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The Impacts of Medium and Benzyladenine on Micropropagation and Micrografting of the Main Commercial Pistachio (*Pistacia vera* L.) Cultivars

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A R T I C L E I N F O

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A B S T R A C T

Micrografting can be used to produce healthy seedlings, assess scion-rootstock compatibility and promote precocity. The first objective of this project was to study the impacts of *in vitro* micropropagation of four major Iranian pistachio cultivars, namely 'Ahmad Aghaei', 'Akbari', 'Badami Sefid' and 'Kalle Ghochi' in two different medium Murashige and Skoog (MS) and Driver and Kuniyuki Walnut (DKW) at different concentrations of benzyl adenine (0, 0.5, 1 and 2 mg L^{-1}). The second objective of this project was to examine the effects of 'Badami-Riz-Zarand' and 'Akbari' rootstocks and micro-scion size (less than 5 and between 5-10 mm) on micrografting of the pistachio cultivars. The results showed that explants of the Akbari cultivar had the highest survival rate of meristems establishment in the DKW medium with 1 mg L^{-1} benzyl adenine. In the proliferation stage, 'Akbari' showed the highest values for proliferation rate, shoot number, shoot length and leaf number whereas, 'Kalle Ghochi' had the lowest values for these traits. Moreover, no significant differences were observed between 'Ahmad Aghaei', 'Akbari' and 'Badami Sefid' plantlets in terms of quality during the growing season. The micrografting results on seedlings showed that the scion of the Badami Sefid cultivar with a 5-10 mm size on the 'Badami-Riz-Zarand' rootstock had the highest grafting success rate and the lowest cultivar; in contrast, was 'Kalle Ghochi' with a scion less than 5 mm on 'Akbari'.

Introduction

The Pistachio (*Pistacia vera* L.) is a member of the Anacardiaceae family, which has dioecious plants with separate male and female trees as well as apetalous and unisexual flowers. The

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Anacardiaceae family is in the major group Angiosperms with 77 genera and 701 species recorded. The genus *Pistacia* has 13 species (The Plant List, 2013). The Pistachio (*Pistacia vera* L.) is the only commercially edible nut among the other species of *Pistacia* genus (Onay *et al.*, 2005). Micrografting has a high potential for reproducing horticultural products, such as improvement and rejuvenation of trees, production of virus-free plants by the meristem tip (Conejero *et al.*, 2013; Hussain *et al.*, 2014); prediction and detection of viruses through micrografting of the sensitive rootstock (Martínez-Gómez and Dicenta, 2000; Pathirana and McKenzie, 2005); rapid recognition of genetic incompatibility (Hussain *et al.*, 2014; Jonard *et al.*, 1990); *in vitro* reproduction of difficult-to-root woody plant species (Onay *et al.*, 2007); species reproduction and production of specific compounds (Puthra and Anil, 2002; Wong *et al.*, 2010); production of plants free of diseases, especially soil-borne diseases (Cambra *et al.*, 2008) and exchange of safe germplasm between countries (Makee *et al.*, 2004).

The current study was conducted to find the most suitable medium and concentration of growth regulators at the establishment and proliferation stages of commercial pistachio cultivars. The results also determines the micrografting success rate of these cultivars on different rootstocks under *in vitro* conditions.

Materials and Methods

In this study, *in vitro* micropropagation and micrografting abilities of four commercial pistachio cultivars (Ahmad Aghaei, Akbari, Badami Sefid and Kalle Ghochi) were evaluated in two factorial experiments with three variables based on the completely randomized design. The experimental work was carried out at the tissue culture laboratory of the Khorasan Razavi Agricultural and Natural Resources Research and Education Center.

