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An improved micropropagation protocol for lentisk (*Pistacia lentiscus* L.)

***Hakan Yildirim*[1]*, Ahmet Onay*[2]*, Kazim Gunduz*[1]*, Sezai Ercisli*[3]**, Firat Ege Karaat*[1]**

[1] Turgul Ozal University, Agriculture Faculty, Horticulture Department, Malatya, Turkey
[2] Dicle University, Faculty of Science, Biology Department, Diyarbakır, Turkey
[3] Ataturk University, Agriculture Faculty, Horticulture Department, Erzurum, Turkey

ABSTRACT

This study presents an efficient improvement in the in vitro propagation protocol for one cloned genotype of lentisk (*Pistacia lentiscus* L.) by assessing the effects of gibberellic acid (GA_3) concentrations, different cytokinins and amino acids and their concentrations on shoot proliferation, the effects of shoot length on rooting and the effects of compost type (sterile and non-sterile) on acclimatization. The best growth medium for multiple shoot induction was the MS medium supplemented with a combination of 1 mg l^{-1} BA, 100 mg l^{-1} tryptophan and 0.5 mg l^{-1} GA_3, which gave a mean shoot length of 1.64 ± 0.07 cm and a mean bud number of 5.46 ± 0.16. The best results in terms of root length, rooting rate and the number of roots per shoot were obtained with 2 cm long shoots. The rooted plantlets were readily acclimatized in the sterile compost. In conclusion, the micropropagation protocol developed in this study can be used for large-scale propagation of *P. lentiscus* L. in reforestation programmes.

Key words: acclimatization, amino acid, cytokinins, gibberellic acid, *in vitro*, rooting rate, shoot proliferation

INTRODUCTION

Some fruit crops are easier to grow and hardy in nature, producing a crop even under adverse soil and climatic conditions. Such crops play a vital role in the nutrition and livelihood of, in particular, rural and ethnic populace for income generation and employment (Vijayan et al., 2008; Ercisli et al., 2012; Cüce and Sökmen, 2017)

Lentisk, *Pistacia lentiscus* L., is one of the 14 species of the genus *Pistacia*. It grows naturally in the Mediterranean countries, especially in Greece, Turkey, Italy and Spain (Onay et al., 2016a). The specific growing areas of the tree in Turkey are along the coastline of the Aegean and Mediterranean parts. Lentisk trees grow at elevations between the sea level and up to 2000 m, having tolerance to drought, cold and high soil lime content (Zohary, 1952; Correia and Catarino, 1994; Ak and Parlakci, 2009; Al-Saghir and Porter, 2012). The lentisk tree is a unique source of mastic gum, which is collected from incisions made in the trunk and thick branches of male trees (Koc et al., 2014). The mastic gum is an important raw material, especially to the food and pharmaceutical industries, and so it significantly supports the areas where lentisk trees are grown economically (Yildirim, 2012). For example, the resin is so commonly produced on Chios Island that the island is identified with the gum and called by its

*Corresponding author.
e-mail: sercisli@gmail.com (Sezai Ercisli).

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name (Onay et al., 2016a). However, there are some difficulties in the cultivation of lentisk because of the lack of effective propagation methods for the species (Akdemir et al., 2013).

Lentisk trees are propagated from seeds or cuttings, but usually seeds are preferred since adventitious root formation in cuttings is usually very poor. On the other hand, when seeds are used for multiplication, the beneficial characteristics of parents are diminished in the next generations because of genetic alterations. Furthermore, when female trees are obtained (about 50%), they are not productive for harvesting mastic gum (Onay et al., 2016a). All those reasons had brought the necessity to develop biotechnological methods for *in vitro* propagation of lentisk. Despite this fact, efforts on the development and improvement of *in vitro* propagation protocols for lentisk had been insufficient until 2004 when the first reports were published (Fascella et al., 2004; Ruffoni et al., 2004). Thereafter, Taskin and Inal (2005) and Mascarello et al. (2007) reported on *in vitro* clonal multiplication of lentisk. An interesting study on an *in vitro* micropropagation protocol set up from seedlings was reported by Yildirim (2012). Recently, Kilinc et al. (2014) tested an *in vitro* micropropagation protocol for four genotypes by assessing the influence of different media types and their strengths, and media supplementation with different carbohydrates. In that study, the full-strength MS medium containing 1 mg l^{-1} BA and 30 g l^{-1} sucrose produced the highest number of shoots per explant (3.62).

More recently, a study was conducted to determine the effects of gibberellic acid (GA_3) and N6-benzyladenine (BA) on *in vitro* micrografting developed to produce *P. lentiscus* plantlets (Onay et al., 2016b). Furthermore, different studies have shown that cytokinins, GA_3 and some amino acids can improve cell growth and enhance organ development (Baskaran and Jayabalan, 2007; Kumar and Kumari, 2010; Unek et al., 2010; Malá et al., 2013; Gonbad et al., 2014; Sarropoulou et al., 2014).

