IN VITRO MICROGRAFTING OF THE ALMOND CULTIVARS "TEXAS", "FERRASTAR" AND "NONPAREIL"

Hakan Yıldırım[1], Hülya Akdemir[2], Veysel Süzerer[2], Yelda Ozden[2], Ahmet Onay[3]
[1]Dicle University, Faculty of Agriculture, Diyarbakır, Turkey
[2]Gebze Institute of Technology, Faculty of Science, Kocaeli, Turkey
[3]Dicle University, Faculty of Science, Diyarbakır, Turkey
Correspondence to: Hakan Yıldırım
E-mail: hakany@dicle.edu.tr, hakanyildirim75@hotmail.com

ABSTRACT

A successful micrografting technique for the almond cultivars (cvs) "Texas", "Ferrastar" and "Nonpareil" was developed using in vitro germinated seedlings as rootstocks and axenic shoot cultures established from mature tree sources as microscions. In vitro germinated seedlings, which developed 14 days after culturing in the modified Murashige and Skoog (MS) medium, were decapitated and used as rootstock. Shoot culture initiation from three almond cvs ("Texas", "Ferrastar" and "Nonpareil") was successfully achieved by culturing mature shoot tips from forced nodal buds, about 4–6 mm, on a modified MS medium containing 1 mg·L⁻¹ benzyl adenin (BA). Slit micrografting on epicotyl and on hypocotyls were equally successful (83.3 % to 100 %). Grafting success was dependent on the rootstock type and lenght of the scion. Grafting success varied between 83.33 % and 100 % depending on the cultivar, when the scion contained 1, 2, and 3 nodes. When almond scions, about 1.5 cm long, were micrografted on germinated seedling and cultured on proliferation medium (PM), the mean shoot length was 19.84 mm, 16.50 mm, 26.93 mm for the cvs "Texas", "Ferrastar" and "Nonpareil" respectively. Micrografts could be easily cultured on a hormone-free semi-solid MS medium and were potted out after 4 to 6 weeks of culture growth. Rooted micrografted plantlets were successfully acclimatized and transferred to potting mix with 100 % survival. Although low percentages of variation were obtained in tested cvs (3.70 %, 6.25 % and 10.2 % in "Texas", "Ferrastar" and "Nonpareil"), molecular analysis showed that the developed micrografting technique produces genetically stable plantlets, at least up to 6 months of sub-culturing in cvs "Ferrastar" and "Nonpareil". The described micrografting technique could be used for rejuvenation of shoot explants of mature elite almond cultivars and it also has potential use in the commercial production of other almond cultivars.

Introduction

Almond (*Prunus dulcis* Mill.) is one of the nut crops cultivated widely in the USA, the Mediterranean region and Australia. The majority of commercial almonds are self-incompatible (autogamy), and typically require cross-pollination (32). However, a continuous genetic variation has occurred across species through the ages, which has led to the formation of different types of trees in terms of crop development, fruit quality and yield, and tolerance to environmental stresses (15). To reduce the problem of immense genetic variation in populations and to obtain genetically identical populations, vegetative propagation via layering, cutting, division, and budding/grafting is applied. However, for almond like many other woody plants, these techniques are inefficient due to the large number of problems faced in this fruit species in *in vivo* rooting of cuttings. Micropropagation techniques are being applied to address the mass propagation needs and to bring about rapid improvement of this important crop species. Although successful tissue culture protocols have been reported for the juvenile explant of some almond cultivars (3, 5, 21, 23, 31), the published reports, especially for mature almond cultivars (1, 3, 7, 23, 33) still need to be improved due to the problems faced with *in vitro* rooting of micropropagated microshoots. Attempts to improve the *in vitro* rooting of "Nonpareil" and "Ne Plus Ultra" cultivars by assessing the influences of several culture requirements was reported by Ainsley et al. (2). Sixty percent rooting have been observed in both "Nonpareil" and "Ne Plus Ultra" cultivars using explants immersed for 12 h in water–agar 6 mg·L⁻¹ with 1.0 mmol·L⁻¹ indole-3-butyric acids (IBA) followed by incubation of microshoots in auxin-free basal media including 100.0 mol·L⁻¹ pholoroglucinol (PG). Effectively overcoming the recalcitrance structure of the rooting can also be overcome by applying a micrografting technique to the almond cultivars in the same manner as the rooting of "Ferragnes" and "Ferraduel" (37). Micrografting is an effective technique especially for rejuvenation, which was investigated in the 1980s (6, 17). It has the potential to combine the advantages of rapid *in vitro* multiplication of healthy plants with the increased productivity that results from grafting superior rootstock–scion combinations (12). This technique is an accommodation of the well-known grafting concept and procedures except that it could only be practiced on a very small scale (microsurgery) and requires exceptional manual dexterity. Micrografting can be achieved either *in vivo* or *in vitro*. *In vivo* micrografting consists of

grafting a relatively small scion originating from a desirable mother plant onto a young rootstock obtained either from a seedling or from an herbaceous cutting grown in a nursery or under aseptic conditions. On the other hand, in the case of *in vitro* micrografting the scion is surface sterilized before use and the rootstock is either a seedling obtained from aseptically germinated seed or a micropropagated microcutting (26, 27).

