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# Rejuvenation of mature lentisk by micrografting and evaluation of genetic stability

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**Abstract:** An in vitro propagation method was established for both male and female genotypes of lentisk using actively growing shoot tips derived from forcefully lignified shoots. The effects of growth regulators on in vitro morphogenesis were investigated. Since rooting of the regenerated shoots for both genotypes was not achieved, an in vitro micrografting method was developed for the production of plantlets. Moreover, genetic stability of 3-, 6-, and 24-times subcultured clones of both genotypes was assessed and compared with the mother plants using fluorescent-based amplified fragment length polymorphism (AFLP) analysis. The set of main plants and the different subcultured clones were divided into two clusters. In the first cluster, the original male and female plants were grouped together with the 3-times subcultured female and the 6-times subcultured male and female groups, whereas the second cluster contained the 24-times subcultured clones and the 3-times subcultured male group. To the best of our knowledge, this is the first report of successfully inducing plantlets from mature lentisk genotypes and the first analysis of clonal fidelity of regenerated mature female and male *P. lentiscus* L. plants by AFLP.

**Key words:** *Pistacia lentiscus* L., plant growth regulator, micropropagation, molecular markers, genetic similarity, AFLP, somaclonal variation

## 1. Introduction

Mastic trees are commonly known as evergreen pistachio or lentisk. In Turkey lentisk shares its habitat with *Pistacia terebinthus* L., olives, carob, and others, but it prefers well-drained to dry sandy or stony alkaline soil conditions (Ak and Parlakcı, 2009). Mastic trees show a peculiar adaptability to several climatic conditions (Zohary, 1952) and have positive biological characteristics, such as drought tolerance (Correia and Catarino, 1994) and protection of soil against erosion (Mulas and Deidda, 1998). However, the area that is suitable for mastic tree cultivation is limited in the world. Today a major limitation facing the widespread expansion of commercial mastic tree plantations is the shortage of superior plants, primarily because of the difficulties experienced in propagating this species using the traditional method of rooting the cuttings. Currently mastic trees are proliferated by seed, with the rational development of hereditary instability and great discrepancy in the formation degree among genotypes because of obstacles such as parthenocarpy and ovary aborticide (Grundwag, 1976). Vegetative propagation by cutting is also difficult due to impoverished or adventitious roots. Conventional propagation methods to improve *Pistacia* species such as *P. lentiscus* L. are limited because species belonging to the genus *Pistacia* have a long stage of juvenility. In addition, plants originating from seeds often do not maintain the genetic purity of the parent plants due to segregation and recombination of genetic characteristics during sexual reproduction. Thus, to improve trade, a vegetative propagation technique must be developed for large-scale propagation. Possible ways to move forward are necessary in order to avoid problems in vegetative propagation and to produce clonal stocks. Micropropagation of lentisk genotypes has been achieved

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through organogenesis (Fascella et al., 2004; Ruffoni et al., 2004; Taşkın and İnal, 2005; Mascarello et al., 2007; Yıldırım, 2012; Kılınç, 2013; Kılınç et al., 2014, 2015), but up until now, no one has successfully propagated lentisk through tissue culture using material from mature trees. The initiation of in vitro cultures from mature trees is one of the well-known obstacles due to their physiological state. It has also been reported that establishment of in vitro cultures of lentisk is greatly hampered by the browning of explants due to leaching of phenolic compounds (Rai et al., 2009; Tilkat et al., 2012, 2013). To overcome these obstacles, several alternative approaches could be the utilization of in vitro technologies that have been demonstrated to induce rejuvenation, including forcing new growth, chemical treatments, shoot tip culture, organogenesis, and micrografting for regeneration of mature lentisk genotypes. Somaclonal variation is a natural problem when in vitro propagation is utilized for large-scale propagation. Therefore, clonal fidelity of micropropagated plants must be screened in order to perceive the new regenerants true to type. In vitro culture may cause variants in regenerated plants (Larkin and Scowcroft, 1981). Owing to this degree of change, the in vitro regenerants may not have the same properties as the donor plants.

Several strategies are available for discovering the true nature of somaclonal variation, including phenotypic identification (Kong et al., 2011), cytological analysis (Konieczny et al., 2010), DNA analysis techniques (Torres Morán et al., 2010; Ai et al., 2011), and protein analysis (Northmore et al., 2012). In recent years, DNA-based molecular markers have served as a significant tool for evaluating heritable homogeneity and the true-to-type nature of in vitro propagated plants. In many cases, random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and inter-simple sequence repeat (ISSR) methods have been utilized to ascertain the heritable fidelity of the regenerated plants (Joshi and Dhawan, 2007; Nookaraju and Agrawal, 2012; Singh et al., 2012). Additionally, the amplified fragment length polymorphism (AFLP) approach, which is an improved approach (Saker et al., 2006; Smykal et al., 2007) that utilizes cleavage and PCR amplification to ascertain point mutations at cleavage sites or deletions and insertions (Vos et al., 1995), could also be used for the detection of plant cell culture-based variations. High levels of reproducibility coupled with a high multiplex ratio compared to other molecular marker techniques (Powell et al., 1996) are the reasons for selecting AFLP markers for the evaluation of genetic fidelity of tissue culture-raised plants in this study. Moreover, unlike other DNA-based markers, it does not need prior DNA sequence information (Vos et al., 1995). Therefore, in the present study, we report an efficient method of in vitro micropropagation for a mature male and female genotype of *P. lentiscus* L. and fluorescent-based DNA profiling for the evaluation of genetic fidelity of in vitro regenerated genotypes. This is the first report on micropropagation using material from mature trees and genetic stability analysis of regenerated plantlets using AFLP markers in mature lentisk genotypes.

## 2. Materials and methods

### 2.1. Plant material and culture conditions

Mature plants were obtained from 20-year-old female and male genotypes of *P. lentiscus* L. growing in the Çeşme region of İzmir Province in western Turkey (Figure 1). First, stem sections of 3–4 cm in length from the base of the explants were trimmed and the freshly cut ends were immersed in a solid or a liquid medium as described below. Unless otherwise specified, a culture initiation medium (CIM) contained mineral nutrients devised in full strength (Murashige and Skoog, 1962) with B5 vitamins (Gamborg et al., 1968), 1.0 mg/L $N^6$-benzyladenine (BA), 6.5 g/L agar (w/v), and 30 g/L sucrose. Apical tips of 10 mm in length were cultured aseptically in a Magenta GA7 vessel (Chicago Corporation, USA) containing 50 mL of medium. Four weeks after culture the proliferated shoots were cut into segments, each containing a node, and these propagules were cultured into a new proliferation medium. By subculturing the new nodal or apical shoots from in vitro regenerated shoots, stock scion cultures were obtained for the production of microscions of mature lentisk trees. Axenically germinated seedlings of the following species were used as rootstocks: *P vera* var. Siirt, *P. atlantica* Desf., *P. terebinthus*, and *P. khinjuk* Stocks. During establishment, shoot initiation, proliferation, and micrografting studies, all cultures were placed in a growth room at 25 °C under irradiance of 40 μmol $m^{-2} s^{-1}$ supplied by white fluorescent lamps with a 16-h photoperiod.

### 2.2. Effects of solid compost on forcing the dormant buds to sprout

Stem sections of both genotypes of 25–30 cm in length were harvested and collected in the middle of May 2011. The base of the cuttings was cut back before the forcing treatment, and the explants were 1) potted in peat, 2) dipped in peat, or 3) potted in perlite, and then placed in a growth room (60 ± 5% relative humidity) at 25 °C under irradiance of 25 μmol $m^{-2} s^{-1}$ supplied by mercury fluorescent lamps (75 W) under a 24-h photoperiod in order to maximize the number of axillary buds to sprout. After 3 weeks of incubation, the survival and the flushing rates of the scions were reported for male and female genotypes.