Here, for the first time, we compare five cytokinins and five amino acids used alone, and different concentrations of GA_3 in combination with the cytokinin BA at 1 mg l^{-1} to determine their effects on micropropagation. In this context, the aim of this study was to improve the previously developed micropropagation protocols for commercial propagation of juvenile shoot tips of *P. lentiscus* L.

MATERIAL AND METHODS

General culture conditions and plant material

Lentisk (*Pistacia lentiscus* L.) fruits were collected from non-cultivated trees grown in the districts of the İzmir Province, Turkey. The *P. lentiscus* fruit consists of a rather small nutmeat (kernel) enclosed in a thin, hard shell surrounded by a fleshy black hull when it has matured. The fleshy black hulls were removed when the fruits were harvested. The kernels contained in the hard shell (endocarp) were germinated according to the protocol reported by Yildirim (2012). Shoot tips or nodal bud segments obtained from the germinated seeds were proliferated in order to obtain stock cultures for the development of the *in vitro* propagation protocol. Cultures were subcultured every 4 weeks and survived for 4 years unde*r vitro* conditions. Morphological studies were performed by visual observation on a 4-week basis. Growth parameters were recorded to document the changes in the developing plantlets. The chemicals used for the experimental assays were high-grade analytical reagents (Sigma Chemical Co., USA).

Unless stated otherwise, the MS (basal MS) medium containing 3% sucrose (w/v) (S5391, Sigma Ltd.) and solidified with agar (0.65%, w/v) (A1296, Sigma Ltd.) was used for all the parameters studied in the following experiments; the explants were regularly subcultured every 4 weeks in a new proliferation medium in order to eliminate the residuals of growth regulators before studying a new parameter. The pH of the medium was adjusted to 5.8 and the medium was autoclaved at 121°C for 20 minutes. Plant growth regulators (PGRs) were added to the medium prior to the adjustment of pH and sterilization. Cultures were maintained at 25°C ± 2°C under a 16/8-h light/dark photoperiod provided by cool-white fluorescent lights (Osram) with an irradiance of 40 µmol photons m^{-2} s^{-1}. The average length of shoots and the number of proliferated shoots were recorded after 28 days of culture.

Shoot proliferation

Effects of different cytokinins

BA (N6-benzyladenine), TDZ (thidiazuron), 2iP (2-isopentyladenine), DiK (dikegulac) and KIN (kinetin) each at 1 mg l^{-1} were tested individually against a control treatment (without cytokinin) in the full strength MS medium to produce numerous shoots and promote further growth and development of these shoots.

Effects of GA_3

MS media were supplemented with GA_3 used in five different concentrations: 0.1, 0.3, 0.5, 0.7, 1.0 mg l^{-1} combined with 1 mg l^{-1} BA. The effects of the different concentrations were used to compare the treatments in terms of shoot number, bud number and shoot length.

Effects of different amino acids and their concentrations

Individual explants 1 cm in height were cultured on the MS medium supplemented with various amino acids (valine, tryptophane, alanine, leucine, methionine), which were added separately at multiple concentrations (0, 25, 50 and 100 mg l^{-1}) with 1 mg l^{-1} BA. After 4 weeks in culture, the total number of shoots, the total number of buds and the length of the shoots per explant were recorded.

Effects of shoot length on rooting

For rooting, healthy developed shoots (1, 2, 3, 4 and 5 cm in length) harvested from the 3rd or 4th sub-culture onward were used. The microcuttings were transferred into a rooting medium consisting of MS medium with 1 mg l^{-1} IBA, 3% (w/v) sucrose and 0.55% (w/v) agar. After 4 weeks in culture, the rooting rate (%), number of roots and root length (cm) per microcutting were recorded.

Acclimatization

The acclimatization experiments were conducted in a growth room to evaluate the effects of sterile and non-sterile composts on the survival percentage of *P. lentiscus* plantlets during the acclimatization stage. The plantlets (4-6 cm in length, with 4-5 leaves) produced *in vitro* were rinsed with running tap water to remove any adhering medium to reduce mortality in the micropropagated lentisk plantlets. Then, they were individually transplanted into 7 cm plastic pots containing a 1:1 (v/v) perlite and peat mixture enriched with a quarter strength MS salt solution, covered with a polyethylene bag or a transparent polythene bag to maintain high relative humidity during hardening for 5 weeks, and placed in the growth room at 25°C ± 2°C under a 16/8-h light/dark photoperiod provided by cool-white fluorescent lights (Osram) with an irradiance of 40 µmol m^{-2} s^{-1} for 5 weeks. Five holes (less than 1 mm in diameter) were made in the transparent polythene bags 2 days after transferring the pots to the growth room, and the number of holes was doubled each day. The five-week period of acclimatization involved progressive reduction in humidity from almost 95% to 65%. The plantlets were irrigated every 2-3 days with water. The hardened plantlets were transferred to earthen pots in the growth room. Each treatment consisted of 20 replicates. After five weeks and also after 2 years, the data on the number of viable regenerants per plantlet were recorded.

