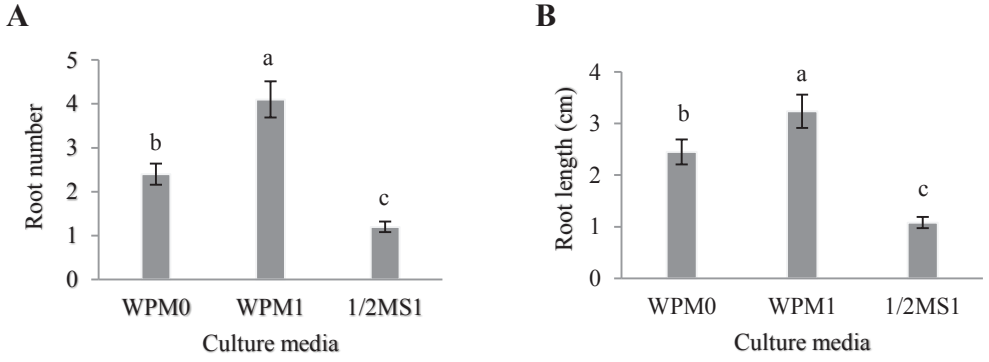

Figure 7. Effect of modified culture media and IBA on **A:** root number and **B:** root length of Q. *brantii* cultured buds.

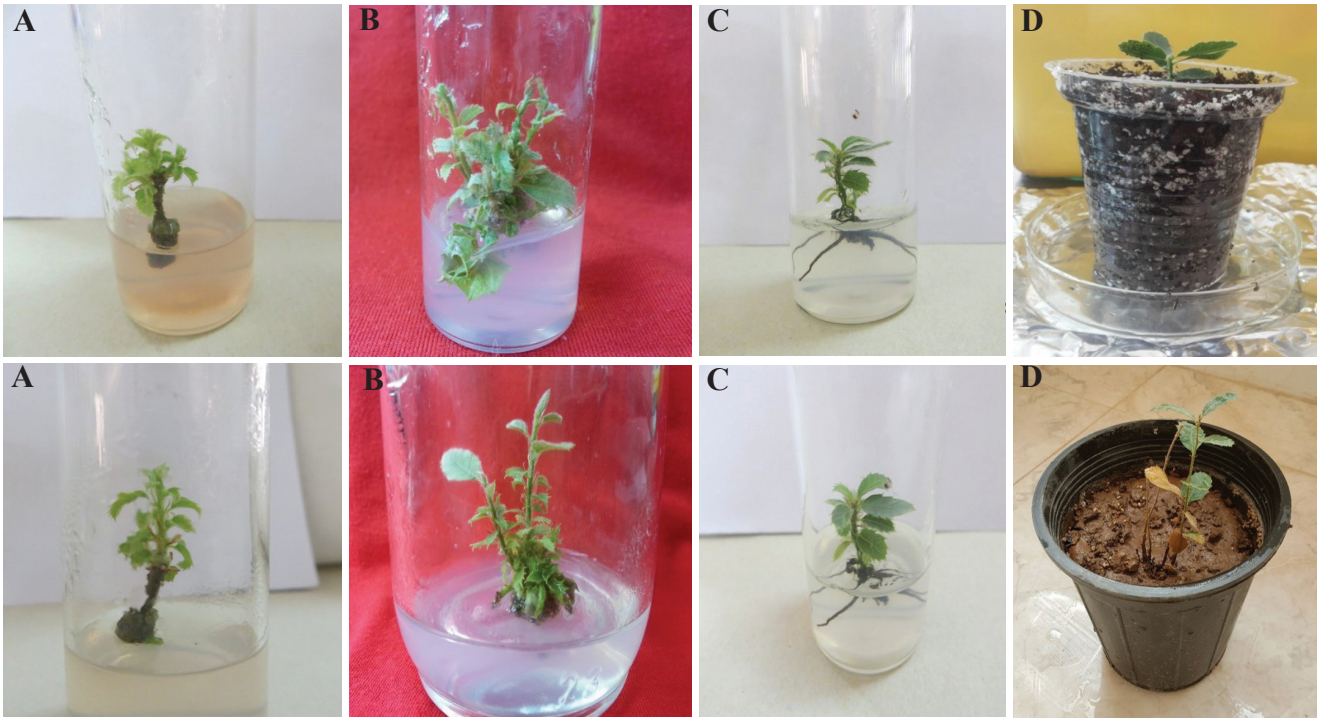

Figure 8. A: Establishment and elongation in WPM containing 0.1 mg/l BA and 0.01 mg/l IBA, **B:** proliferation in WPM culture containing 0.7 mg/l BA and 0.01 mg/l IBA, **C:** rooting in WPM containing 1 mg/l IBA and **D:** adaptation stage in peat and perlite at 2:1 ratio of Q. *brantii* cultured buds.

et al. (1985), Evers *et al*. (1993), Chalupa (1988), and Fayyaz *et al*. (2014), Zamani *et al*. (2012).

In this study, combinations of BA with 0.7 mg/l and IBA with 0.01 mg/l provided suitable solution of proliferation of Persian oak with the average of 4 shoots per explant, although cytokinin is said to be the appropriate plant growth regulator for proliferation of oak in many studies such as Ostroluck *et al*. (2007) and Zamani *et al*. (2012).

In low concentration of auxin improper rooting is usual, though in high concentrations no roots will grow (Pierik, 2011). Auxin with 0.01–10 mg/l concentration is added to the culture (Pierik, 2011). Based on the results of this study, using IBA had a successful rooting in WPM culture for Persian oak. The rooting in WPM had a significant advantage compared to half-strength MS culture medium. About 80% of explants in WPM containing 1 mg/l IBA started rooting. Fayyaz *et al*. (2014) obtained no results on the proliferation and reproduction of Persian oak. Zamani *et al*. (2012) reported that using 0.3 mg/l IBA produced the longest roots in *Quercus castaneifolia* species.

Totally, the results of this study showed an adequate potential of Persian oak for mass production by tissue culture in order to reconstruct Zagros oak forests and also for breeding tasks.

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