### 2.3. Effects of liquid forcing solution on forcing the axillary buds to sprout

Stem sections of 25–30 cm long of both male and female genotypes of lentisk were collected in the middle of May

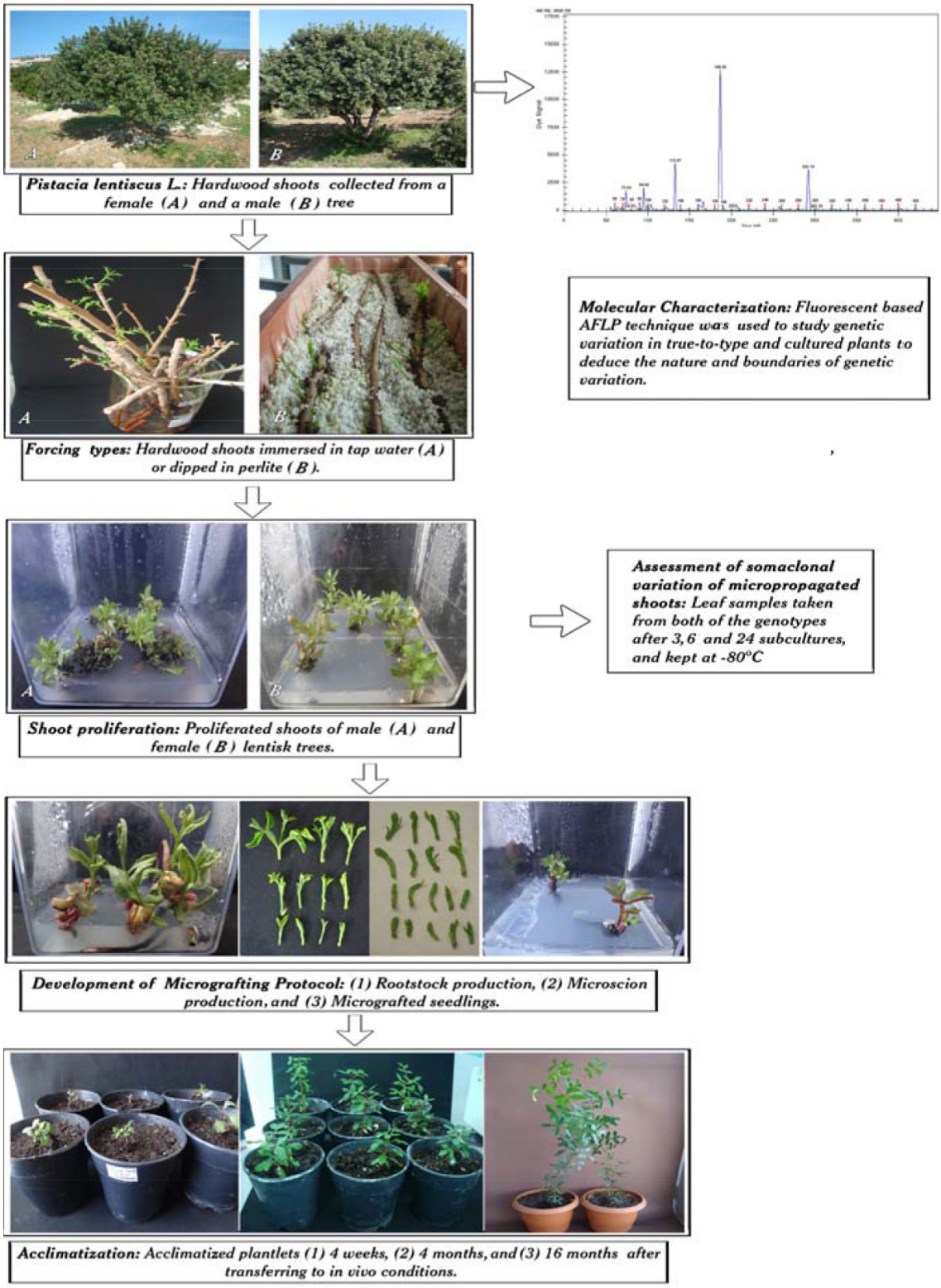


**Figure 1.** Schematic diagram illustrating the complete process of plant regeneration in lentisk, *P. lentiscus* L.

2011 and immersed in the following forcing solutions: 1) tap water, 2) tap water containing 30 g/L sucrose, and 3) tap water containing full-strength Murashige and Skoog (MS) medium. Three weeks after culturing, the survival and flushing rates of the scions were reported for male and female genotypes.

**2.4. Establishment of sterile shoot cultures**

Five weeks after culturing, newly formed leafy shoots of 1.0 cm long from the forced stem sections were isolated and disinfested following the protocol developed by Çalar (2013) in order to obtain axenic cultures.

**2.4.1. Shoot proliferation in the CIM**

**2.4.1.1. Effects of BA**

Effects of 4 concentrations (0.3, 0.5, 0.7, and 1.0 mg/L) of BA on shoot proliferation from regenerated shoot tips of lentisk were examined.

**2.4.1.2. Effects of inclusion of different $GA_3$ concentrations with applied BA concentration**

Using 1 mg/L BA as the control, the effects of 5 different concentrations (0.1, 0.3, 0.5, 0.7, and 1.0 mg/L) of $GA_3$ were examined for shoot proliferation in both the female and male cultures.

**2.4.2. Development of micrografting protocol**

A micrografting technique developed on juvenile materials of lentisk was applied to mature genotypes (Çalar, 2013).

**2.4.2.1. Rootstocks production**

Open pollinated seeds of the following species were used as rootstocks: *P vera* var. Siirt, *P. atlantica* Desf., *P. terebinthus*, and *P. khinjuk* Stocks. Mature kernels of *P. vera* var. Siirt and *P. khinjuk* Stocks were disinfested by shaking in a 10% (v/v) NaOCl solution for 10 min, followed by washing thoroughly with sterile distilled water at least twice. The seeds of *P. atlantica* Desf. and *P. terebinthus* L. were disinfested with their hulls because they were too small to isolate from their shells. Surface-sterilized kernels of different *Pistacia* species were cultured in Magenta vessels containing 50 mL of MS medium, including B5 vitamins, 1.0 mg/L BA, 6.7 g/L agar, and 30 g/L sucrose (Sigma, USA). Culture tubes were cultured in a growth room under irradiance of 40 µmol $m^{-2}$ $s^{-1}$ supplied by mercury fluorescent lamps (75 W) under a photoperiod of 16 h at 25 ± 2 °C. Rootstocks for grafting were 14-day-old seedlings for *P. vera* L. and *P. khinjuk* Stocks and 40- to 50-day-old seedlings for *P. atlantica* Desf. and *P. terebinthus* L. At the end of the in vitro culture periods for rootstock development, observations were made on a total of at least 30 seedlings regarding the percentage of seeds germinated, stem height, stem diameter, and length of roots.

**2.4.2.2. Proliferation and maintenance of in vitro shoot cultures for scion source**

Regenerated shoot tips (10 mm in length), explanted from in vivo grown trees (after surface sterilization), were used as microscions in all micrografting studies. The regenerated shoot tips of both male and female genotypes were cultured in culture tubes containing 50 mL of MS medium supplemented with 50 mg/L valine, 30 g/L sucrose, 0.7 mg/L BA, and 6.7 g/L agar to induce stock cultures in order to be used as a source of scions. The proliferated new shoots that originated from the mature explants were excised and subcultured every 4 weeks in the same medium for more than a 2-year period.