The first attempts to rejuvenate mature trees by micrografting of *in vitro* mature almond scions onto hybrid almonds was carried out by Martinez-Gomez and Gradziel (20), who grafted buds of "Nonpareil" seedlings onto different rootstocks with bud survival varying from 30 % to 90 %, while Ghorbel et al. (13) micrografted apical buds from *in vitro* shoots of the same cultivar with 60 % to 80 % success. *In vitro* micrografting in which almond scions were grafted on rootstock stems and cultured on a rooting medium with 50 % and 65 % survival for "Nonpareil 15-1" and "Ne Plus Ultra", respectively, was also investigated by Channuntapipat et al. (8). Micrografting of seedling shoot tips and buds on *in vitro* germinated rootstock has also been described by Ramanayake and Kovoor (29). Although several studies have been published regarding *in vitro* micrografting of almond cultivars, until now, none of these publications may be used for mass commercial production of selected almond cultivars. The regeneration protocols described in these publications tend to be difficult to replicate and no use of the protocol described in these publications may be made for any other commercially important almond cultivars. Recently, Yıldırım et al. (37) reported a successful micrografting technique using embryo-germinated seedlings as a roostock and a mature regenerated shoot as a scion of the almond cvs "Ferragnes" and "Ferraduel". The described micrografting technique resulted in 100 % regeneration in both of the tested almond cultivars, indicating that the success of the protocol was not cultivar dependent.

In the present paper the previously developed micrografting technique was applied on different commercially variable almond cvs "Texas", "Ferrastar" and Nonpareil. In addition, the genetic stability of the clones was also assessed, as somaclonal variation can be a real problem when *in vitro* propagation is used for mass propagation. Since in the almond micrografting process no adventitious formation of new buds is involved, and only the natural system of axillary bud re-growth is used for the production of new buds, no genetic changes are expected to occur in the micrografted trees. Therefore, the objectives of this study were: 1) to determine the applicability of the previously developed micrografting technique to different almond cultivars; 2) to reveal the optimum development conditions for the almond cvs "Texas", "Ferrastar" and "Nonpareil"; and 3) to test if the developed propagation methodology is useful for producing true-to-type planting material.

Materials and Methods

Plant Materials
The details of the rootstock development were reported by Yıldırım et al. (37). In short, dehusked mature almond seeds were surface-sterilized for 15 min in commercial bleach (1.2% sodium hypochlorite, NaOCl) at a volume fraction of 10 %.

Then, the seed coats were removed and the kernels were washed three times with sterile distilled water. After washing the intact kernels, the half of the kernels was cut and the kernels with the embryogenic axes were cultured in Magenta GA-7 vessels (Chicago Corp.) containing 50 ml plant growth regulator-free MS medium. Fourteen-day-old *in vitro* seedlings were decapitated above the cotyledons, and used as rootstocks for grafting.

In vitro shoot cultures of almond scions (*Prunus dulcis* cvs "Texas", "Ferrastar" and "Nonpareil") were initiated from 6-year-old almond trees grown in the Diyarbakır Province of southeastern Turkey. Fifteen to 20 mm shoot tips were surface-sterilized for 10 min in 10 % commercial bleach. After four to five rinses in sterile distilled water, shoot tips were cultured in Magenta GA 7 vessels containing 50 mL of Murashige and Skoog (24) medium with Gamborg vitamins (11) supplemented with 1.0 mg·L^{-1} BA and 30 g·L^{-1} sucrose to induce shoot proliferation. Stock cultures of "Texas", "Ferrastar" and "Nonpareil" were maintained in a fresh MS medium supplemented with 1.0 mg·L^{-1} BA.

Effects of scion type on the success of the micrografts
In order to evaluate the effect of the type of scions on the grafting success and shoot length, microscions of the following types were used: shoot tip, shoot tip plus 1 node, shoot tip plus 2 nodes, and shoot tip plus 3 nodes. Semi-solid MS medium supplemented with 30 g·L^{-1} sucrose was used to maintain the micrografts *in vitro*.

Effect of rootstock type on micrografting
Successful slit micrografting was developed for the almond cvs "Ferragnes" and "Ferraduel" by Yıldırım et al. (37). In order to further develop this method, the following rootstock types were used: 1) seedlings were decapitated (1–1.5) cm above the cotyledonary joint and the expanded cotyledons were excised, and 2) the rootstock seedling was decapitated 0.5 cm below the cotyledonary joint and a vertical slit was made on the stump. The base of the shoot tip or shoot tip plus 2 nodes, cut in v-shape, was then fitted into the slit.

Effects of media on the micrograft development
Due to the results of the experiments regarding the above mentioned two micrografting procedures, shoot tip plus 1 node was used in this micrografting trial. Micrografts were cultured in three different media: 1) proliferation medium (PM) with Gamborg vitamins containing 0.5 mg·L^{-1} BA and 0.1 mg·L^{-1} IBA, 30 g·L^{-1} sucrose and 7 g·L^{-1} agar; 2) rooting medium (RM) with Gamborg vitamins containing 0.5 mg·L^{-1} IBA and 0.1 mg·L^{-1} BA, 30 g·L^{-1} sucrose and 7 g·L^{-1} agar; and 3) a hormone-free medium consisting of Gamborg vitamins, 30 g·L^{-1} sucrose and 7 g·L^{-1} agar.

All the media used throughout the above experiments were prepared by a modified MS medium solidified with 7 g·L^{-1} agar and adjusted to pH 5.7 with NaOH and HCl before autoclaving at 121 °C. All cultures were maintained in Magenta GA-7 vessels containing 50 ml culture media with subculturing every 3 or 4 weeks. All the cultures

were placed and maintained for at least 3 weeks in a growth room under 16 h photoperiod (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with day and night temperatures of (25 ± 2) °C. Fifteen to 20 mm shoot tips were micropropagated and subcultured every 4 weeks for more than a 1-year period.

Acclimatization

The successful micrografts were carefully removed from the culture vessels, and rinsed with tap water to remove the remaining agar from the root system. Then, they were transplanted into individual commercial plastic pots filled with an autoclaved mix of equal volumes of peat and perlite (1:1), enriched with a ¼ strength MS salt solution, covered with a plastic bag to maintain high relative humidity, and transferred to a growth room at (25 ± 2) °C. During the first 2 weeks, plants were gradually acclimatized by making holes in a plastic bag. The pots were grown in a culture room with 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) at a photoperiod of 16 h. Following a 4- or 5-week period of acclimatization by progressive reduction of the humidity approximately from 95 % to 65 %, the micrografted plantlets were maintained under *in vitro* conditions for another 4 weeks in the earthen pots and kept in a shade house for further growth and observation.