Statistical analysis

Statistical analysis was performed by using SPSS Statistics version 13.0 software package. Experiments were carried out in a completely randomized block design. Unless otherwise stated, all of the experiments were repeated twice and with at least 25 shoot tips in the shoot proliferation experiments and 15 shoots in the rooting experiment in each repetition. Data on the number and length of shoots and the number of buds obtained through shoot tip proliferation, and the percentage of rooted explants, the number of roots as well as root length were expressed as means ± standard error. Comparisons between groups were performed with Duncan's multiple range tests as a post-hoc comparison of statistical significance (p values ≤ 0.05). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Influence of cytokinins on shoot proliferation

Among the five cytokinins tested at the same concentration, the cytokinin BA was more effective than TDZ, DiK or kinetin, as it induced better results in terms of shoot number (2.64 ± 0.41) and shoot length (1.44 ± 0.06 cm), and the quality of developing shoots (Tab. 1). Similarly, in other *in vitro* studies which had been performed by using either juvenile or mature axenic material of other

Table 1. Effects of different cytokinins on shoot proliferation*

Cytokinin type (1.0 mg l^{-1})	Shoot number**	Shoot length** (cm)
Control	1.18 ± 0.10 d	0.85 ± 0.03 d
BA	2.64 ± 0.41 a	1.44 ± 0.06 a
Kinetin	1.61 ± 0.16 bc	1.20 ± 0.03 b
TDZ	2.20 ± 0.13 ab	1.16 ± 0.04 bc
2iP	1.50 ± 0.14 c	1.05 ± 0.04 c
Dikegulac	1.77 ± 0.17 b	1.10 ± 0.04 bc

*Data were collected by using at least 25 shoot tips per treatment on day 28 of culture

**Differences between values followed by the same letters in a column are not significant according to Duncan's multiple comparison test at $p \leq 0.05$

Table 2. Effects of different concentrations of GA_3 and 1 mg l^{-1} BA on the proliferation of lentisk shoots*

Treatment	Shoot number**	Shoot length** (cm)	Bud number**
Control (1 mg l^{-1} BA)	2.72 ± 0.22 b	1.35 ± 0.06 ab	5.40 ± 0.17 ab
1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3	3.16 ± 0.26 ab	1.33 ± 0.05 ab	5.45 ± 0.13 ab
1 mg l^{-1} BA + 0.3 mg l^{-1} GA_3	3.36 ± 0.23 ab	1.18 ± 0.05 b	5.36 ± 0.11 ab
1 mg l^{-1} BA + 0.5 mg l^{-1} GA_3	3.56 ± 0.30 a	1.43 ± 0.06 a	5.87 ± 0.16 a
1 mg l^{-1} BA + 0.7 mg l^{-1} GA_3	3.24 ± 0.15 ab	1.32 ± 0.05 ab	5.41 ± 0.15 ab
1 mg l^{-1} BA + 1.0 mg l^{-1} GA_3	3.12 ± 0.16 ab	1.22 ± 0.04 b	5.29 ± 0.11 b

*Data were collected by using at least 25 shoot tips per treatment on day 28 of culture
**Differences between values followed by the same letters in a column are not significant according to Duncan's multiple comparison test at $p \leq 0.05$

species of the genera *Pistacia*, among which lentisk was also included, the effects of different cytokinins and their concentrations had been examined. *In vitro* propagation studies of axenic shoots obtained from *P. vera* seeds were performed by Barghchi (1982). In the proliferation studies performed by Barghchi (1982), Abousalim (1990) and Onay (2000), it was reported that BA gave better results when compared with KIN, and that 4 mg l^{-1} was an optimal dose for the proliferation of *P. vera* shoots. In a study reported by Tilkat et al. (2008a), among the cytokinin types which were tested for maximum proliferation of shoots obtained from apical and lateral buds of 25-year-old male pistachio trees, BA performed better than kinetin and TDZ, and in shoot proliferation studies 0.25-2.0 mg l^{-1} BA gave optimum shoot propagation. Martinelli (1988) had suggested that low concentrations of BA would be enough for the proliferation of explants taken from 4-year-old *P. vera* plants. Another researcher, Pontikis (1984), had determined that low concentrations of BA (0.7 mg l^{-1} in *P. atlantica*, 1 mg l^{-1} in *P. integerrima* and 2.5 mg l^{-1} in mature *P. terebinthus*) were suitable for shoot proliferation. According to Barghchi (1982), Barghchi and Alderson (1985), Abousalim (1990) and Onay (1996, 2000), the cytokinin BA had been reported as an ideal PGR for shoot proliferation. According to Bustamante-Garcia (1984), the optimal concentration of BA was 1 mg l^{-1} in *P. atlantica.* In this study, newly formed shoots were cut into segments containing a node, and these explants were transferred to a fresh MS medium supplemented with 1.0 mg l^{-1} BA. By repeating the process of cutting and subculturing twice the shoot tips from *in vitro* generated shoots, a large number of shoots were proliferated to optimize a novel approach for rapid propagation of *P. lentiscus* L.