**2.4.2.3. Effects of time the explants were harvested on the success of micrografts**

Scions that originated from cultures initiated in February, May, July, and October were used to determine the effect of the time of the year on micrografting. The scions were 10-mm sterile shoot tips of both male and female genotypes. The micrografts were cultured in full-strength MS medium supplemented with 6.7 g/L agar and 30 g/L sucrose.

**2.4.2.4. Effects of scion type**

To determine the effect of the type of scions of both genotypes on micrografting success, shoot length, and root development, 3 microscion types were used: 1) in vitro regenerated shoot tips, 2) in vitro forced shoot tips, and 3) in vivo grown shoot tips. MS medium supplemented with 30 g/L sucrose, 50 mg/L valine, and 6.7 g/L agar was utilized.

**2.4.2.5. Effects of medium type**

On account of the results of previously reported micrografting methods concerning almond (Yıldırım et al., 2013) and pistachio (Onay et al., 2004), slit micrografting was used in this study. Three different culture media were tested for optimum micrograft development: 1) MS medium containing 30 g/L sucrose and 6.7 g/L agar; 2) shoot proliferation MS medium containing 30 g/L sucrose, 1.0 mg/L BA, and 7 g/L agar; 3) rooting MS medium containing 30 g/L sucrose, 1.0 mg/L indole-3-butyric acid (IBA), and 6.7 g/L agar.

**2.5. Acclimatization**

In vitro micrografted lentisk plantlets were taken out of the Magenta vessels and washed in tap water. The micrografts were then potted in a mixture of perlite and sand [1:2 (v/v)], covered with a beaker glass, and placed in a growth room for development. The pots containing the micrografted plantlets were covered with beaker glasses for the purpose of successful acclimatization to the in vivo conditions. Two weeks after the transfer, the micrografts were gradually ventilated by opening the beaker glasses each day, doubling the previous day's ventilation period, to achieve a successful acclimatization. The culture tubes were placed in a growth room under irradiance of 50 µmol $m^{-2}$ $s^{-1}$, supplied by mercury fluorescent lamps (75 W) under a photoperiod of 16 h at 25 ± 2 °C. The complete regeneration process, from the forcing of the hardwood shoots of the mother plants to the micropropagated

plantlets assessed by using AFLP markers, is summarized in Figure 1.

### 2.6. Assessment of somaclonal variation of micropropagated plantlets

Genomic DNA was extracted from the leaves according to the protocol of Lodhi et al. (1994). The AFLP reactions were achieved following Vos et al. (1995), as modified by Myburg et al. (2000). AFLP fingerprints were obtained using fluorescently labeled primers of AFLP amplification (Genome Lab GeXP Genetic Analysis System, USA) according to the manufacturer's instructions. AFLP analyses were performed on 3-, 6-, and 24-times subcultured cultures of the female and the male genotypes, together with the donor plants, which were axillary buds forced from lignified stem sections of 20-year-old female and male trees. Reproducibility was assured by performing 3 technical repetitions using 3 individuals for each culturing time, as well as donor plants.

Selective amplifications were carried out with fluorescently labeled primer pair combinations (*Eco*RI AGG*D4 - *Mse*I CAA*D4, *Eco*RI AGG*D4 - *Mse*I CTT, EcoRI AGG*D4 - *Mse*I CAT, *Eco*RI AGG*D4 - *Mse*I CAC, *Eco*RI AGG*D4 - *Mse*I CCA). These primers were selected because of the maximum number of polymorphisms detected between the different species. Amplification was achieved with the following program setting: 1 cycle at 94 °C for 2 min; 13 cycles of 30 s at 94 °C, 30 s ramping from 65 to 56 °C (0.7 °C per cycle), and 1 min at 72 °C; 24 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C; and 1 cycle at 60 °C for 30 min. Then 2.5 µL of amplification product was added to 17.5 µL of sample loading solution mixed with the DNA size standard and overlaid with mineral oil. The Genome Lab GeXP Genetic Analysis System (Beckman Coulter, USA) was used for analysis of fluorescently labeled final PCR products. All of the reactions were performed twice and only reproducible peaks were taken into consideration.

### 2.7. Data analysis

Morphological changes were recorded by visual observation. Lignified shoots of the current year (including last year's growth) from female and male 20-year-old *P. lentiscus* L. trees growing in a Mediterranean continental climate were utilized for flushing buds. Treatments in all statistically analyzed experiments were arranged in a completely randomized design. Experiments were repeated 2 or 3 times and on 2 or 3 separate plots. The number of explants utilized for each treatment in regeneration experiments is given in the legend of the relevant tables. Unless otherwise acknowledged, the measured parameters are reported at the end of a 4-week period. The survival rates and flushing rates of the scions are reported for both male and female genotypes for forcing axillary buds to sprout. At the end of the 4-week culture period, the data on survival, as well as on contaminated and necrotic explants, were reported for the establishment of surviving axenic cultures. The data on the average number of shoots and average shoot length obtained through shoot tip proliferation were reported. The mean values of the percentages of successful micrografts, displaced micrografts, and micrografts with elongating axillary shoots were reported. Analysis of variance was carried out and data from factorial and nonfactorial experiments were subjected to Duncan's multiple range test at the $P \leq 0.05$ probability level to separate the means of different treatments. In the case of proportional data, the chi-square ($\chi^2$) test was used whenever appropriate.

Polymorphic bands were scored automatically according to the GeXP system as present (1) or absent (0). All fragments were given equal weights. In order to reduce scoring problems resulting from excess primer peaks and decreased signals for fragments longer than 500 bp, only fragments between 54 and 451 bp in length were recorded. In order to assess the nature of somaclonal variation in AFLP data, three different methods were employed. First, binary AFLP data were analyzed using the Bayesian approach implemented in the software program structure (Pritchard et al., 2000) in order to evaluate possible differentiation among all eight combinations. The obvious advantage of using a Bayesian approach is that no superimposed model is assumed. To deduce the optimal value of K, each value of K = 1–5 was run subsequently. Admixture was allowed and the allele frequencies were assumed to be correlated, since common ancestry was evident. The data were collected from 100,000 Markov chain Monte Carlo (MCMC) replications after the burn-in of 10,000 MCMC replications. The optimal number of K was determined using the method developed by Evanno et al. (2005). The second method used to investigate genetic variation was principal component analysis (PCA), which allows to visualize the distribution of groups and the pair-wise total genetic distance matrix. Both the genetic distance matrix and the PCA were obtained from GenAlEx 6.4 (Peakall and Smouse, 2001). Genotypic values were plotted for the first two principal component estimates. For the final analysis, we computed a genetic distance matrix (Saitou and Nei, 1987) and created a neighbor-joining dendrogram using the software program PowerMarker version 3.23 (Liu, 2002).

## 3. Results

### 3.1. Rejuvenation of mature lentisk genotypes

An experiment was performed to determine the optimal solution for forcing herbaceous shoot production from lateral buds of hardwood cuttings.

#### 3.1.1. The effects of solid compost and liquid forcing solution on the flushing of dormant buds

Significant differences were observed in the rates of sprouting buds of the lignified hardwood cuttings when the cuttings were cultured on solid compost (Table 1).