DNA extraction and PCR amplification

The pair clones of 3 (3-1 and 3-2), 6 (6-1 and 6-2) and 9 (9-1 and 9-2) months subcultured plants from micrografted *P. dulcis* cvs "Nonpareil" and "Ferrastar" donor plants (D) and the pair clones of 6 (6-1 and 6-2) and 9 (9-1 and 9-2) months subcultured plants from micrografted cultivar (cv.) "Texas" donor plants (D) were selected randomly for genetic stability analysis by RAPD markers. Genomic DNA was extracted from frozen leaves by using i-genomic Plant DNA extraction Mini Kit (Intron Biotechnology, Inc.) following the manufacturer's instructions and stored at -20 °C until further use. The DNA was quantified by both agarose gel electrophoresis by using λ-DNA as a marker and spectrometric measurements. Totally, 15 primers were assessed for their reproducibility in donor plants and only 10 primers that gave clear and reproducible bands were used in RAPD PCR analysis for each cultivar (**Table 1**). The optimized RAPD protocol was carried out in a volume of 25 µl of reaction mixture containing 50 ng template DNA, 50 ng primer, 1×PCR reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 2.5 mM $MgCl_2$, 2 mM dNTP mix and 2 units of Taq DNA polymerase (Intron Biotechnology, *i*-Taq™). The amplification reaction consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles for 30 s at 95 °C for denaturation, 1 min at 35 °C for primer annealing, 1 min 40 s at 72 °C for the extension step. The final extension step was done for 8 min at 72 °C and reactions were kept at 4 °C.

The RAPD amplification products were electrophoresed in 1 $g \cdot L^{-1}$ agarose with 1×TAE buffer (Tris-asetic acid-EDTA buffer) at a constant voltage (80 V) and detected with ethidium bromide. The amplified products were visualized and photographed digitally on a UV transilluminator.

Statistical analysis

All the experiments were conducted using a completely randomized block design. Each experiment was carried out using at least 12 replicants and repeated at least twice. Significance was determined by analysis of variance, with the least significant ($P \leq 0.05$) differences, among mean values of grafting success (%), mean shoot length (mm), and the percentage of rooting, the percentage of viable regenerants (%) 4 weeks and 52 weeks after transplanting were estimated by using Duncan's new multiple range tests. For RAPD analysis, only clear and reproducible amplified bands were scored. Data was scored as "1" for the presence and "0" for the absence of a given DNA band of each plant material. The similarity matrix and dendogram were constructed using MVSP 3.1 software package (18). Genetic similarities between samples were measured by the Jaccard's similarity coefficient (16). Similarity coefficients were used to construct a dendogram using the UPGMA (unweighted pair-group method with arithmetical average) (30).

Results and Discussion

In vitro morphogenic responses of almond microscions ("Texas", "Ferrastar", "Nonpareil") and rootstocks

Dehusked mature almond seeds were harvested from one tree (**Fig. 1A**). *In vitro* germinated almond seedlings (**Fig. 1B**) derived from the embryos with half kernels were decapitated and used as rootstocks. The *in vitro* regenerated mature shoots were initiated according to Yıldırım et al. (37) from the forced shoot tips of mature almond cultivars and the proliferated microshoots were micropropagated, and subcultured every 4 weeks for more than a 1-year period. Shoot formation was observed within 10 days of explanting of the apical tips from the forced shoot tips (**Fig. 1C**). On the modified MS medium, microshoots were micropropagated (**Fig. 1D**), and subcultured every 4 or 5 weeks. After 2 or 3 weeks, the newly formed leafy shoot tips (shoot tip alone, shoot tip plus 1 node, shoot tip plus 2 nodes, and shoot tip plus 3 nodes) were removed from the shoots before they were used as microscions. The scion base of shoot tips was cut in the form of a v-shape (**Fig. 2A**). The upper portion of the rootstock was decapitated in order to use the germinated seeds as rootstocks and a vertical slit was made on the stump (**Fig. 2B**). Grafts where the scion was semi-hard produced very successful grafts depending on the cultivar 3 days after micrografting to the seedling rootstocks (**Fig. 2C**). Successful grafts occurred when scions with stems were used. Two weeks after micrografting, apical or axillar shoot development was evident on the micrografts (**Fig. 2D**). Then, the micrografts were transferred to pots and acclimatized under a transparent nylon-bag cover for 4 to 6 weeks (**Fig. 3A**). Micrografts also exhibited substantially new shoot growth with several new leaves after six months of the acclimatization period (**Fig. 3B**). However, slow growth and a lack of shoot development were noticed when the micrografts were grown in a hormone-free MS medium.
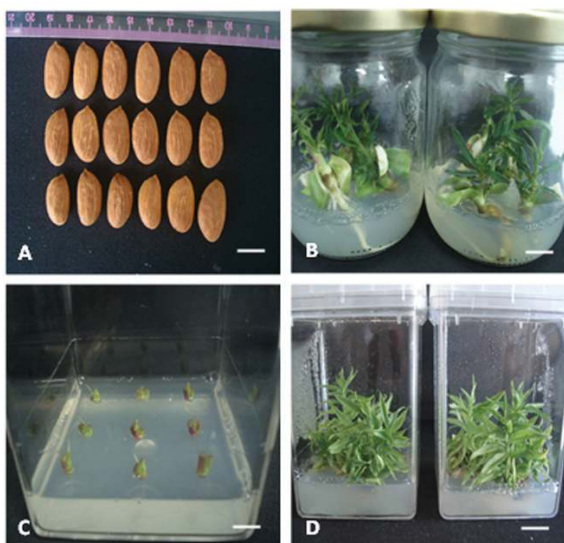
Fig. 1. *In vitro* production of almond rootstocks and microscions: dehusked mature-wild almond seeds, bar = 12 mm (**A**); 14-day-old *in vitro* propagated seedlings on a MS medium containing 1 mg·L^{-1} BA, bar = 9.5 mm (**B**); *in vitro* culture initiation from mature shoot tips of the almond cultivars, bar = 8.5 mm (**C**); multiple shoots differentiated on MS medium supplemented with 1 mg·L^{-1} BA, bar = 12 mm (**D**).
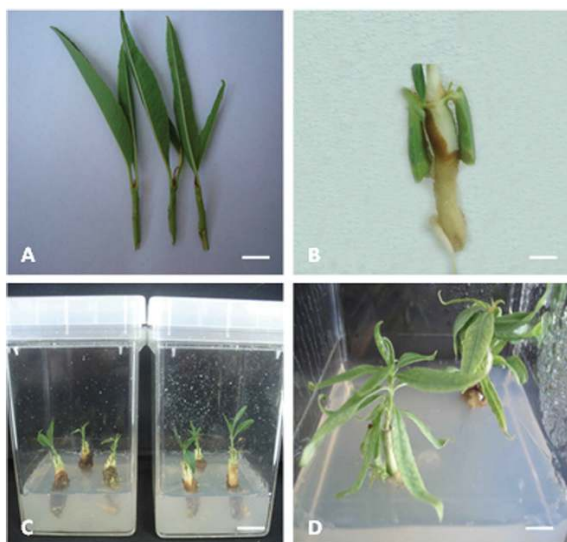