Preparation of plant and disinfectant explants

The shoot tip of all the pistachio cultivars was cut (2 to 3cm in length) from trees with about 10-15 years of age at Faizabad Pistachio Research Station Orchards, Khorasan Razavi Province, Iran, and transferred to the lab. The explants were immersed under running tap water for 20 min and then re-immersed in water with a few drops of dishwashing liquid for 15 min. The buds were then placed under a laminar air flow for 60 seconds in 70% ethanol and rinsed three times with sterile distilled water. For surface disinfection, the buds were immersed in 10% sodium hypochlorite (NaClO) solution (1% active chlorine) for 10 minutes and then rinsed three times with sterile distilled water. In the final disinfection step, the buds were immersed in a 0.1% mercuric chloride solution for 5 min and then rinsed four times with sterile distilled water to remove any residual particulate matter (Benmahioul *et al.*, 2016; Titov *et al.*, 2006).

Preparation of culture medium

The MS (Murashige and Skoog, 1962) and DKW (Driver and Kuniyuki, 1984) medium, which was already successfully used for walnut (Hassankhah *et al.*, 2014; Ashrafi *et al.*, 2010), were used. In all the medium, pH was adjusted to 5.7 and sucrose was applied in 30 g L^{-1} and 6 g L^{-1} agar. The culture medium was sterilized in an autoclave at 121°C and 1.2 bar for 15min. To reduce phenol production by buds in the culture medium in addition to subculture, 200 mg L^{-1} ascorbic acid solution (Garcia *et al.*, 2012), was used after sterilization of the medium and before gelling and distribution in tubes by sterile syringe filter 0.22 μm.

Experiment 1: micropropagation of pistachio cultivars

A three-factorial experiment was laid out in a completely randomized design with five replications. The first factor was commercial pistachio cultivars at

four levels ('Ahmad Aghaei', 'Akbari', 'Badami Sefid' and 'Kalle Ghochi'), the second factor was culture medium at two levels (MS and DKW) and the third factor was BA concentrations at four levels (0, 0.5, 1 and 2 mg L^{-1}). In this experiment, the bud-scale leaves were removed using a scalpel, and meristem explants (apical meristem with 2-3 leaf primordial) were cultured in various medium at different BA concentrations. The test tubes were completely sealed with parafilm to prevent contamination and placed in a growth chamber at 25°C ± 2°C under a 16/8 light/dark photoperiod provided by cool-white fluorescent lamps with 2000 lux light intensity. The percentage of explants survival (ratio of active meristem to total explants) was recorded four weeks after cultivation.

The established meristems of the pistachio cultivars, obtained in the first stage, were cultured in the MS and DKW medium at different BA concentrations of 0, 0.5, 1 and 2 mg L^{-1}. The cultivation conditions were the same as previously described. After three subcultures (20, 40 and 60 days), quality explants (3= normal green leaves, 2=light green leaves and 1=green-yellow leaves) (Abbasi *et al.*, 2018), leaf number, shoot number, shoot length and proliferation rate (number of small samples produced per primary active meristem) were recorded every 20 days.

Experiment 2: micrografting success rate

In this experiment, the first factor was the type of scion obtained from the plantlet of the cultivars in the previous stage. The second factor was the rootstock type consisting of the seedling of two rootstocks ('Akbari' and 'Badami-Riz-Zarand'), which was obtained through *in vitro* culture. The third factor was scion size at two levels less than 5 mm and between 5-10 mm. The culture medium and concentrations of growth regulators used in this stage were prepared according to the results of the first experiment in the proliferation stage.

Mature dry seeds of the Akbari and Badami-Riz-Zarand cultivars, after the removal of exocarps and endocarp shells, were disinfected, as in the first experiment. Then, the half kernel with an embryo was placed in a MS medium and closed.

Micrografting was initiated after the stem reached the midpoint of the tube. After cutting the rootstock with sterile scissors and removing the leaves and buds, the scion base, cut in V-shape and was fitted into the slit. The grafted plants were placed in a growth chamber at 25°C ± 2°C under a 16/8 light/dark photoperiod provided by cool-white fluorescent lamps with 2000 lux light intensity. After four weeks, the grafting success rate was evaluated.

Statistical analysis

The experiments were carried out in a completely randomized design with three treatment factors in a factorial design with five replications, each containing six explants. The obtained data were analyzed using the SAS 9.4 statistical software. Comparisons between means were performed with Duncan's multiple range tests at p values ≤ 0.05. A value of $p < 0.05$ was considered statistically significant.