**Table 1.** Effects of solid compost on inducing outgrowth of lateral buds in cuttings collected from 20-year-old male and female *P. lentiscus* L.*

| Treatments | Sprouting buds (%) | |
|---|---|---|
| | Female | Male |
| Dipped in peat | 6.7 b | 10.0 b |
| Potted in peat | 13.3 b | 6.6 b |
| Dipped in perlite | 80.0 a | 63.3 a |

*Values represent the averages as determined from 30 replicated cuttings per treatment. For each treatment, different letters indicate that percentages are significantly different at $P \leq 0.05$. Data were recorded on the 21st day.

**Table 2.** Effects of forcing solution type on inducing outgrowth of lateral buds in cuttings collected from 20-year-old male and female *P. lentiscus* L.*

| Type of forcing solution | Sprouting buds (%) | |
|---|---|---|
| | Female | Male |
| Tap water containing 3% sucrose | 16.7 b | 23.3 b |
| MS medium | 23.3 b | 33.3 b |
| Tap water | 50.0 a | 60.0 a |

*Values represent the averages as determined from 30 replicated cuttings per treatment. For each treatment, different letters indicate that percentages are significantly different at $P \leq 0.05$. Data were recorded on the 21st day.

After 3 weeks of culture, 80% of the female explants dipped in perlite and 6.7% of the explants dipped in peat induced actively growing axillary shoots. Only 13.3% of the explants induced actively growing axillary shoots when the hardwood shoots of a female tree were potted in peat. Similar to the female genotype, 63.3%, 10%, and 6.6% of the explants (cuttings) of a male tree induced growing shoots when dipped in perlite, dipped in peat, and potted in peat, respectively. The percentage of sprouting buds in both genotypes differed significantly in different types of forcing solution (Table 2). Sprouting bud rates of the female genotypes were significant among the different types of forcing solutions. The percentage of sprouting buds obtained from tap water, tap water containing 30 g/L sucrose, and MS medium was found to be 50%, 16.7%, and 23.2%, respectively (Table 2). Quantitative evaluation of the results reported for the male genotypes also showed that the type of forcing solution had an effect on the sprouting buds. Tap water produced the highest rate (60%) among the treatments tested. Taking into account the observations reported, dipping in perlite appeared to be a promising process for solid compost, while tap water is the most suitable type of forcing solution for forcing nodal segments. Forced shoots (Tables 1 and 2) were utilized as the source of explants from the 20-year-old *P. lentiscus* L. trees. After 3 weeks of culturing, the newly formed shoots from dormant buds were excised and subjected to surface sterilization, as described by Çalar (2013), and cultured for the establishment of in vitro cultures.

### 3.1.2. Shoot initiation and proliferation

Among the cytokinins tested, better and faster shoot formation was observed when 1.0 mg/L of the cytokinin BA was used in MS medium within 28 days of culture (Kılınç et al., 2015). The axenic regenerated shoot tips were further multiplied by subculturing on new MS medium with 1.0 mg/L of BA and maintained for more than 2 years.

#### 3.1.2.1. The effects of different BA concentrations

Results presented in Table 3 show the superiority of 0.3 mg/L BA to all other concentrations tested for the female genotype. This medium had more shoots per explant than those on the rest of the BA concentrations tested. Average shoot lengths of various BA treatments for the female genotype were not significantly different from each other after 4 weeks of culture period. In the case of the male genotype, the highest number of shoots was observed in the 0.5 mg/L BA treatment. The lowest number of shoots was recorded from the explants on the MS medium supplemented with 0.3 mg/L BA.

#### 3.1.2.2. Effects of the inclusion of different $GA_3$ concentrations with the applied BA concentration

The lowest level of average shoot number and length was observed in the control treatments of both genotypes (Table 4). The highest shoot number per explant in the female genotype (2.2) was obtained with 1.0 mg/L $GA_3$ + 1 mg/L BA (Table 4). The mean shoot length was also positively affected by $GA_3$ concentration, and the optimum result (1.4 cm in length) was obtained with 1.0 or 0.5 mg/L $GA_3$ + 1 mg/L BA treatment. In the case of the male genotype, the highest mean shoot number (2.0) was obtained with 1 mg/L BA + 0.7 mg/L $GA_3$. The mean shoot length was also positively affected by $GA_3$ concentrations up to 0.5 mg/L. Shorter shoots were produced by the addition of 0.7 or 1.0 mg/L $GA_3$ to the MS medium containing 1.0 mg/L BA (Table 4). Concerning the average shoot number and length, 0.5 mg/L $GA_3$ + 1 mg/L BA treatment was superior to the rest of the treatments tested on the male genotype.

### 3.2. Development of micrografting protocol

#### 3.2.1. In vitro microscion production

In vitro culture was established from forced shoot tips according to the method described by Onay et al. (2013). These micropropagated shoots were subcultured monthly

**Table 3.** Effects of BA on shoot length and number of cultured explants from mature female and male shoot tips of lentisk on proliferation medium.*

| BA (mg/L) | Female | | Male | |
|---|---|---|---|---|
| | Shoot number | Shoot length | Shoot number | Shoot length |
| 0.3 | 2.4 a | 1.0 a | 1.2 b | 1.1 ab |
| 0.5 | 1.7 ab | 1.0 a | 1.6 a | 0.9 b |
| 0.7 | 1.6 b | 1.0 a | 1.5 a | 1.2 a |
| 1.0 | 1.4 b | 1.0 a | 1.4 ab | 1.2 a |

*Values represent the averages as determined from 20 replicated cuttings per treatment. For each treatment, different letters indicate that percentages are significantly different at $P \leq 0.05$. Data were recorded on the 28th day.

**Table 4.** The effect of different $GA_3$ concentrations with 1 mg/L BA on the proliferation of cultured mature female and male explants of lentisk.*

| Treatments | Female | | Male | |
|---|---|---|---|---|
| | Shoot number | Shoot length | Shoot number | Shoot length |
| Control (1 mg/L BA) | 1.6 b | 1.1 c | 1.4 c | 1.2 c |
| 1 mg/L BA + 0.1 mg/L $GA_3$ | 1.7 ab | 1.2 bc | 1.6 bc | 1.2 c |
| 1 mg/L BA + 0.3 mg/L $GA_3$ | 1.9 ab | 1.3 abc | 1.6 bc | 1.5 ab |
| 1 mg/L BA + 0.5 mg/L $GA_3$ | 1.9 ab | 1.4 ab | 1.9 ab | 1.6 a |
| 1 mg/L BA + 0. 7 mg/L $GA_3$ | 2.1 a | 1.3 ab | 2.0 a | 1.4 b |
| 1 mg/L BA + 1.0 mg/L $GA_3$ | 2.2 a | 1.4 a | 1.2 ab | 1.4 b |

*Values represent the averages as determined from 25 replicated cuttings per treatment. Different letters indicate statistical significance at $P \leq 0.05$ by Duncan's multiple range test. Data were recorded on the 28th day.

for more than 2 years. Shoot tips, 10 mm in length, were used as microscions.