Fig. 2. Stages of micrografting: scion bases cut in a v-shape, bar = 9 mm (**A**); germinated seeds used as rootstock and a vertical slit was made on the stump, bar = 4 mm (**B**); cv. "Nonpareil", 3 days old after micrografting to the wild seedling rootstocks, bar = 15 mm (**C**); micrografted plant (arrow: the point of graft union) with new emerged axillary shoots, 3 weeks of culture, bar = 6 mm (**D**).

The rate of successful grafted shoots was influenced by the type of scions (**Table 2**). The scions of shoot tips plus 3 nodes gave the best results with 100 % successful grafts for the cvs "Ferrastar" and "Nonpareil", whereas only 85.71 % successful grafts were obtained for the scion of the cv. "Texas". Scions of a single shoot tip also gave 100 % successful grafts for the cvs "Texas" and "Nonpareil". Similarly, there were significant differences in the shoot length between the treatments tested. In cvs "Texas" and "Ferrastar" the single shoot tip plus 2 nodes

produced the highest mean shoot number, but a single shoot tip also gave relatively longer shoots for cv "Nonpareil". In general, the growth and development for the cv "Texas" was very poor when a single shoot tip and a single shoot tip plus one node was used, but the cv "Nonpareil" gave better results for the development of the shoot length among the cultivars studied.
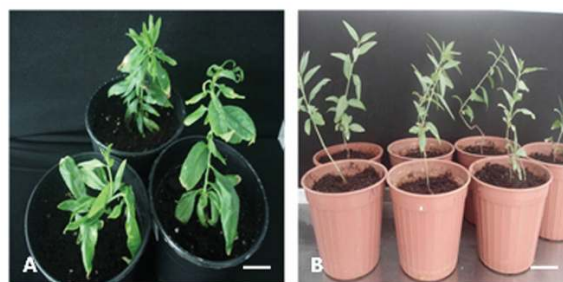

Fig. 3. The growth of micrografts of cv. "Ferragnes" under *in vitro* and *in vivo* conditions: micrografted plantlets successfully acclimatized 4 weeks after potting under a transparent nylon cover, bar = 16.5 mm (**A**); plantlets with in pots 1 year after transfer to *in vivo* conditions, bar = 42 mm (**B**).

The grafting success of both the tested rootstock types varied from 83.33 % to 100 % (**Table 3**). Relatively higher grafting success was observed in shoot tip grafting on epicotyl grafts than in shoot tip grafting on hypocotyls for all the cultivars tested. The increased success achieved in the former may be due to better cambial contact and absence of competition from buds that are present in the latter. Similarly, the length of the scion showed a significant effect on the graft. The shoot length was the highest (17.66 mm) when shoot tip plus 2 nodes was used for the cv "Ferrastar", and the lowest (8.8 mm) for the cv "Nonpareil".

Table 4 shows the effects of the medium in which the grafted plantlets were cultured on growth of micrografted scions. After 4 weeks significant differences in mean shoot length (mm) and root development of successful micrografts were observed between the treatments. Four weeks after growth, the highest mean shoot length of the micrografts were 19.84 mm, 16.50 mm and 26.93 mm for the cvs "Texas", "Ferrastar" and "Nonpareil" grown in the PM medium, respectively. In this medium, however, the secondary root development was not as good as the shoot length development. Micrografts grown in the hormone-free medium gave a mean shoot length of 15.25 mm, 14.37 mm and 26.53 mm for "Texas", Ferrastar" and "Nonpareil", respectively (**Table 4**). The micrografts in the MS0 media also exhibited the highest root development percentages with some new axillary roots but shoot formation was reduced within 4 weeks of the development period. In general, relatively shorter shoots were obtained when the RM media was used for micrograft development. However, new root development in the RM media was as high as that in the PM media.