In this study, explants containing segments of node and shoot tips were cut into segments containing a node or an apical tip, and those explants were cultured on a proliferation medium containing 30 g l^{-1} sucrose and 1.0 mg l^{-1} BA. A large stock of shoots was raised from the primary culture by sequential subculturing in order to optimize the rapid propagation protocol for *P. lentiscus* L. Onay (2000) had reported that an optimum concentration of BA to be used in subcultures of mature regenerated shoots of *P. vera* was 1.0-2.0 mg l^{-1}. In another study, Parfitt and Almehdi (1994) had suggested that 0.1 mg l^{-1} TDZ was the best plant growth regulator (PGR) in the proliferation studies of 1- to 3-year-old *P. atlantica* × *P. integerrima* hybrids. When most of the *in vitro* micropropagation studies are evaluated, it can be concluded that, in general, the most effective cytokinin in shoot proliferation, as was shown in this study, is BA.

Effects of supplementation with different concentrations of GA_3 on shoot proliferation

The effects of five concentrations of GA_3 (0.1, 0.3, 0.5, 0.7 and 1.0 mg l^{-1}) in combination with 1 mg l^{-1} BA on twice-subcultured seedling materials were examined by comparing them with the use of 1 mg l^{-1} BA as the control. The mean length of shoots, the mean number of shoots, and the mean number of buds were measured (Tab. 2).

According to the results of analysis of the data obtained at the end of the 4-week culture period, significant differences were found between the groups tested by adding GA_3 to the cultures in terms of shoot length, number of shoots, and number of buds (Tab. 2, $p < 0.05$). It was ascertained that, among the different GA_3 concentrations added to the medium in addition to 1 mg l^{-1} BA, 0.5 mg l^{-1} GA_3 gave the best results in terms of shoot length, number of shoots, and number of buds. This suggests that there

is a high sensitivity in *P. lentiscus* to the addition of GA_3. In a previous study, it had been reported that shoot proliferation and growth did not occur after adding GA_{4+7} or 0.25-4.00 mg l^{-1} GA_3 to a culture medium containing 4 mg l^{-1} BA in *P. terebinthus* and *P. integerrima* (Pontikis, 1984). In the same study, it was suggested that shoot production under optimum conditions could be achieved by transferring fresh tips together with their original buds to a culture medium supplemented with BA + GA_3 + IBA every two weeks. As is known, GA_3 addition to culture medium increases shoot length by extending the internodes of growing shoots (Reeves et al., 1985). According to the results presented in Table 2, the best responses in respect of shoot length and mean bud number were noted in the combination of GA_3 at 0.5 mg l^{-1} with BA at 1 mg l^{-1} (Tab. 2), which gave a mean bud number of 5.87 ± 0.16 and a mean shoot length of 1.43 ± 0.06 cm per explant on day 28 of culture.

Effects of amino acids and their concentrations on shoot proliferation

The results presented in Table 3, obtained at the end of the 4-week culture period, clearly show that there were significant differences among the tested concentrations of each amino acid type (tryptophane, valine, leucine, alanine and methionine) added to culture media supplemented with 1 mg l^{-1} BA in terms of shoot length, shoot number and bud number during the multiplication stage (Tab. 3).

The highest number of shoots was obtained from the medium supplemented with 100 mg l^{-1} valine (3.48), which was followed by 100 mg l^{-1} alanine (3.40). In terms of shoot length, 100 mg l^{-1} alanine and tryptophane gave the longest shoots (1.59 and 1.64 cm, respectively), whereas 50 mg l^{-1} tryptophane and alanine gave the highest bud numbers (6.10 and 6.39, respectively). The lowest numbers of shoots or buds were obtained with 25 mg l^{-1} alanine in the medium. The effects of amino acids on inhibiting or stimulating the growth of plant tissue cultures depend on explant types, the kind of amino acids, and genotypes. For example, Orlikowska (1992) observed the effects of twelve amino acids added to the cultures of two dwarf apple rootstocks, P 2 and P 60, in the concentration of 200 mg l^{-1} and found ornithine, arginine, glycine and glutamic acid as enhancing; lactalbumin hydrolysate and proline

Table 3. Effects of different amino acids and their concentrations on shoot proliferation*