**3.2.2. In vitro rootstock production from various *Pistacia* species**

In vitro seed germination response of 4 *Pistacia* species was tested for best rootstock production using rootstock production medium. The percentage of germinated seeds from *P. vera* var. Siirt, *P. khinjuk* Stocks, *P. atlantica* Desf., and *P. terebinthus* L. was found to be 80%, 92%, 60%, and 48%, respectively (Table 5). Significantly taller shoots (2.45 cm) were developed from the seeds of *P. vera* L. on the 14th day of culture. Seeds of *P. atlantica* Desf. had significantly lower shoot length among the species. With regard to root length, *P. vera* L. seeds had longer roots after 2 weeks of culture, while seeds of *P. atlantica* Desf. produced the shortest roots even after 6 weeks of culture. The largest stem diameter (2.7 mm) was produced from seeds of *P. vera* L. among the species studied (Figure 1), while the lowest stem diameter (1.62 mm) was produced from seeds of *P. terebinthus* L. even after 6 weeks of culture. Considering the observations made, *P. vera* var. Siirt or *P. khinjuk* Stocks appeared to be a promising rootstock for in vitro micrografting. Shoot necrosis in both *P. khinjuk* Stocks and *P. vera* var. Siirt was observed in most of the shoot tips from the 3rd week of culture; shoot necrosis was evident during 7 or 8 weeks of culture on the shoot tips of *P. atlantica* Desf. and *P. terebinthus* L. (data not shown). Adventitious root formation was observed after 3 or 4 weeks of culture in parallel to the development of shoot tip necrosis in almost all of the species studied. Aseptically germinated seedlings of *P. vera* var. Siirt (Figure 1) produced the largest stem diameter among the species tested for micrografting, and *P. terebinthus* and *P. atlantica* Desf. reached the grafting stage 6 weeks after culturing.

**Table 5.** Effects of *Pistacia* species on in vitro rootstock production.*

| *Pistacia* species** | Germination (%) | Shoot length (cm) | Root length (cm) | Grafting point stem diameter (mm) |
|---|---|---|---|---|
| *P. vera* var. Siirt | 80.0 b | 2.5 a | 0.8 b | 2.7 a |
| *P. khinjuk* Stocks | 92.0 a | 2.1 b | 0.6 b | 2.0 b |
| *P. atlantica* Desf. | 60.0 c | 1.4 c | 0.8 b | 1.8 c |
| *P. terebinthus* L. | 48.0 d | 2.0 b | 1.6 a | 1.6 c |

*Values represent the averages as determined from 50 replicated cuttings per treatment. Different letters indicate statistical significance at $P \leq 0.05$ by Duncan's multiple range test. Data were recorded on the 14th day.
**Results for *P. atlantica* Desf. and *P. terebinthus* L. were reported after 6 weeks of culture.

**3.2.3. Influence of the time of the year on micrografting success**

Significant differences were evident in the frequencies of successful micrografts when microscions regenerated from different times of the year in both genotypes were used (Table 6). The frequency of successful micrografts was significantly higher in all explanting times of the year when regenerated shoot tips were used in female and male lentisk (90%, 85%, 85%, 90%, 95%, 85%, 90%, and 80%, respectively). Contrary to the regenerated shoot tips, shoot tips when used directly produced significantly less successful micrografts at all explanting times of the year in both genotypes (20%, 15%, 25%, 25%, 10%, 0.0%, 0.0%, and 0.0%, respectively). The results presented in Table 6 show that the regenerated shoot tips can be used to study rejuvenation of mature shoot tips of lentisk with a success rate ranging from 85% to 95% in both genotypes.

**3.2.4. Effect of scion type on micrografting success**

The type of scion affected the rate of successfully grafted shoots significantly (Table 7) and 100% successful grafts were recorded from in vitro regenerants of both genotypes, whereas only 80% and 70% successful grafts were obtained from male and female genotypes, respectively. However, in vivo grown scions gave 50% and 40% successful grafts in both genotypes. A similar trend of growth differences was observed in the mean shoot length between the treatments tested. The highest mean shoot number was produced from in vitro regenerated shoot tips in the male and female genotypes. In vitro forced explants gave significantly lower shoots than in vitro regenerated shoots in both male and female genotypes. However, among the scion types studied, the shortest mean shoot length was obtained from in vivo grown scions. Root development of micrografts was observed in all treatments.

**3.2.5. Effects of culture medium on micrograft development**

The type of medium had an effect on micrografting. Significant differences between the three types of media were observed. Micrografting was higher in plant growth regulator (PGR)-free medium (100%) and rooting medium (100%) compared to that in the proliferation medium (80%) for the male genotypes tested (Table 8). Similarly, the female genotype also gave high graft fusions in the three types of media tested. Fused grafts developed further by elongation of scions and formation of new leaves. Development of shoots from the scions of the micrografts was evident during the first week of culturing in all treatments, but shoot development was slow in the rooting medium. A good root system was developed from micrografts in the rooting medium, but this medium

**Table 6.** Effect of the time of the year at which shoot tips were harvested on micrografting success.*

| Scion types | Successful micrografts (%) at time of the year | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | February | | May | | July | | October | |
| | Female | Male | Female | Male | Female | Male | Female | Male |
| Shoot tips used directly | 20.0 b | 15.0 b | 25.0 b | 25.0 b | 10.0 b | 0.0 b | 0.0 b | 0.0 b |
| Regenerated shoot tips | 90.0 a | 85.0 a | 85.0 a | 90.0 a | 95.0 a | 85.0 a | 90.0 a | 80.0 a |

*Values represent the averages as determined from 20 replicated cuttings per treatment. For each treatment, different letters indicate that percentages are significantly different at $P \leq 0.05$. Data were recorded on the 28th day.

**Table 7.** Effects of different scion types of mature male and female lentisk micrografted onto seedling rootstocks of *Pistacia vera* L.*

| Type of scion | Grafting success (%) | | Shoot length (cm) | | Roots development (%) | |
|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female |
| In vivo grown | 50.0 c | 40.0 c | 0.9 c | 0.9 c | 100.0 a | 90.0 b |
| In vitro forced | 80.0 b | 70.0 b | 1.3 b | 1.0 b | 90.0 b | 100.0 a |
| In vitro regenerated | 100.0 a | 100.0 a | 1.7 a | 1.6 a | 100.0 a | 100.0 a |

*Values represent the averages as determined from 20 replicated cuttings per treatment. For each treatment, different letters indicate percentages significantly different at $P \leq 0.05$. Data were recorded on the 28th day.

**Table 8.** Effects of culture medium on micrograft development after 4 weeks of culture.*

| Culture medium | Successful micrografts (%) | | Axillary shoot development (%) | | Root development (%) | |
|---|---|---|---|---|---|---|
| | Female | Male | Female | Male | Female | Male |
| PGR-free medium[a] | 100.0 a | 100.0 a | 100.0 a | 80.0 b | 70.0 b | 80.0 a |
| Proliferation medium[b] | 100.0 a | 80.0 b | 100.0 a | 90.0 a | 20.0 c | 0.0 c |
| Rooting medium[c] | 90.0 b | 100.0 a | 40.0 b | 60.0 c | 90.0 a | 70.0 b |

*Values represent the averages as determined from 20 replicated cuttings per treatment. For each treatment, different letters indicate that percentages are significantly different at $P \leq 0.05$. Data were recorded on the 28th day.
[a]MS + 30 g/L sucrose + 7 g/L agar + 50 mg/L valine.
[b]MS + 30 g/L sucrose + 7 g/L agar + 1.0 mg/L BA + 50 mg/L valine.
[c]MS + 30 g/L sucrose + 07 g/L agar + 1.0 mg/L IBA + 50 mg/L valine.

reduced shoot formation in both genotypes by up to 40% and 60% in the female and the male genotypes, respectively.

### 3.3. Acclimatization

Acclimatization of in vitro micrografted shoots of mature lentisk material was readily achieved in the growth room with a 100% success rate for both of the genotypes grown in different culture mediums. However, the micrografts grown in the PGR-free medium exhibited growth of 3 to 5 new leaves within 4 weeks of the transplanting period (data not shown). These results demonstrate the ease with which lentisk micrografts can be acclimatized in compost mixtures in the growth room. Acclimatization of micrografted seedlings was satisfactory because the micrografted lentisk plantlets resumed their growth after 2 years. Micrografted seedlings showed an orthotropic (vertical) growth within a 2-year growth period.