Acclimatization

Four weeks after acclimatization, the survival rates from the micrografts were 100 %, 85 % and 90 % for the cvs "Texas", "Ferrastar" and "Nonpareil", respectively, when sterile

Total number and size range of amplified fragments and number of polymorphic fragments generated by RAPD with 10 random primers in P. dulcis cvs "Ferrastar", "Nonpareil", "Texas", donor plants and their clones

Primer	Primer sequence (5′- 3′)	Cultivars											
		Ferrastar				Nonpareil				Texas			
		Size range (bp)	Number of amplified fragments	Number of polymorphic fragments	Polymorphism percentage (%)	Size range (bp)	Number of amplified fragments	Number of polymorphic fragments	Polymorphism percentage (%)	Size range (bp)	Number of amplified fragments	Number of polymorphic fragments	Polymorphism percentage (%)
K-01	CAT TCG AGC C	-	-	-	-	380-1050	7	0	0.0	-	-	-	-
K-02	GTC TTC GCA A	190-1300	11	1	9.1	-	-	-	-	195-880	9	0	0.0
K-03	CCA GCT TAG G	120-830	8	2	25.0	220-850	6	1	16.6	240-780	7	0	0.0
K-05	TCT GTC GAG G	230-1100	11	2	18.1	210-1290	12	3	25.0	-	-	-	-
K-08	GAA CAC TGG G	200-1100	12	1	8.3	370-1100	8	0	0.0	350-1100	9	0	0.0
K-09	CCC TAC CGA C	180-1200	10	0	0.0	190-1670	11	1	9.1	380-1250	7	2	28.6
K-11	ATT GCC CCA G	220-1200	9	0	0.0	330-1400	12	2	16.6	470-1550	10	0	0.0
K-12	TGG CCC TCA C	320-1200	11	0	0.0	-	-	-	-	-	-	-	-
K-13	GGT TGT ACC C	-	-	-	-	-	-	-	-	140-1560	12	0	0.0
K-14	CCC GCT ACA C	-	-	-	-	380-1200	8	0	0.0	-	-	-	-
K-15	CTC CTG CCA A	-	-	-	-	290-960	9	0	0.0	280-980	8	0	0.0
K-16	GAG CGT CGA A	220-1250	7	0	0.0	-	-	-	-	250-1250	8	1	12.5
K-17	CCC AGC TGT G	190-1300	11	0	0.0	185-1250	14	3	21.4	-	-	-	-
K-18	CCT AGT CGA G	-	-	-	-	-	-	-	-	210-1050	6	0	0.0
K-19	CAC AGG CGG A	240-1000	6	0	0.0	250-1100	11	0	0	-	-	-	-
K-20	GTG TCG CGA G	-	-	-	-	-	-	-	-	360-760	5	0	0.0
		Total	96	6	6.25	Total	98	10	10.2	Total	81	3	3.70

Effect of the type of scions on grafting success and shoot length on the grafts in MS medium containing 30 g·L⁻¹ sucrose*

Explant type (Length of scion)	Grafting success (%)			Mean Shoot Length (mm)		
	Texas	Ferrastar	Nonpareil	Texas	Ferrastar	Nonpareil
Shoot tip	100.0 ± 0.00 a	83.33 ± 11.23 b	100.0 ± 0.00 a	5.80 ± 0.24 c	5.00 ± 0.25 d	16.00 ± 0.68 a
Shoot tip plus 1 node	100.0 ± 0.00 a	100.0 ± 0.00 a	83.33 ± 11.23 b	8.25 ± 0.79 b	7.75 ± 0.21 c	10.90 ± 0.48 c
Shoot tip plus 2 nodes	85.71 ± 9.70 b	83.33 ± 11.23 b	100.0 ± 0.00 a	9.83 ± 0.54 a	16.70 ± 1.22 a	12.83 ± 0.63 b
Shoot tip plus 3 nodes	85.71 ± 9.70 b	100.0 ± 0.00 a	100.0 ± 0.00 a	9.25 ± 0.37 a	13.58 ± 0.63 b	10.66 ± 0.68 c

* Different lowercase letters above any two columns indicate that the means are statistically different at $P \leq 0.05$ according to the Duncan multiple test. Data are means of 2 experiments with 12 replicates.

compost was used for the acclimatization (**Table 5**). The micrografts from the three cultivars acclimatized in the non-sterile compost gave the lowest level of viable regenerants. The micrografts grown in the semi-sterile compost gave survival rates of 75.0 %, 70.0 % and 75 % for the cvs "Texas", "Ferrastar" and "Nonpareil", respectively. Micrografts also exhibited substantially new shoot growth with at least three to five new leaves within 4 weeks of the acclimatization period. After 4 weeks of successful acclimatization, the micrografts were transferred to bigger pots containing a mix composed of peat and perlite. There was no evidence of a significant difference in the frequencies of viable regenerants

between those that had undergone treatments after one year of transplanting. This compost was found to be satisfactory for survival of plantlets from *in vitro* cultures of micrografted plantlets. At least 85 % of the plants propagated *in vitro* were recovered and grown as normal plants (**Fig. 3B**).

Analysis of genetic stabilities in regenerants of *P. dulcis* cvs "Nonpareil", "Ferrastar" and "Texas" by RAPD