Amino acid (25 mg l^{-1})	Shoot number** Avg. ± SE	Shoot length** Avg. (cm) ± SE	Bud number** Avg. ± SE
Control	2.40 ± 0.19 bc	0.84 ± 0.04 c	4.04 ± 0.16 cd
Valine	3.00 ± 0.23 a	1.40 ± 0.06 a	4.96 ± 0.15 a
Tryptophane	2.56 ± 0.16 ab	1.43 ± 0.09 a	5.29 ± 0.18 a
Alanine	1.92 ± 0.16 c	1.16 ± 0.07 b	3.91 ± 0.18 d
Leucine	2.36 ± 0.16 bc	1.32 ± 0.07 ab	4.42 ± 0.18 bc
Methionine	2.56 ± 0.17 ab	1.39 ± 0.07 a	4.87 ± 0.14 ab
(50 mg l^{-1})			
Control	2.24 ± 0.18 b	0.85 ± 0.03 b	4.14 ± 0.17 e
Valine	2.87 ± 0.17 ab	0.86 ± 0.04 b	5.00 ± 0.21 d
Tryptophane	2.92 ± 0.17 ab	1.42 ± 0.08 a	6.10 ± 0.19 ab
Alanine	3.24 ± 0.27 a	1.44 ± 0.07 a	6.39 ± 0.21 a
Leucine	3.17 ± 0.34 a	1.27 ± 0.07 a	5.73 ± 0.16 bc
Methionine	2.79 ± 0.17 ab	1.01 ± 0.05 b	5.50 ± 0.17 cd
(100 mg l^{-1})			
Control	2.80 ± 0.17 ab	0.93 ± 0.10 c	4.16 ± 0.17 c
Valine	3.48 ± 0.31 a	1.25 ± 0.04 b	5.08 ± 0.11 b
Tryptophane	3.16 ± 0.25 a	1.64 ± 0.07 a	5.46 ± 0.16 ab
Alanine	3.40 ± 0.23 a	1.59 ± 0.06 a	5.60 ± 0.14 a
Leucine	2.24 ± 0.16 b	1.23 ± 0.08 b	4.08 ± 0.16 c
Methionine	2.32 ± 0.17 b	0.96 ± 0.03 c	4.05 ± 0.10 c

*Data were collected by using at least 25 shoot tips per treatment on day 28 of culture
**Differences between values followed by the same letters in a column are not significant according to Duncan's multiple comparison test at $p \leq 0.05$

as neutral; and tyrosine, methionine, asparagine, glutamine and cysteine as lowering the number of roots of the P 60 rootstock. On the other hand, aspartic acid, glutamic acid and ornithine were found as enhancing, arginine and tryptophane as only slightly enchancing, asparagine as neutral, and proline as reducing the number of roots and rooted shoots of the P 2 rootstock. Even though some of the amino acids are known to have positive effects in tissue culture applications, almost all of the current regeneration protocols are lacking in the utilization of those benetifs (Kumar and Kumari, 2010). There are even no studies reported on the effects of amino acids on lentisk tissue culture in the previous studies. Studies conducted on different species, which have been reported, mostly indicate positive effects which are similar to our results. Benson (2000) reported that exogenously added amino acids were found to play an important role in the micropropagation of some species, but culture media of the existing regeneration protocols are rarely supplemented with amino acids. The efficacy of various amino acids in stimulating sugarcane plant regeneration was developed by adding amino acids to the regeneration medium (Asad et al., 2009).

Effects of shoot length on rooting

In order to determine the effects of shoot length on rooting, 1, 2, 3, 4 and 5 cm long shoots subcultured regularly on the MS medium supplemented with 1 mg l^{-1} BA were cultured and the number of roots per shoot, root length (cm) and rooting rate (%) were recorded on day 28 of culture.

The results presented in Table 4 show that shoot length significantly affected root length (cm), rooting rate (%) and root number per shoot. It was observed that among the different shoot lengths, the best results were obtained with 2 cm long shoots in terms of root length, rooting rate, and the number of roots per shoot. In general, the cultured shoots started to take root three weeks after the culture had been initiated.

The rooting rate for 2 cm shoots was 91%, while the rooting rates for 4 and 5 cm shoots were 50% and 66%, respectively. In terms of the mean number of roots per shoot, better results were obtained with 3 and 2 cm long shoots, with the values of 2.62 and 2.74, respectively. In terms of the average length of roots, it was observed that the results obtained with 1 cm shoots were the best (3.80 cm), while 2, 3 and 4 cm shoots were statistically in the same group and the root lengths obtained with them were 3.2 cm, 2.8 cm and 3.34 cm long, respectively. Rooting is one of the most important steps of micropropagation stages, especially for the adaptation of plantlets to natural conditions. There had been many previous studies on the rooting of both juvenile and mature materials of species included in the genus *Pistacia*. In those previous studies, the shoot tips that were used for *in vitro* rooting of pistachio were generally 1.5-2.0 cm long and originated from 1- to 4-year-old plants (Barghchi and Alderson, 1983; Pontikis, 1984; Abousalim, 1990; Onay, 1996). Tilkat (2006) used 4 cm long shoot tips propagated from buds taken from 25-year-old mature pistachio trees for *in vitro* rooting. Barghchi and Alderson (1983) found IBA as the most satisfactory auxin for rooting. In another study, Barghchi (1982) reported that rooting was achieved by transferring roots to a medium without the auxin when they reached a length of 1-2 cm after incubation of explants in the MS medium prepared with macronutrients in ½ concentrations and under dark conditions for the first seven days of culture. Barghchi and Alderson (1985) observed that the rooting of shoots grown from explants taken from *P. vera* before the age of two years was not significantly affected by 2.5, 3.0 or 3.5 mg l^{-1} IBA, and the rooting rate was 80% in all the concentrations. On the other hand, Abousalim (1990) reported that optimum rooting responses were obtained when the culture medium was supplemented with IBA in the