### 3.4. Assessment of somaclonal variation

The usage of seven AFLP primer pairs (Table 9) for selective amplification to assess the genetic fidelity of 3-, 6-, and 24-times subcultured mature female and male genotypes provided different profiles that were analyzed separately. For each primer combination the fragment size ranged from 54 to 451 bp in length (Table 10) with an average of 58 fragments per primer pair. The highest number of bands (98) was achieved with the *Eco*RI AGG*D4 - *Mse*I CAA primer combination, whereas the *Eco*RI AGG*D4 - *Mse*I CAT primer combination produced the lowest number of bands (27). A total of 406 polymorphic bands were scored. The *Eco*RI primer used in this study is known as insensitive to CpNpG methylation and triggers clustering in hypermethylated regions with low recombination frequency, such as centromeres (Young

**Table 9.** Selective primer combinations used in this study.

| Primer name | Sequence (5'–'3) |
|---|---|
| E- AGG*D4 (E) | CTGCGTACCAATTCAAGG |
| M-CAG (1) | GATGAGTCCTGAGTAACAG |
| M-CAA (2) | GATGAGTCCTGAGTAACAA |
| M-CTT (3) | GATGAGTCCTGAGTAACTT |
| M-CGT (4) | GATGAGTCCTGAGTAACGT |
| M-CAT (5) | GATGAGTCCTGAGTAACAT |
| M-CAC (6) | GATGAGTCCTGAGTAACAC |
| M-CCA (7) | GATGAGTCCTGAGTAACCA |

**Table 10.** AFLP selective primer codes, total fragments, and minimum and maximum fragment lengths in micropropagated *P. lentiscus* L. genotypes.

| Primer pair combinations | Primer code | Minimum fragment length (bp) | Maximum fragment length (bp) | Number of fragments |
|---|---|---|---|---|
| *Eco*RI AGG*D4 - *Mse*I CAG | E1 | 54.72 | 451.97 | 82 |
| *Eco*RI AGG*D4 - *Mse*I CAA | E2 | 61.29 | 369.12 | 98 |
| *Eco*RI AGG*D4 - *Mse*I CTT | E3 | 60.73 | 400.5 | 37 |
| *Eco*RI AGG*D4 - *Mse*I CGT | E4 | 54.6 | 345.58 | 32 |
| *Eco*RI AGG*D4 - *Mse*I CAT | E5 | 54.4 | 403.22 | 27 |
| *Eco*RI AGG*D4 - *Mse*I CAC | E6 | 54.53 | 445.64 | 70 |
| *Eco*RI AGG*D4 - *Mse*I CCA | E7 | 56.73 | 450.62 | 60 |
| Total | | | | 406 |

et al., 1999). The number of polymorphic fragments was 57 (14%), 59 (14.5%), 19 (4.6%), and 24 (25.6%) in the female donor plants and 3-, 6-, and 24-times subcultured clones, respectively. For the male genotypes, the number of polymorphic fragments was 44 (10.8%), 193 (47.5%), 81 (19.9%), and 130 (32%) in the male donor plants and 3-, 6-, and 24-times subcultured clones, respectively. Male subcultured clones showed higher polymorphism than female subcultured clones in comparison to their donor plants. Second-order statistics developed by Evanno et al. (2005) for structure suggested the optimal value of K = 2 (Figures 2a and 2b). Based on this, a set of main plants and different subcultured clones were divided into two clusters. In the first cluster, the original male and female plants along with 3-times subcultured female and 6-times subcultured male and female groups were included, whereas the second cluster contained the 24-times subcultured clones and the 3-times subcultured male group. PCA results (Figure 3) and total pairwise genetic distances (Table 11) were similar to the structure results. The first principal component explained 35% and the second principal component explained 21% of the AFLP variation among the 8 groups. The 3-times subcultured male group showed a higher genetic distance from the rest of the groups and was clearly differentiated in PCA. One obvious pattern in PCA was an increase in the genetic distinctiveness of the subcultures from the original plants in parallel to the increase in the number of subcultures, with the exception of the 3-times subcultured male group. As in the third analysis, the neighbor-joining dendrogram showed the same pattern as in the other two analyses (Figure 4). The 3-times subcultured male group exhibited an unusual distinction in relation to the other groups.

## 4. Discussion

Large-scale mastic tree production has not been reported for any reforestation program, although a high rooting percentage (76.6%) was obtained from cuttings taken in

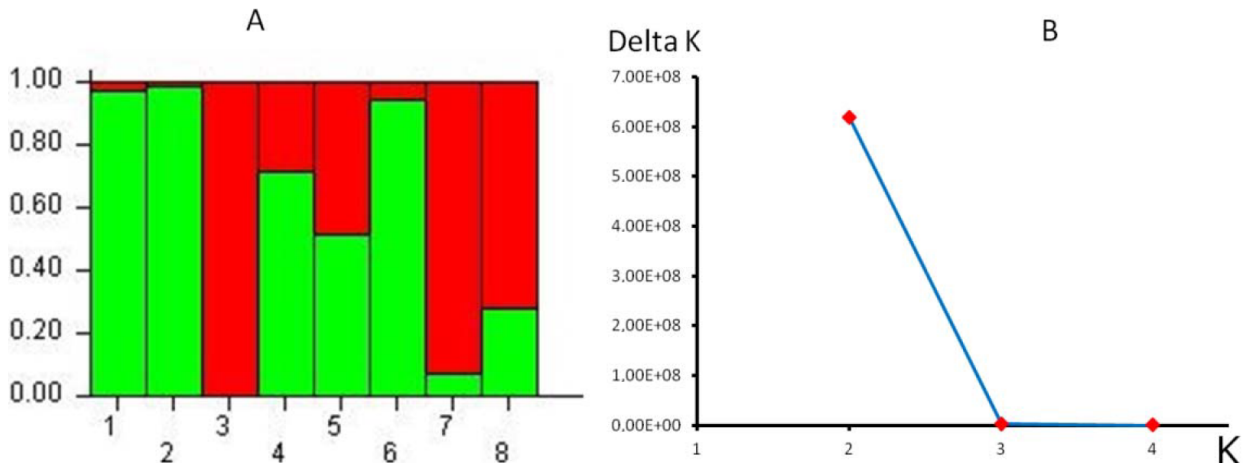


**Figure 2.** The pattern of genetic differentiation among male and female lentisk genotypes (*P. lentiscus* L.) and their somaclonal variants using fluorescent-based AFLP markers: (A) assuming two clusters and (B) determining optimal value of K based on second-order statistics (DK) developed by Evanno et al. (2005).