Amplified DNA bands were scored with all of the selected RAPD primers (**Table 1**) and each primer yielded amplification products ranging in size from 185 bp to 1670 bp in cv. "Nonpareil", from 120 to 1300 in cv. "Ferrastar", and from 140 to 1560 in cv. "Texas". The number of bands for each primer varied from 6 in primer K-03 to 14 in primer K-17 in cv. "Nonpareil", from 6 in primer K-19 to 12 in primer K-08 in cv. "Ferrastar", and from 5 in primer K-20 to 12 in primer K-13 in cv. "Texas". These 10 primers used in RAPD analysis yielded a total of 98 scorable bands with an average of 9.8 bands per primer in cv. "Nonpareil", 96 scorable bands detected in cv. "Ferrastar" with an average of 9.6 bands and 81 scorable bands detected in cv. "Texas", with an average of 8.1 bands per each primer. Totally, 88 monomorphic and 10 polymorphic (10.2 %) bands in cv. "Nonpareil", 90 monomorphic and 6 polymorphic (6.25 %) bands in cv. "Ferrastar" and 78 monomorphic and 3 polymorphic (3.70 %) bands in cv. "Texas" were produced with all the tested primers. The highest number of three polymorphic bands was detected by primer K-3 and K-17, whereas primers K-01, K-08, K-14, K-15 and K-19 resulted in monomorphic bands in cv. "Nonpareil". In the case of cv. "Ferrastar", the highest number of polymorphic bands (two) was obtained by primer K-03 and K-05; and the lowest (no bands), by primers K-09, K-11, K-12, K-16, K-17, and K-19. The highest number of polymorphic bands (two) was obtained by primer K-09 and, except for primer K-16, the other primers did not give any polymorphic bands in cv "Texas". RAPD amplification patterns of *P. dulcis* cvs "Nonpareil" and "Ferrastar" are shown by representative gel profiles of primers K-05 and K-09 (**Fig. 4**), K-03 and K-05 (**Fig. 5**), respectively. Cluster analysis was carried out on the basis of Jaccard's similarity coefficients between 7 individuals (6 micropropagated and 1 donor plant) for cv. "Nonpareil" and "Ferrastar" and 5 individuals (4 micropropagated and 1 donor plant) for cv. "Texas" by means of UPGMA. The similarity values ranged from 0.907 to 1.000 with a mean of 0.945 in cv. "Nonpareil", they ranged from 0.948 to 1.000 with a mean of 0.976 in cv. "Ferrastar", and from 0.963 to 1.000 with a mean of 0.982 in cv. "Texas". As shown in the dendogram for cv. "Nonpareil" (**Fig. 6**), the donor plant and 3 month subcultured plants (3-1 and 3-2) clustered together, while the other clones (6-1, 6-2, 9-1 and 9-2) were closest related with a similarity value of 0.968 and generated another group. In cv. "Ferrastar", although the donor plant and all clones had 97 % level of similarity, clone 6-1 had the lowest similarity value (94 %) and formed another group. Clones 9-1 and 9-2 were in the same group with the donor plant, but clones 3-1, 3-2 and 6-2 were more close to the donor plant (**Fig. 7**). In the case of

cv "Texas", the donor plant and all the other clones clustered in two groups and clone 6-2 had the highest similarity value (97.5 %) to the donor plant. Other clones (6-1, 9-1 and 9-2) had the same similarity value (96.3 %) with the donor plant (**Fig. 8**). Overall, the molecular analysis showed that the genetic instability of the micrografted clones, although with a small percentage, started after 6 months of subculturing in cvs "Ferrastar" and "Nonpareil".
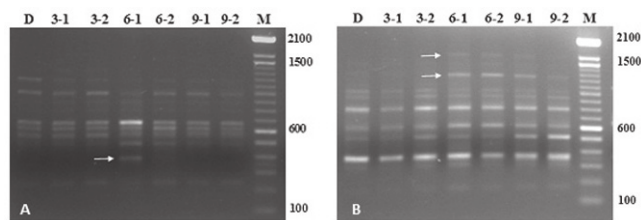

Fig. 4. RAPD analysis using primers K-05 (**A**) and K-09 (**B**) in *P. dulcis* cv. "Nonpareil" donor plant (**D**) and its clones. M: molecular size marker (100 bp + 1 kb, Invitrogen).
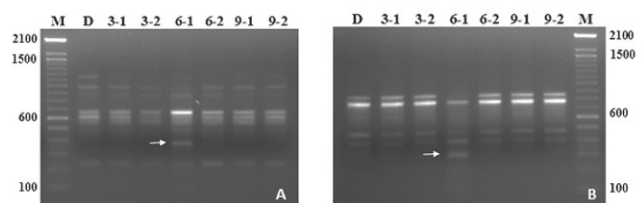

Fig. 5. RAPD analysis using primers K-03 (**A**) and K-05 (**B**) in *P. dulcis* cv. "Ferrastar" donor plant (**D**) and its clones. M: molecular size marker (100 bp + 1 kb, Invitrogen).

Effects of scission type, rootstock type and media on the micrografts

Micrografting was applied successfully to some woody species such as *Pistacia vera* Onay et al. (26), *Hevea brasiliensis* Perrin et al. (28) and *Quercus* Ewald and Naujoks (9) and some almond cultivars, i.e. (8, 13, 20, 37), for the rejuvenation of adult tissues. Among them, Ghorbel et al. (13) achieved 60 % to 80 % success with micrografting "Nonpareil" apical buds excised from *in vitro* shoots. Later, Martinez-Gomez and Gradziel (20) grafted seedlings of the same cultivar onto different rootstock with 30 % to 90 % bud survival. When 1.5 cm long almond scions were micrografted on rootstock stems and cultured on a rooting medium, 60 % and 65 % survival ("Nonpareil 15-1" and "Ne-Plus Ultra", respectively) were also achieved (8). Very recently, Yıldırım et al. (37) micrografted apical buds (approximately 1 cm) from *in vitro* shoots of the almond cvs "Ferragnes" and "Ferraduel" onto rootstocks derived from zygotic embryos of the cultivars with 100 % success. In order to assess the applicability of this technique to different almond cultivars, *in vitro* shoots obtained from elite mature trees of "Texas", "Ferrastar" and "Nonpareil" were micrografted on *in vitro* germinated rootstock. In this study scions of a shoot tip plus 3 nodes gave the best results with 100 % successful grafts for the cvs "Ferrastar" and "Nonpareil", whereas only 85.71 % successful grafts were obtained for the scion of the cv. "Texas". As in the previous publication (37), in this

TABLE 3

Effect of micrografting (rootstock type) on grafting success and shoot length on the grafts in MS medium containing 30 g·L⁻¹ sucrose and 0.5 mg·L⁻¹ BA + 0.1 mg·L⁻¹ IBA*