Table 4. Effects of shoot length on *in vitro* rooting of lentisk shoots*

Shoot length (cm)	Rooting rate** (%)	Root number**	Root length** (cm)
1	23.33 ± 7.85 c	1.71 ± 0.18 b	3.80 ± 0.57 a
2	91.18 ± 4.93 a	2.74 ± 0.13 a	3.20 ± 0.31 ab
3	38.10 ± 10.85 c	2.62 ± 0.30 a	2.80 ± 0.40 ab
4	50.00 ± 13.86 bc	1.85 ± 0.26 b	3.34 ± 0.56 ab
5	66.67 ± 12.59 ab	1.70 ± 0.15 b	1.94 ± 0.16 b

*Data were collected from 15 explants in two replicates
**Differences between values followed by the same letters in a column are not significant according to Duncan's multiple comparison test at $p \leq 0.05$

concentrations of 1 mg l^{-1} or 2 mg l^{-1}. As a result of their experiments on rooting 2-3 cm long shoot tips obtained by the micropropagation of shoots grown *in vitro* from mature seeds of *Pistacia khinjuk*, a rootstock of pistachio, Tilkat et al. (2005) reported that the best results, with a 100% rooting rate, were obtained from explants cultured in the MS medium containing 0.5 mg l^{-1} IBA. Dolcet-Sanjuan and Claveria (1995) had reported that in their study on the micropropagation of 3-year-old *P. paleastina* rootstock shoot tips, root formation occurred in 62% of 1-3 cm long shoots cultured in a medium containing 6.0 mg l^{-1} NAA. When previous studies were evaluated, in contrast with the results of Dolcet-Sanjuan and Claveria (1995), parallel with the results reported by Martinelli (1988), Barghchi and Alderson (1989), Parfitt and Almehdi (1994), Onay (2000) and Onay et al. (2003), among all the auxin types (IBA, IAA, NAA and 2,4-D tested for *in vitro* rooting of shoots taken from pistachio trees), the most satisfactory auxin and its concentration were IBA and 2.0 mg l^{-1}, respectively, with 2 cm long shoots, giving a 65% rooting rate. Additionally, Tilkat (2006) improved this rate to 73% by using 4 cm long explants. Those results, reported in the literature, show that the rooting rate under *in vitro* conditions was influenced by not only the age of the explant used and the genotype but also by the length of the explant used for rooting. For this reason, the entire culture development environment for each species should be optimized.

Acclimatization

Healthy and strongly developed plantlets were planted into pots containing peat and perlite (1:1 v/v), and transferred to a growth room for a five-week acclimatization period in order to wean the plantlets towards ambient environmental conditions such as low relative humidity and high illumination levels. In this compost, plantlets tended to resume shoot growth very quickly and there were at least two pairs of new leaves on each plant after 5 weeks of acclimatization, and a total of 80.00% of the plantlets survived in the sterile compost, but this rate was reported to be only 36.67% in the non-sterile compost (Tab. 5). After the 5-week period of transitional development, the hardened plantlets were transferred to earthen pots in the growth room. The described protocol for plant acclimatization was satisfactory because a high plant survival percentage (92%) in the growth room was obtained after two years. This success rate shows that the described acclimatization protocol included a transitional development in which both anatomical characteristics and physiological performance of the micropropagated lentisk plantlets. Similar results had also been reported by Tilkat et al. (2005; 2008a; 2008b), who used mature and juvenile regenerated materials of *P. vera*.

Table 5. Effects of compost type (1:1 v/v peat and perlite mixture) on the acclimatization of lentisk plantlets*

Compost type	Viable regenerants** after 5 weeks (%)	Viable regenerants*** after 2 years (%)
Sterile	80.00 ± 7.42 a	92.00 ± 5.53 a
Non-sterile	36.67 ± 8.94 b	85.71 ± 9.70 a

*Means in each column followed by different letters are significantly different according to Duncan's multiple range test ($p < 0.05$). Data are means of at least 20 replicates per treatment

**Data was recorded five weeks after transfer to growth room

***Data was recorded two years after transfer to growth room

CONCLUSIONS

A major improvement over previous protocols (Yildirim, 2012; Kilinc et al., 2014; Koc et al., 2014; Kilinc et al., 2015; Onay et al., 2016b) has been achieved for the percentage of multiplication and acclimatization, which increases the efficiency of the steps required in the micropropagation of lentisk. Optimum shoot/bud development in the one cloned genotype of *P. lentiscus* was solely dependent upon BA as a plant growth regulator in *in vitro* culture. This study also affirmed that the MS medium containing 0.5 mg l^{-1} GA_3 or 100 mg l^{-1} valine or alanine combined with 1 mg l^{-1} BA produced the highest shoot/bud number compared with the results of previously reported studies. The use of one cloned genotype culture in the rooting of lentisk microcuttings also reaffirmed the quality of the rooted plantlets and consequently improved plant survivability compared to the available protocols. High percentages of plantlet acclimatization (80%) five weeks after transfer to the growth room and also two years after transfer to the growth room, and of viable plantlets (92%), demonstrate the reliability of the described protocol. This optimized protocol has resulted in a considerably improved micropropagation approach for *P. lentiscus* with a great potential for large scale propagation in reforestation programmes.