Results

Micropropagation of the pistachio cultivars

The results showed that the difference between the cultivars was statistically significant in the establishment stage of the pistachio cultivars' shoot tips. After four weeks, the highest survival rate of meristems was observed in the Akbari cultivar on the DKW medium containing 1 mg L^{-1} BA. Moreover, the DKW medium yielded better results than the MS medium. The results also indicated that the use of the BA growth regulator at 1 mg L^{-1} concentration had the highest effect among all

the regulators on the survival rate of meristems. In comparison, the BA concentrations of 0.5 and 2 mg L^{-1} revealed no significant difference (Table 1).

Among all the cultivars, the Akbari and Kalle Ghochi cultivars had the highest and lowest proliferation rates of 5.4% and 4.5%, respectively (Table 1). Moreover, the MS medium containing 1 mg L^{-1} BA showed better results in producing more plantlets (Fig. 1).Based on the obtained results, the Akbari, Badami Sefid, Ahmad Aghaei, and Kalle Ghochi cultivars have the highest to lowest effect on the shoots number, in the order of their appearance. Accordingly, the Akbari cultivar had the highest number of shoots among all the cultivars with an average of 2.25 shoots per plantlet. Moreover, the MS medium showed better results compared to the DKW medium. The results also revealed that the BA growth regulator was more effective in producing shoots at the 2 mg L^{-1}concentration compared to other levels (Table 1).

Table 1. The influence of cultivar, medium, and BA on the survival rate of meristem, quality, leaf number, shoot number, shoot length, and proliferation rate.

Treatment	Survival rate of meristem (%)	Proliferation rate	Shoot number	Shoot length (mm)	Leaf number	quality
Cultivar						
Ahmad Aghaei	45.41 ab	4.78 bc	2.12 bc	22.52 b	4.38 b	3.05 ab
Akbari	48.32 a	5.42 a	2.25 a	23.82 a	4.95 a	3.20 a
Badami sefid	42.90 bc	4.85 b	2.18 b	22.70 b	4.42 b	3.15 ab
Kalle Ghochi	39.98 c	4.48 c	2.08 c	20.82 c	3.70 c	2.90 b
Medium						
MS	41.24 b	5.21 a	2.31 a	23.76 a	4.68 a	3.32 a
DKW	47.08 a	4.55 b	2.00 b	21.18 b	4.05 b	2.82 b
BA						
0	34.98 c	2.45 d	1.00 d	10.72 d	2.32 c	1.00 c
0.5	41.66 b	4.70 c	2.12 c	23.55 c	4.08 b	3.00 b
1	59.17 a	6.95 a	2.50 b	25.50 b	5.58 a	4.20 a
2	40.82 b	5.42 b	3.00 a	30.10 a	5.48 a	4.10 a
Significance						
Cultivar	**	**	**	**	**	NS
Medium	**	**	**	**	**	**
Cultivar* Medium	**	NS	**	*	**	*
Cultivar* Medium*BA	**	NS	**	NS	**	NS

Significance: Significant at 1% level if shown by **, at 5% level if shown by *, and no significant if shown by NS.
Mean separation within columns in each group of treatments by DUNKAN at 5% level or less.

According to the results obtained in terms of the length of shoots in the proliferation stage, the Kalle Ghochi cultivar on the DKW medium in the absence of BA (Fig. 2a), and 'Akbari' on the MS medium containing 2 mg L^{-1} BA (Fig. 2b), had the lowest (8 mm) and highest (34 mm) shoot length, respectively. In this regard, similar to that for shoot number, the MS medium with 2 mg L^{-1} BA generated the highest shoot length (Fig. 3).