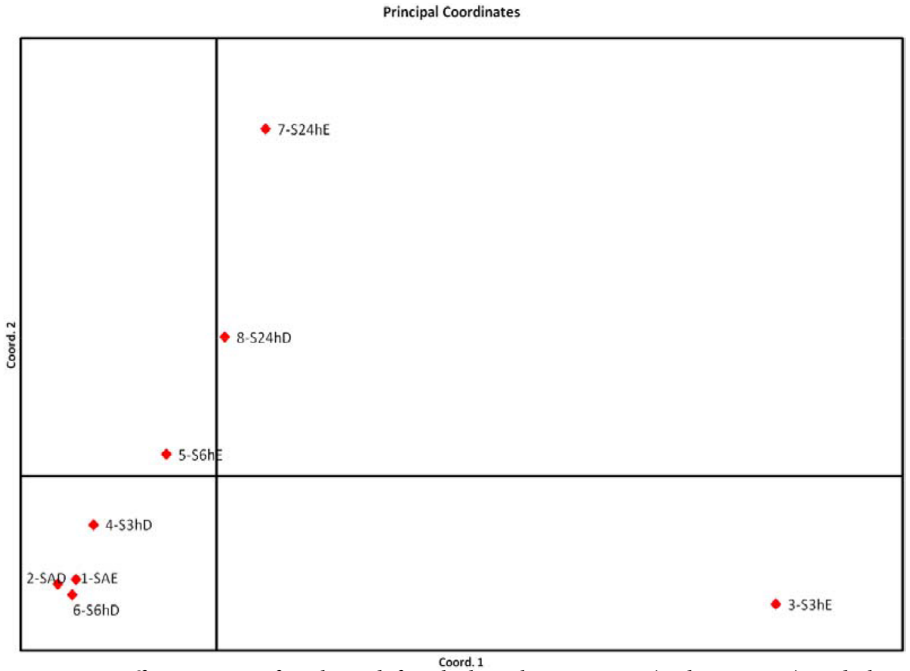


**Figure 3.** Differentiation of male and female lentisk genotypes (*P. lentiscus* L.) and their somaclonal variants using fluorescent-based AFLP markers based on the first two principal components. 1. SAE: Male plant, 2. SAD: female plant, 3. S3hE: 3-times subcultured male clone, 4. S3hD: 3-times subcultured female clone, 5. S6hE: 6-times subcultured male clone, 6. S6hD: 6-times subcultured female clone, 7. S24hE: 24-times subcultured male clone, 8. S24hD: 24-times subcultured female clone.

February and treated with 20 g/L IBA in perlite (Karakır and İsfendiyaroğlu, 1999). Likewise, other researchers who attempted to micropropagate mature lentisk trees have also encountered several difficulties (Ruffoni et al., 2004; Mascarello et al., 2007). Phenolic compounds are frequently exudated from explants cultured in vitro from mature lentisk, and these cultures often suffer from necrosis. New growth of shoots from the mature *P. vera* L. plants may be stimulated by pruning, grafting, micrografting, or BA and $GA_3$ spray treatments (Barghchi and Martinelli, 1984; Barghchi, 1986; Gonzales and Frutos, 1990). The forcing of shoots of mature *Pistacia* species has been conducted by researchers for years. In the present study, several forcing techniques were utilized to force

**Table 11.** Pairwise total genetic distance values among male and female lentisk (*Pistacia lentiscus* L.) and their somaclonal variants.

| | 1-SAE | 3-S3hE | 5-S6hE | 7-S24hE | 2-SAD | 4-S3hD | 6-S6hD | 8-S24hD |
|---|---|---|---|---|---|---|---|---|
| 1-SAE | 0 | | | | | | | |
| 3-S3hE | 227 | 0 | | | | | | |
| 5-S6hE | 106 | 219 | 0 | | | | | |
| 7-S24hE | 152 | 241 | 146 | 0 | | | | |
| 2-SAD | 51 | 234 | 103 | 153 | 0 | | | |
| 4-S3hD | 80 | 235 | 80 | 160 | 83 | 0 | | |
| 6-S6hD | 59 | 232 | 111 | 157 | 50 | 97 | 0 | |
| 8-S24hD | 126 | 227 | 130 | 154 | 129 | 126 | 139 | 0 |

SAE: Male plant; S3hE: 3-times subcultured male clone; S6hE: 6-times subcultured male clone; S24hE: 24-times subcultured male clone; SAD: female plant; S3hD: 3-times subcultured female clone; S6hD: 6-times subcultured female clone; S24hD: 24-times subcultured female clone.

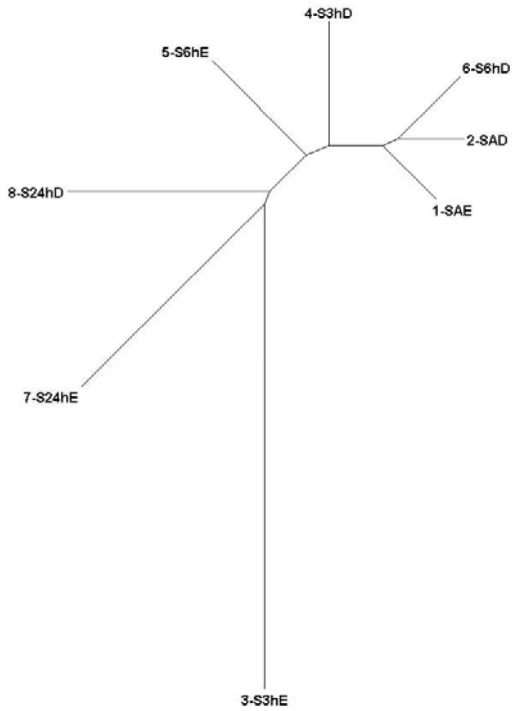


**Figure 4.** Neighbor-joining dendrogram of male and female lentisk genotypes (*P. lentiscus* L.) and their somaclonal variant genotypes. 1. SAE: Male plant, 2. SAD: female plant, 3. S3hE: 3-times subcultured male clone, 4. S3hD: 3-times subcultured female clone, 5. S6hE: 6-times subcultured male clone, 6. S6hD: 6-times subcultured female clone, 7. S24hE: 24-times subcultured male clone, 8. S24hD: 24-times subcultured female clone.

new shoot growth in a growth room during the dormant season for culture establishment of explants of mature lentisk genotypes. Among the treatments applied (Tables 1 and 2), dipping in perlite appeared to be a potential solid compost, while tap water was the most suitable type of solution for forcing nodal segments of both genotypes. Fresh herbaceous shoots were obtained from current-year hardwood shoots dipped in perlite or in tap water (forcing solution) in the growth room under a 24-h photoperiod. The explants were isolated, disinfected, and cultured in vitro when fresh growing shoots were sufficiently large enough to excise.

A surface sterilization method described by Çalar et al. (2012) for mature lentisk explants was utilized for culture initiation and in vitro shoot proliferation. Regarding shoot proliferation of juvenile lentisk materials, contradictory results have been reported by Fisichella et al. (2000), Fascella et al. (2004), Mascarello et al. (2007), Yıldırım (2012), and Kılınç et al. (2014). Earlier studies focused on the effects of carbohydrates, medium types and their strength, and concentration of BA on shoot multiplication of juvenile shoots (Yıldırım, 2012; Kılınç et al., 2014), while no study has reported on the effects of any nutrient composition for mature regenerants of lentisk. In the present study, results of the effects of different BA concentrations (Table 3) and the inclusion of different $GA_3$ concentrations with the applied BA concentration on shoot proliferation showed that statistically significant differences in average shoot number and average shoot length of both of genotypes were evident (Table 4). With regard to average shoot number and length, 1 mg/L BA + 0.5 mg/L $GA_3$ treatment was superior to the rest of the treatments tested on both genotypes. Additionally, lowest levels of mean shoot number and mean shoot length were reported in the control treatments of both genotypes. Therefore, an optimized $GA_3$ application is necessary for the proliferation of shoots of both genotypes.