Effect of micrografting (rootstock type) on grafting success and shoot length on the grafts in MS medium containing 30 $g \cdot L^{-1}$ sucrose and 0.5 $mg \cdot L^{-1}$ BA + 0.1 $mg \cdot L^{-1}$ IBA*

Micrografting type	Grafting success (%)			Mean Shoot Length (mm)		
	Texas	Ferrastar	Nonpareil	Texas	Ferrastar	Nonpareil
On hypocotyl						
Shoot-tip grafting	83.33 ± 11.23 b	100.00 ± 0.00 a	100.00 ± 0.00 a	10.00 ± 0.47 b	12.33 ± 0.68 b	12.25 ± 0.57 b
Shoot tip plus 2 nodes	100.00 ± 0.00 a	100.00 ± 0.00 a	83.33 ± 11.23 b	13.16 ± 0.32 a	17.66 ± 0.68 a	8.80 ± 0.35 c
On epicotyl						
Shoot-tip grafting	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a	11.91 ± 0.41 b	13.58 ± 0.75 b	12.41 ± 0.58 b
Shoot tip plus 2 nodes	83.33 ± 11.23 b	100.00 ± 0.00 a	100.00 ± 0.00 a	14.10 ± 0.69 a	9.58 ± 0.39 c	16.83 ± 0.53 a

* Different lowercase letters above any two columns indicate that the means are statistically different at P ≤ 0.05 according to the Duncan multiple test. Data are means of 2 experiments with 12 replicates.

TABLE 4

Effect of culture medium on micrograft development*

Culture medium	Mean shoot length (mm)			Root development (%)		
	Texas	Ferrastar	Nonpareil	Texas	Ferrastar	Nonpareil
PM**	19.84 ± 1.72 a	16.50 ± 1.33 a	26.93 ± 1.64 a	66.67 ± 12.59 a	66.67 ± 12.59 a	46.67 ± 13.33 b
RM***	14.13 ± 1.27 b	16.33 ± 1.88 a	14.97 ± 1.89 b	66.67 ± 12.59 a	66.67 ± 12.59 a	60.00 ± 13.09 ab
MS0****	15.25 ± 1.28 b	14.37 ± 2.06 a	26.53 ± 2.07 a	80.00 ± 10.69 a	73.33 ± 11.81 a	86.67 ± 9.08 a

* Data are mean values from at least 20 micrografts. Different lowercase letters above any two columns indicate that the means are statistically different at P ≤ 0.05
according to the Duncan multiple test (P < 0.05). Data were taken 4 weeks after culture to the micrografting development medium.
** PM + 3 % sucrose + 0.7 % agar + 0.5 mg·L⁻¹ BA + 0.1 mg·L⁻¹ IBA;
*** RM + 3 % sucrose + 0.7 % agar + 0.5 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ BA;
**** MS0, Hormone-free MS+ 3 % sucrose + 0.7 % agar.

TABLE 5

The effects of compost on acclimatization of almond plantlets in sand peat and perlite (1:1) mixture*

Compost	Viable regenerant (%)					
	4 weeks after transplanting**			52 weeks after transplanting***		
	Texas	Ferrastar	Nonpareil	Texas	Ferrastar	Nonpareil
Sterile	100.00 ± 0.00 a	85.00 ± 8.19 a	90.00 ± 6.88 a	95.00 ± 5.00 a	90.00 ± 6.88 a	100.00 ± 0.00 a
Semi-sterile	75.00 ± 9.93 b	70.00 ± 10.51 a	75.00 ± 9.93 a	90.00 ± 6.88 a	85.00 ± 8.19 a	90.00 ± 6.88 a
Non-sterile	30.00 ± 10.51 c	25.00 ± 9.93 b	40.00 ± 11.23 b	100.00 ± 0.00 a	85.00 ± 8.19 a	90.00 ± 6.88 a

* Data are mean values from at least 20 micrografts. Different lowercase letters above any two columns indicate that the means are statistically different at P ≤ 0.05 according to the Duncan multiple test (P < 0.05).
** Data taken 4 weeks after transplanting to growth room conditions;
*** Data taken 52 weeks after transplanting to glass house conditions.

study the successful micrografts were obtained without need of pre-treatment of the microscions. This result was also in agreement with the results of Navarro (25) and Wu et al. (36), who obtained higher micrografting success with several woody species by using untreated microscions. It should also be noted that the micrografting success rates obtained in this study with the three cvs "Texas", "Ferrastar" and "Nonpareil" microscions gave similar results in comparison with the previous study of Yıldırım et al. (37), in which 100 % micrografting success rate was achieved with the "Ferragnes" and "Ferraduel" almond cultivars by utilizing mature shoot tip microscions. There was significant difference between the two

types of micrografting, as the grafting success was higher in the shoot tip grafting on epicotyl grafts than that in the shoot tip grafting on hypocotyls for all the cultivars tested (**Table 3**). The increased shoot length in the hypocotyls may be due to cambial contact and absence of competition from axillary buds that are present in the epicotyls. Although several authors have previously reported that in the micrografting of different plant species, e.g., cashew (34), pistachio (26), cherry (4) and King Protea (36), including almond (13), the size of the scion affects the success rate of micrografts, no significant difference in the frequency of successful micrografts was obtained in the

three of the almond cultivars tested when the microscion was applied to different sizes.
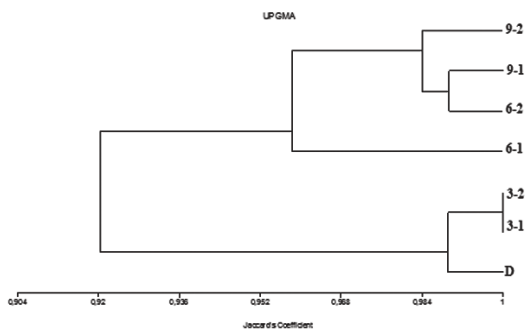