The results demonstrated that the highest and lowest numbers of leaves belonged to the Akbari and Kalle Ghochi cultivars, respectively, Moreover, the MS medium showed better results compared to the DKW medium in leaf number (Table 1). BA at the concentration of 1 mg L^{-1} showed a higher effect than other levels on this variable, leading to the production of 5.6 leaves per plantlet. In contrast, the lack of using BA had an adverse effect on leaf generation in plantlets, causing to generate only 2.3 leaves per plantlet (Fig. 4).

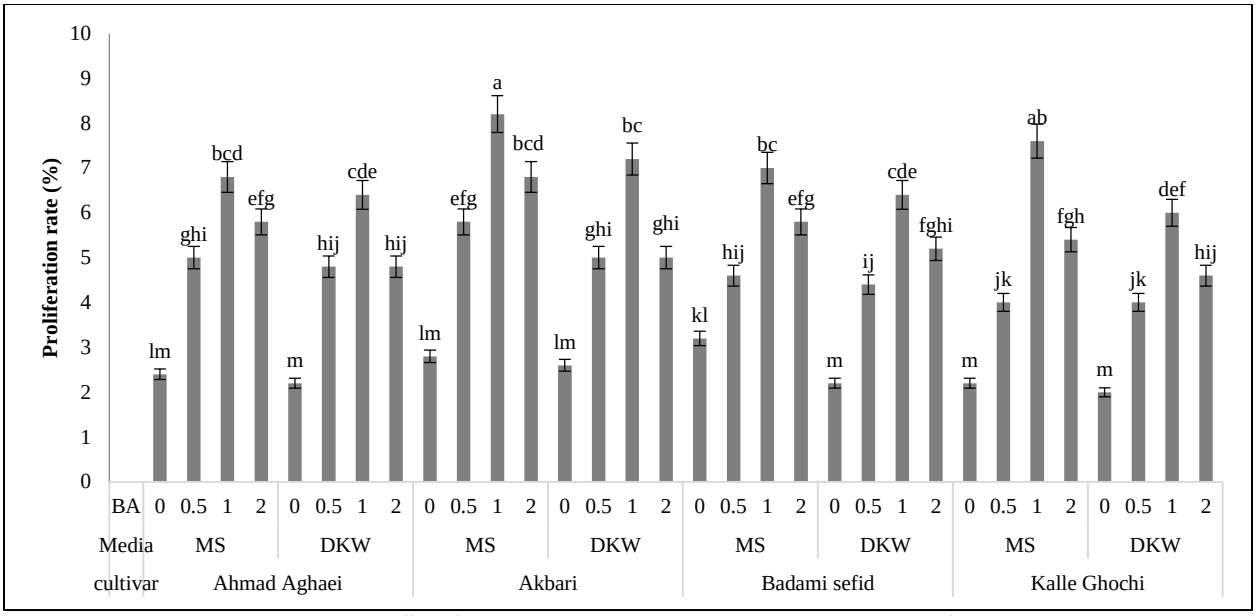

Fig 1. The interaction effect of cultivar, medium and BA concentration on the average proliferation rate.
(Mean values followed by the same letter are not significantly different at P≤0.05, as shown by Duncan's multiple range test.)

Observations of the quality of plantlets showed that they had high quality and natural green leaves after the use of the MS medium containing 1 mg L^{-1} BA. However, plantlets had yellowish-green leaves in the absence of BA, which turned yellow. In addition, the Ahmad Aghaei, Akbari and Badami Sefid cultivars showed no significant differences in terms of plantlet quality (Table 1).