Regarding the rooting of cuttings of mature lentisk, the main problem encountered in vegetative propagation is the recalcitrant nature of this species. Like other *Pistacia* species, *P. lentiscus* L. is also a natural outbreeder. Possible ways to overcome the rooting problems of mature lentisk genotypes are needed. A need for a working micrografting protocol that may be used to invigorate mature lentisk is therefore evident. Different *Pistacia* species, including *P. vera* var. Siirt, *P. khinjuk* Stocks, *P. atlantica* Desf., and *P. terebinthus* L. were assessed for in vitro production of rootstock. Germination rates of 80%, 92%, 60%, and 48% were obtained for *P. vera* L., *P. khinjuk* Stocks, *P. atlantica* Desf., and *P. terebinthus*, respectively.

Grafting point stem diameter is one of the important factors for early and successful grafting under in vitro conditions. It was reported that there were grafting point stem diameter differences among rootstocks developed in vitro by Onay et al. (2003b, 2004, 2007). In this study, *P. vera* var. Siirt had the largest grafting point stem diameter, while the kernels of *P. khinjuk* Stocks produced the second largest stem diameter among the species tested. The kernels of *P. terebinthus* L. and *P. atlantica* Desf. showed significantly smaller grafting point stem diameters (Table 5). Similar results were reported for the production of rootstocks from different *Pistacia* species in other previous publications (Onay et al., 2004). As reported in this study, there were also significant shoot length records of the seedlings of *Pistacia* species (Table 5). Concerning the length of roots and shoots, a 2-week culture period was enough for the development of roots and shoots of *P. vera* L. and *P. khinjuk* Stocks seedlings, but when they were cultured for more than 2 weeks on the culture medium, both shoot length and especially root length developed further. However, *P. terebinthus* L. and *P. atlantica* Desf. seedlings developed slowly during the 2-week period of culture. A couple of adventitious roots and long shoots were observed after 6 weeks of culture. These results suggest that *P. vera* L. and *P. khinjuk* Stocks were the most suitable rootstocks (fast

growing) for in vitro micrografting of mature regenerants of lentisk. The micrografting success of lentisk was influenced by the time of year in which shoot tips were used and the scion type (Table 6). Grafting success ranged between 80% and 95% when regenerated explants were used for the micrografting, but this changed depending on the genotype and the time of year during which shoot tips were used after the forcing treatment. Grafting success was highest (25% in both genotypes) in May but decreased to zero in October when the shoot tips were directly used for micrografting. This season-dependent response was not only reported in the pistachio (Onay et al., 2004), but also in other woody species (Navarro et al., 1975; Mosella-Chancel et al., 1979; Huang and Millican, 1980; Poessel et al., 1980). Table 7 shows the effects of different scion types of mature male and female lentisk micrografted onto seedling rootstocks of *P. vera* var. Siirt. After 4 weeks of culture, the grafting success was 100% in both genotypes when the in vitro regenerated shoots were used for micrografting, but it was only 50% and 40% for in vivo grown shoots of the male and female genotypes, respectively. As expected, root development was good in all types of scions studied, but shoot development was not as sufficient as root development because all the rootstocks utilized for micrografting were juvenile. The effects of culture medium on micrograft development were reported after 4 weeks of culture (Table 8). A PGR-free medium resulted in a 100% rate of graft fusions in both genotypes, but in the proliferation medium the male genotype achieved only 80% success rate. Successful grafts developed further with the formation of new leaves and the elongation of the scions, especially on the female micrografts, in both the proliferation and the PGR-free medium. An adequate root system was developed from micrografts in the rooting medium, but shoot formation was reduced; 40% and 60% of the micrografts of the male and female lentisk plants respectively showed visible development of new shoots. New root development was not observed in male micrografts when proliferation medium was used, but 20% of female micrografts developed further roots. Several researchers reported that the composition of the medium was one of the important factors for micrografting success of almond cultivars (Fotopoulos and Sotiropoulos, 2005; Yıldırım et al., 2010, 2013) and of pistachio (Onay et al., 2003a, 2004). Similarly, in this study significant differences were reported in the mean rates of micrografting success, as well as in axillary shoot and root development in both genotypes for the three types of media tested. However, it should be noted that a PGR-free medium was superior to the other types of media for further development of micrografts.

The results indicate that *P. lentiscus* micrografts can be easily and successfully acclimatized in compost mixtures in growth room conditions, and no problems were encountered with the establishment of micrografted plants in vivo.

Plant cell culture has the possibility to result in genetic alteration and variation in long-term regenerated plants (Larkin and Scowcroft, 1981). Due to this variation, there is always a risk that the resulting plant may not possess the same properties as the donor plant (Bairu et al., 2011). In addition to the potential infidelity of the clones, the deviations were also noted to be unpredictable in nature and extent (Skirvin et al., 1994). Therefore, the genetically diverse pattern of the 3-times subcultured male group is not surprising. The possibility of occurrence of somaclonal variation is not recommended for any micropropagation system, except for tissue culture of ornamental plants. PCR-based techniques would be required to ascertain the genetic fidelity of the regenerated plants (Venkatachalam et al., 2007). AFLP is probably the most robust molecular technique for variability analysis (Hale and Miller, 2005). Indeed, AFLP is a powerful tool for detecting tissue culture-induced changes with its high reproducibility and the possibility to multiplex 50–100 loci per primer. Several studies have revealed the stability or instability of genomes using this technique (Hashmi et al., 1997; Arencibia et al., 1999; Vendrame et al., 1999; Wilhelm, 2000; Polanco and Ruiz, 2002; Labra et al., 2004; Li et al., 2007; Martelotto et al., 2007). Therefore, AFLP markers were used in the present study to analyze the possible genetic variation in the micropropagated female and male plantlets of *P. lentiscus* L. Samples were collected from the 3-, 6-, and 24-times subcultured clones and 7 fluorescently labeled primer pair combinations were selected for the testing of genetic fidelity. As a general rule, the frequency of somaclonal variation has been found to increase in parallel with the number of subcultures and their duration (Reuveni and Israeli, 1990; Rodrigues et al., 1998). In addition to the number of subcultures, long-term cultures are also referred to as the cause of somaclonal variation (Israeli et al., 1995; Cote, 2001).

Statistical modeling efforts have not been very successful in completely predicting the extent of variations, mainly due to the complexity of the mechanisms behind them (Leva et al., 2012). The results presented here show increased diversity and differentiation when the number of subcultures is increased. An exception to the this generalization was observed in the explants of the male group from the third subculture. The 3-times subcultured male group could be considered an acceptable example of deviation from the norm.

Genetic variability induced by in vitro conditions (somaclonal variation) is not limited to any particular group of plants. AFLP analysis has been shown to be a reliable and efficient method for identification of

somaclonal variation in *P. lentiscus* regenerants. Since in the lentisk micrografting process there is little new adventitious bud formation, and only the natural system of axillary bud regrowth up to a certain number of subcultures is used for the production of new buds, no genetic changes are expected in the micrografted trees. AFLP can be used to control the quality of tissue culture seedlings for *P. lentiscus* L.

An in vitro micropropagation and micrografting method using in vitro raised seedlings as rootstock and forced shoot scions was successfully applied to mature elite lentisk trees. To the best of our knowledge, this is the first report of successfully inducing plantlets from mature lentisk genotypes, and the first analysis of the clonal fidelity of regenerated mature female and male *P. lentiscus* plants by AFLP. The establishment of an in vitro micrografting protocol for clonally propagating true-to-type mature lentisk genotypes may be an efficient technique for overcoming conventional lentisk propagation problems, where male trees are preferred for their economic value. The methods described in the present paper may be commercially viable considering the successfully high regeneration frequency and the low frequency of genotypic variation in the regenerated plants.

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