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AUTHOR CONTRIBUTIONS

All of the authors: H.Y., A.O., K.G, S.E. and F.E.K. – contributed equally to this manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

Abousalim A., 1990. Micropropagation and micrografting of pistachio (*Pistacia vera* L. and *Pistacia atlantica* Desf.). PhD Thesis, Imperial College London, University of London, London, UK.

Ak B.E., Parlakci H., 2009. *Pistacia lentiscus* in the Mediterranean Region in Turkey. Acta Hortic. 818, 77-81.

Akdemir O.F., Tilkat E., Onay A., Kilinc M.F., Suzerer V., Ciftci Ozden Y., 2013. From past to date *Pistacia lentiscus* L. Batman Univ. J. Life Sci. 3, 1-28.

Al-Saghir M.G., Porter D.M., 2012. Taxonomic revision of the genus *Pistacia* L. (Anacardiaceae). Am. J. Plant Sci. 3, 12-32.

Asad S., Arshad M., Mansoor S., Zafar Y., 2009. Effect of various amino acids on shoot regeneration of sugarcane (*Sacchrum officinarum* L.). Afr. J. Biotechnol. 8(7), 1214-1218.

Barghchi M., 1982. In vitro propagation of *Pistacia* species. PhD Thesis, University of Nottingham, Nottingham, UK, 117.

Barghchi M., Alderson P.G., 1983. In vitro propagation of *Pistacia* species. Acta Hortic. 131, 49-60.

Barghchi M., Alderson P.G., 1985. In vitro propagation of *Pistacia vera* L. and the commercial cultivars Ohadi and Kalleghochi. J. Hortic. Sci. 60, 423-430.

Barghchi M., Alderson P.G., 1989. Pistachio (*Pistacia vera* L.). In: Biotechnology in Agriculture and Forestry. Vol. 5. Trees II. Y. P. S. Bajaj (Ed.), Springer-Verlag, Berlin, Germany, 68-98.

Baskaran P., Jayabalan N., 2007. Rapid micropropagation of *Psoralea corylifolia* L. using nodal explants cultured in organic additive-supplemented medium. J. Hortic. Sci. Biotechnol. 82, 908-913.

Benson E.E., 2000. *In vitro* plant recalcitrance: An introduction. In Vitro Cell Dev. Biol. Plant 36, 141-148.

Bustamante-Garcia M.A., 1984. Micropropagation and rejuvenation of *Pistacia* species and the mechanism by which light influences root initiation. PhD Thesis, University of California, Davis, US.

Correia O.A., Catarino F.M., 1994. Seasonal changes in soil-to-leaf resistance in *Cistus* sp. and *Pistacia lentiscus*. Acta Oecol. 15, 289-300.

Cüce M., Sökmen A., 2017. In vitro production protocol of *Vaccinium uliginosum* L. (bog bilberry) growing in the Turkish flora. Turk. J. Agric. For. 41, 294-304.

Dolcet-Sanjuan R., Claveria E., 1995. Improved shoot-tip micropropagation of *Pistacia vera* L. and the beneficial-effects of methyl jasmonate. J. Amer. Soc. Hort. Sci. 120, 938-942.

Ercisli S., Tosun M., Karlidag H., Dzubur A., Hadziabulic S., Aliman Y., 2012. Color and antioxidant characteristics of some fresh fig (*Ficus carica* L.) genotypes from Northeastern Turkey. Plant Foods Hum. Nutr. 67, 271-276.

Fascella G., Airo M., Zizzo G.V., Ruffoni B., 2004. Prime osservazioni sula coltivazione in vitro di Lentisco (*Pistacia lentiscus* L.). Italus Hortus 11, 141-143.

Gonbad R.A., Sinniah U.R., Abdul Aziz M., Mohamad R., 2014. Influence of cytokinins in combination with GA_3 on shoot multiplication and elongation of tea clone Iran 100 (*Camellia sinensis* (L.) O. Kuntze). Sci. World J., ID 943054.

Kilinc F.M., Süzerer V., Çiftçi Y.Ö., Onay A., Yildirim H., Uncuoğlu A.A., et al., 2015. Clonal micropropagation of *Pistacia lentiscus* L. and assessment of genetic stability using IRAP markers. Plant Growth Regul. 75(1), 75-88.

Kilinc F.M., Süzerer V., Özden Çiftçi Y., Koc I., Akdemir H., Yildirim H., Tilkat E., Onay A., 2014. Improved shoot multiplication of lentisk (*Pistacia lentiscus* L.) using different carbohydrates and media. Acta Hortic. 1028, 153-160.