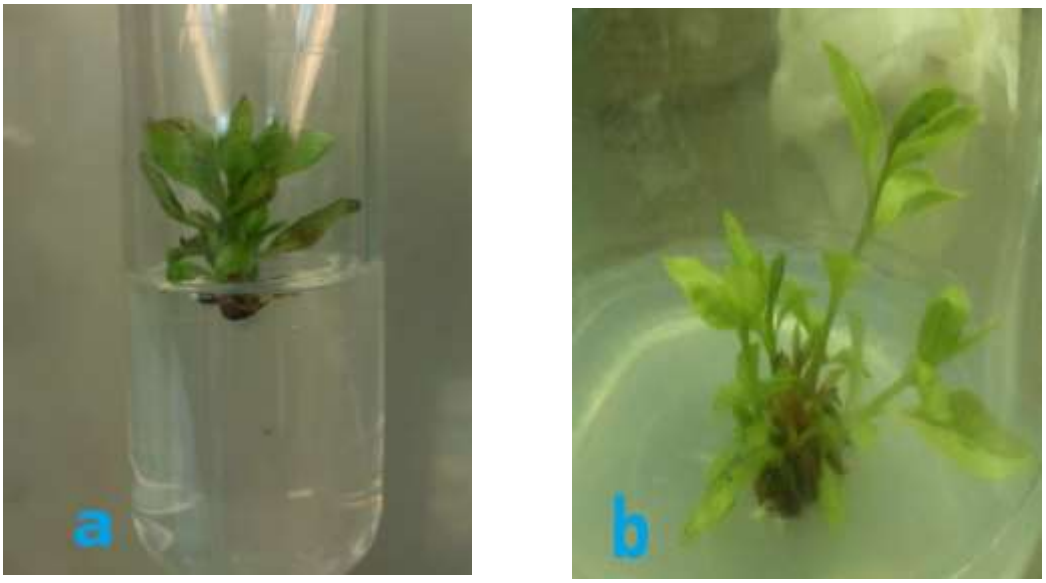

Fig. 2. The proliferation of pistachio cultivars (shoots number and shoots length)
a) DKW medium in the absence of BA b) MS medium containing 2 mg L^{-1} BA

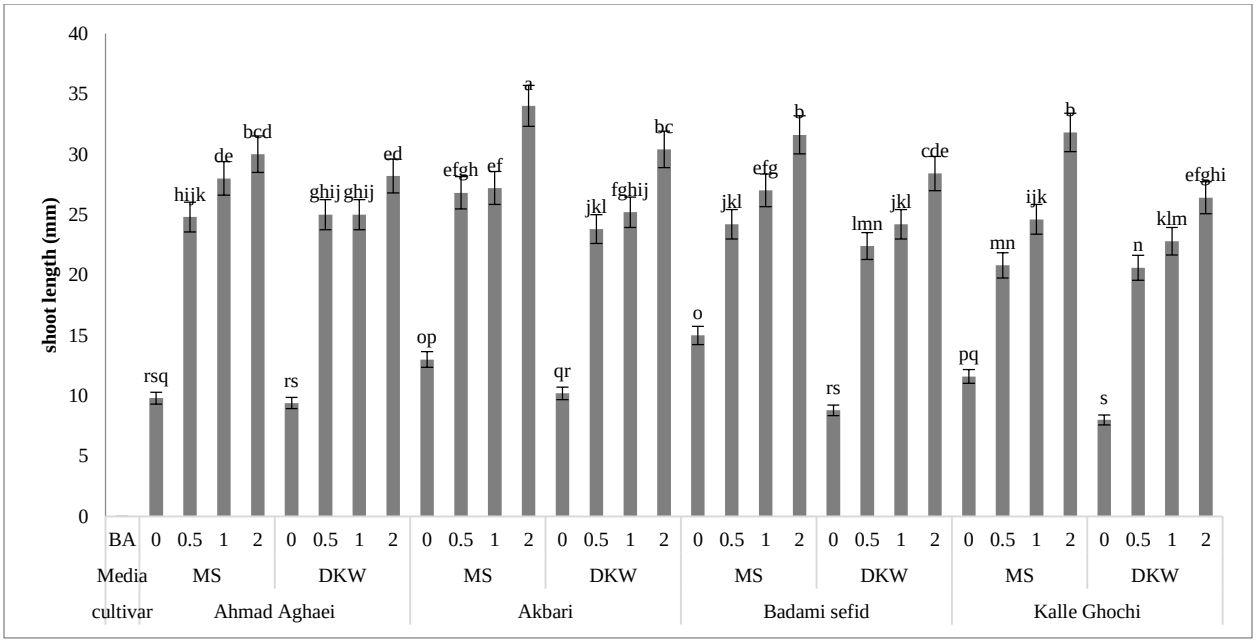

Fig. 3. The interaction effect of cultivar, medium, and BA concentration on the average shoot length.
(Mean values followed by the same letter are not significantly different at P≤0.05, as shown by Duncan's multiple range test.)