Fig. 6. Dendogram illustrating the similarity coefficients among 6 micropropagated plantlets and the donor plant of *P. dulcis* cv. "Nonpareil" by UPGMA analysis derived from a total of 98 bands produced by the 10 RAPD primers.
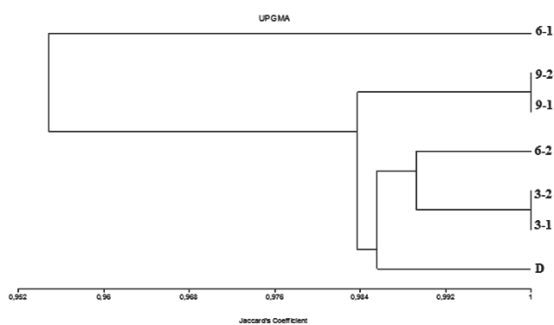

Fig. 7. Dendogram illustrating the similarity coefficients among 6 micropropagated plantlets and the donor plant of *P. dulcis* cv. "Ferrastar" by UPGMA analysis derived from a total of 96 bands produced by the 10 RAPD primers.
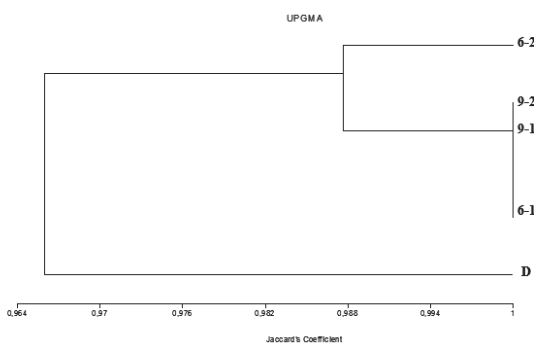

Fig. 8. Dendogram illustrating the similarity coefficients among 4 micropropagated plantlets and the donor plant of *P. dulcis* cv. "Texas" by UPGMA analysis derived from a total of 81 bands produced by the 10 RAPD primers.

The culture medium also showed a significant effect on the graft success (**Table 4**). In the present study, the micrografts in rooting medium showed a good root system but reduced shoot formation. New shoot elongation was observed when the PM medium was used for the development of the grafts. Similarly, Yıldırım et al. (37) reported that MS0 permitted good growth and branching of the roots and resulted in the development of axillary branching of the scion of cvs "Ferragnes" and "Feraduel". Moreover, it was also previously stated that the

composition of the media that grafts are established in can also have an effect on micrografting success (10). In this study, there was no significant difference in the means of micrografting success in the three cultivars, as 100 % frequency was obtained in a hormone-free MS medium, as well as in PM and in RM (data not shown). However, it should also be noted that relatively lower plant survival was obtained in micrografts established in MS0 medium during acclimatization. Hormone-free semi-solid MS medium with 3 % sucrose was found to be ideal for culturing of micrografts. Similarly, Onay et al. (26, 27) in pistachio, Mantell et al. (19) and Ramanayake and Kovoor (29) in cashew, and Yıldırım et al. (37) in almond have used solid medium for the establishment of micrografts. This result probably shows that the transfer of endogenous cytokinins or auxins to the scion on the part of the rootstock was not adequate in the hormone-free medium and absorption of plant growth regulators from the medium and transfer to the microscion is needed for a higher survival after transfer to *in vivo* conditions (37).

To the best of our knowledge, this is the first report which includes assessment of the genetic integrity in micrografted almond cultivars. However, genetic analysis of almond plantlets propagated with axillary branching (21) and adventitious regeneration and meristem culture (22) showed genetic integrity of the plantlets. But it is well-known that tissue culture conditions result in physiological stress that can lead to impairment of normal developmental controls and trigger the mechanism of somaclonal variation (14). Somaclonal variation can be detected by using different molecular techniques. Among them, randomly amplified polymorphic DNA (RAPD) (35) is one of the mostly used molecular markers as it is relatively fast, easy and cheap. Thus, in order to confirm that micrografting is a reliable propagation method, RAPD molecular marker technique was carried out in *P. dulcis* cvs "Ferrastar", "Nonpareil" and "Texas" donor plants and their regenerants. In this study, five of the 10 primers in cv. "Nonpareil" and six of 10 primers in cv. "Ferrastar" generated monomorphic patterns among the donor plants and its clones, confirming the genetic uniformity of the micropropagated materials. However, other primers produced polymorphism (**Fig. 4** and **Fig. 5**) although at a low percentage. RAPD analysis showed that subculturing of micrografted plantlets especially more than 6 months lead to increase of polymorphism for cvs "Ferrastar" and "Nonpareil". In the case of cv. "Texas", as somaclonal variation was observed after a 6 months' subculturing period, in cvs "Ferrastar" and "Nonpareil" evaluation of only 6 and 9 months subcultured plants was assessed in this study. The overall polymorphism frequency detected in *P. dulcis* cv. "Texas" was relatively lower (3.70 %) in comparison with cv. "Ferrastar" (6.25 %) and cv "Nonpareil" (10.2 %). We showed that the developed micrografting protocol can be safely applied in almond cultivars up to 6 months of subculture but a longer subculture period may result in somaclonal variation although at low level in almond cultivars.

Conclusions

A standardized micrografting technique using *in vitro* raised seedlings as rootstock and shoot scions from mature elite trees was successfully applied to almond cvs "Texas", "Ferrastar" and "Nonpareil". The results of the RAPD analysis strongly suggest that in the three tested almond cultivars, the optimized micrografting protocol may not induce genetic variability until 6 months. To the best of our knowledge, this is the first study on assessment of the genetic stability of micrografted almonds by RAPD analysis.

Acknowledgements

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