Koc I., Onay A., Ciftci Y.O., 2014. *In vitro* regeneration and conservation of the lentisk (*Pistacia lentiscus* L.). Turk. J. Biol. 38, 653-663.

Kumar S.P., Kumari B.R., 2010. Effect of amino acids and growth regulators on indirect organogenesis in *Artemisia vulgaris* L. Asian J. Biotechnol. 2, 37-45.

Malá J., Máchová P., Cvrčková H., Karady M., Novák O., Mikulík J., et al., 2013. The role of cytokinins during micropropagation of wych elm. Biol. Plantarum 57, 174-178.

Martinelli A., 1988. Use of in vitro techniques for selection and cloning of different *Pistacia* species. Acta Hortic. 227, 436-437.

Mascarello C., Fascella G., Zizzo G.V., Mantovani E., Ruffoni B., 2007. In vivo and in vitro propagation of *Pistacia lentiscus* L. Acta Hortic. 764, 299-306.

Onay A., 1996. *In vitro* organogenesis and embryogenesis of pistachio, *Pistacia vera* L. PhD Thesis, University of Edinburgh, Edinburgh, UK.

Onay A., 2000. Micropropagation of pistachio from mature trees. Plant Cell Tissue Organ Cult. 60, 159-162.

Onay A., Pirinc V., Adiyaman F., Isikalan C., Tilkat E., Basaran D., 2003. In vivo and in vitro micrografting

of pistachio, *Pistacia vera* L. cv. Siirt. Turk. J. Biol. 27, 95-100.

Onay A., Yildirim H., Yavuz M.A., 2016a. *Pistacia lentiscus* L. growing techniques mastic gum production. Batman Univ. J. Life Sci. 6, 133-144.

Onay A., Tilkat E., Suzerer V., Metin K., Ozden Ciftci Y., Kilinc F.M., et al., 2016b. Rejuvenation of mature lentisk by micrografting and evaluation of genetic stability. Turk. J. Biol. 40, 781-796.

Orlikowska T., 1992. Effect of amino acids on rooting of apple dwarf rootstocks *in vitro*. Biol. Plantarum 34, 39-44.

Parfitt D.E., Almehdi A.A., 1994. Use of a high CO_2 atmosphere and medium modifications for the successful micropropagation of pistachio. Sci. Hortic. 56, 321-329.

Pontikis C.A., 1984. In vitro propagation of *Pistacia terebinthus* L. The Plant Propagator 31, 14-15.

Reeves D.W., Couvillon G.A., Horton B.D., 1985. Effect of gibberellic acid (GA_3) on elongation and rooting of 'St. Julien A' rootstock in vitro. Sci. Hortic. 2, 253-259.

Ruffoni B., Mascarello C., Fascella G., Airó M., Amoretti M., 2004. La micro-propagazione del Lentisco (*Pistacia lentiscus* L.). Atti II Convegno nazionale Piante Mediterranean. Italus Hortus 11, 141-143.

Sarropoulou V., Dimassi-Theriou K., Therios I., 2014. L-arginine impact on cherry rootstock rooting and biochemical characteristics in tissue culture. Turk. J. Agric. For. 38, 887-897.

Taskin T., Inal A., 2005. In vitro micropropagation of *Pistacia lentiscus* var. chia Duhamel. Aegean Agricultural Research Institute (Turkey) 5(1), 1-14.

Tilkat E., Isikalan C., Onay A., 2005. *In vitro* propagation of khinjuk pistachio (*Pistacia khinjuk* stocks) through seedling apical shoot tip culture. Propag. Ornam. Plants 5, 124-128.

Tilkat E., 2006. Micropropagation of male *Pistacia vera* L. via apical shoot tip culture. PhD Thesis, Institute of Science University of Dicle, Turkey (in Turkish).

Tilkat E., Onay A., Yildirim H., Ozen H.C., 2008a. Micropropagation of mature male pistachio *Pistacia vera* L. J. Hortic. Sci. Biotechnol. 83, 328-333.

Tilkat E., Onay A., Ozden Tokatli Y., 2008b. In vitro rooting improvement of adult pistachio, *Pistacia vera* L. 'Atli'. Acta Hortic. 839, 215-221.

Unek C., Tanriver E., Kuden A.B., 2010. The effects of different cytokinins on micropropagation of Garnem rootstock. Acta Hortic. 923, 195-197.

Vijayan K., Chakraborti S.P., Ercisli S., Ghosh P.D., 2008. NaCl induced morpho-biochemical and anatomical changes in mulberry (*Morus* spp.). Plant Growth Regul. 56, 61-69.

Yildirim H., 2012. Micropropagation of *Pistacia lentiscus* L. from axenic seedling-derived explants. Sci. Hortic. 137, 29-35.

Zohary M.,1952. A monographic study of the genus *Pistacia*. Palest. J. Bot. 5, 187-226.

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