Micrografting

The results of micrografting of the cultivars showed that Badami Sefid (69.0%) and Kalle Ghochi (30.2%) cultivars had the highest and lowest grafting percentage, respectively. *In vitro* seedlings of Badami-Riz-Zarand and Akbari cultivars used as rootstocks showed significant differences in grafting, with the highest (56.6%) and lowest micrografting (40.9%) success rates, respectively (Table 2). Comparison of the means showed that Badami Sefid cultivar with the 5-10 mm scion size grafted onto Badami-Riz-Zarand rootstock with 83.3% grafting percentage and Kalle Ghochi cultivar with the scion size of less than 5 mm grafted onto Akbari cultivar with 16.7% grafting percentage had the highest and lowest micrografting success rates, respectively (Table 3).

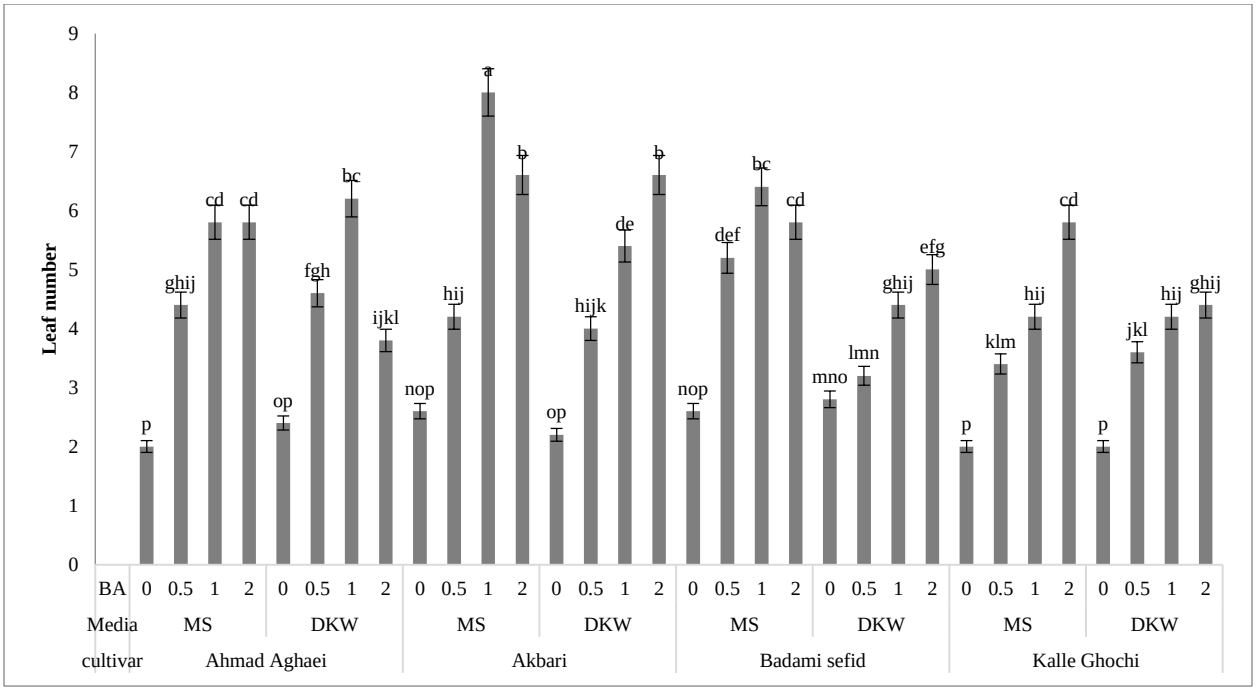

Fig. 4. The interaction effect of cultivar, medium, and BA concentration on the average leaf number.
(Mean values followed by the same letter are not significantly different at P≤0.05, as shown by Duncan's multiple range test.)

Table 2. The influence of cultivar, rootstock, and micrografting on success rate.

Treatment	success rate
Cultivar	
Ahmad Aghaei	39.66 c
Akbari	56.19 b
Badami sefid	69.03 a
Kalle Ghochi	30.22 d
Rootstock	
Akbari	40.89 b
Badami Riz Zarand	56.65 a
Scion size	
≤ 5 mm	43.36 b
5-10 mm	54.18 a
Significance	
Cultivar	**
Rootstock	**
Cultivar* Rootstock	*
Cultivar* Rootstock * Scion size	*

Significance: Significant at 1% level if shown by **, at 5% level if shown by *, and no significant if shown by NS.
Mean separation within columns in each group of treatments by Dunkan at 5% level or less.

Table 3. The interaction effect of cultivar, rootstock and scion length on the average of micrografting success rate.

Cultivar/rootstock	'Akbari'		'Badami Riz Zarand'	
	≤ 5 mm	5-10 mm	≤ 5 mm	5-10 mm
Ahmad Aghaei	30.0 hi	36.6 fg	42.0 ef	50.0 cd
Akbari	47.0 de	53.3 c	54.4 c	70.0 b
Badami sefid	50.0 cd	69.3 b	73.5 b	83.3 a
Kalle Ghochi	16.7 j	24.2 i	33.3 gh	46.7 de

(Mean values followed by the same letter are not significantly different at P≤0.05, as shown by Duncan's multiple range test.)

Discussion

In pistachio orchard development, the use of seedlings during cultivation and grafting in the following years will cause delayed fruiting and return on capital, non-uniform orchard, etc. Despite some studies conducted on pistachio micropropagation (Onay *et al.*, 2016, 2004; Tilkat *et al.*, 2008; Yildirim *et al.*, 2019), the current study attempted to fill the gap by finding an appropriate protocol for pistachio micropropagation and determining the most successful scion-rootstock combination in the micrografting of cultivars.

The results showed that the survival rate of meristems (establishment stage) was higher in the DKW medium than in the MS medium; however, the MS medium was more effective in the propagation of plantlets in the proliferation stage compared to the DKW medium. The difference may be due to the concentrations of substances present in the two medium. The results also revealed that the concentrations of NO^{3-}, NH^{4+}, K^{+}, and Cl^{-} ions were higher in the MS medium than in its counterpart medium. In comparison, in the DKW medium, the concentrations of SO_4^{2-} and Ca^{2+} ions were more than six and about three times those in the MS medium (PhytoTechnology Laboratories, 2016). Moreover, the Ca^{2+}/K^{+} ratio was 6.7 and 2.1 in the MS and DKW medium, respectively (Garoosi *et al.*, 2016). The MS makes a good plant regeneration medium because of the high levels of nitrogen in both nitrate and ammonium forms, with a relatively high ratio of ammonium to nitrate (Phillips and Garda, 2019). It appears that the number of minerals in the culture medium can improve the growth rate and prevent the necrosis of leaves and shoots of pistachio cultivars (Dolcet-Sanjuan and Claveria, 1995). Beside minerals, the effect of carbon source on micropropagation of woody plants has been also reported (Moradnezhad *et al.*, 2017). Our results are consistent with the findings of those who reported that the MS medium was more effective in proliferation (Husain and Anis, 2009; Yıldırım *et al.*, 2018). However, DKW was reported to perform better than MS in pear and walnut micropropagation (Bell *et al.*, 2009; Ashrafi *et al.*, 2010; Hassankhah *et al.*, 2014).

Results obtained from the effects of different BA levels in the medium demonstrated that the 1 mg L^{-1} concentration had a higher impact than the other levels of variables, including survival rate, leaf number, proliferation rate, and plantlet quality. This result is consistent with the results of (Benmahioul *et al.*, 2016; Yıldırım *et al.*, 2018). In contrast, (Abbasi *et al.*, 2018), reported that 2 mg L^{-1} concentration of this medium contributed to the further generation of leaves and improved plantlet quality. Based on the results of the present study, the 2 mg L^{-1} concentration led to the generation of more shoots with greater length, in agreement with the results of (Shi, 2014). However, in other studies the MS medium with 1 mg L^{-1} BA generated the highest number of shoots with maximum length in micropropagation of lentisk (*P. lentiscus*),

(Yildirim *et al.*, 2019) and Iranian Melon (*Cucumis Melo* L. 'Samsoori') (Naderi and Mahmoudi, 2017). BA, as the most widely used cytokine in the last five years (Phillips and Garda, 2019), stimulates shoot growth and production by breaking apical dominance and completely or partially inhibiting root formation. However, one study reported that although elevating BA concentration in pistachio proliferation would increase shoot number, it would lead to vitrification of plantlets (Onay, 2000), The effect of BA on vitrification of explants was also reported in micropropagation of *Gerbera jamesonii* (Nazari *et al.*, 2016). In this regard, various factors such as source of explant, type of culture medium, growth regulators, type and concentration of elements, different types of vitamin and salt in the culture medium, temperature etc., are involved in the success rate of the proliferation stage (Avestan *ET AL.*, 2018; Benmahioul, 2017; Zarei *et al.*, 2017; Pourkhaloee and Khosh., 2015; Leng *et al.*, 2009).

The response of cultivars was different in this experiment. Based on the obtained results, Akbari cultivar had the best results in terms of meristem survival rate, proliferation rate, shoot number, shoot length, leaf number, and quality. In comparison, the Kalle Ghochi cultivar had the least impact such that it was four times less effective compared to the Akbari cultivar (the proliferation rate of 2 against 8.2). The best results were observed in the proliferation stage of the Akbari cultivar in the MS medium containing 1 mg L^{-1} BA. The results of the establishment and proliferation stages indicated various responses of the cultivars to micropropagation, depending on the genetic differences of each cultivar and their ability to absorb substances. According to (Kishore *et al.*, 2015), the plant's ability to absorb substances is more important than the number of elements existing in the medium.

The results of pistachio micrografting showed that selecting proper rootstocks and scions with appropriate sizes had a significant effect on grafting efficiency and would contribute to a higher micrografting success rate. In this experiment, the highest micrografting success rate was found to be 83.3%, which belonged to the Badami Sefid cultivar with the scion size of 5-10 mm grafted onto the Badami-Riz-Zarand cultivar. A key to micrografting success is the enhancement of rootstock-scion compatibility (Hussain *et al.*, 2014; Jonard *et al.*, 1990; Onay, 2003). Other researchers also believe that micrografting success depends on the type of rootstocks (Kanwar *et al.*, 2019). Evaluating different rootstocks for a certain scion enhances the chance of grafting success, which will greatly contribute to commercializing the micrografting method for mass production. By changing rootstocks, (Tangolar *et al.*, 2003) succeeded in increasing the grape micrografting rate from 26.1% to 80%. In addition to rootstock type, scion size will also enhance the chance of micrografting success (Onay *et al.*, 2004; Wu *et al.*, 2007), although some researchers do not have a similar view (Yıldırım *et al.*, 2010). Other results showed that the best scion size for pistachio was greater than 6 mm (Onay *et al.*, 2007), which is consistent with the results of our experiments.

Conclusions

According to the results, pistachio cultivars can be propagated and grafted *in vitro*. The use of a DKW medium in the establishment stage and an MS medium in the proliferation stage with a 1 mg L^{-1} BA, will result in pistachio micropropagation success. The results also revealed the Akbari cultivar as the best cultivar for the *in vitro* propagation of pistachios. In addition, the grafting percentage showed significant differences in micrografting concerning the type of cultivar and rootstock as well as scion size. To achieve a high micrografting success rate, it is recommended using the Badami Sefid cultivar with the scion size of 5-10 mm grafted onto the Badami-Riz-Zarand cultivar.

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