

Micrografting of almond (*Amygdalus communis*) cultivar 'Nonpareil'

Çiğdem Işıkalan^{1*}, Süreyya Namli¹, Filiz Akbas², Bekir Erol Ak³

¹Dicle University, Faculty of Science, Department of Biology, 21280, Diyarbakır-Turkey

²Batman University, Faculty of Science and Arts, Department of Biology, Batman-Turkey

³Harran University, Faculty of Agriculture, Department of Horticulture, Urfa-Turkey

*corresponding author: ahrar@dicle.edu.tr

Abstract

Effects of plant growth regulators (PGRs) were investigated on micropropagation of scions and micrografting of almond (*Amygdalus communis*) cultivar 'Nonpareil'. *In vitro* germinated wild almond seedlings developed from seeds were used as rootstocks. The mature apical or subapical shoot tips of almond cultivar 'Nonpareil' were used as material for establishment of the microscions cultures. The shoot tips were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 4.0 mg l⁻¹) of N⁶-benzylaminopurine (BAP). The data showed that the increase in BAP concentration resulted in significant reduction at the shoot regeneration rate. Among all tested groups, the highest regeneration rate was obtained on medium containing 1.0 mg l⁻¹ BAP. The regenerated adventitious shoots from *in vitro* cultures were cultured on media containing BAP (0.5, 1.0, 1.5 mg l⁻¹) combined with 0.2 and 0.4 mg l⁻¹ indole butyric acid (IBA) separately for development of shoots. The best respond was observed from MS medium supplemented with 1.0 mg l⁻¹ BAP + 0.2 mg l⁻¹ IBA. The effects of BAP and IBA (1.0 mg l⁻¹) were studied on development of micrografted plantlets. Regenerated shoots tips, which were micrografted onto *in vitro* germinated wild almond seedlings. The results indicated that the most graft rate and new shoots formation were obtained 1.0 mg l⁻¹ BAP. *In vitro* micrografted plantlets were successfully transferred into commercial plastic pots for acclimatization.

Keywords: Almond, Nonpareil, Micrografting, Rootstocks, Microscions.

Abbreviations: PGRs; Plant growth regulators, BAP; N⁶-benzylaminopurine, IBA; Indole-3-butyric acid, MS; Murashige and Skoog (1962) medium

Introduction

Modern biotechnology techniques can hasten the production of new genotypes and broaden the gene pool available for improvement of woody fruit species (Ainsley et al., 2001). However, fruit trees are amongst the most recalcitrant for *in vitro* culture, and regeneration of adventitious shoots from adult explants has proven difficult (Miguel et al., 1996). Conventional breeding of woody fruit species is a slow and difficult process due to high levels of heterozygosity and long generation cycles (Sriskandarajah et al., 1994). To minimize the problem of enormous genetic variation and to obtain genetically identical populations, vegetative propagation via layering or cutting is applied. However, these techniques are inefficient due to the large number of problems faced in this fruit species in *in vivo* rooting of cutting. Because of the difficulties encountered with rooting of cuttings of almond cultivars, grafting and budding onto seedling rootstocks is the usual method used for vegetative propagation (Yıldırım et al., 2010). Microshoots that are difficult to root, or do not form roots at all *in vitro*, can be micrografted onto rootstocks to obtain rooted plantlets (Thimmappaiah Putra and Anil 2002). *In vitro* micrografting has been reported in many plants such as cashew (Ramanayake and Kovoov 1999; Mneney and Mantell 2001; Thimmappaiah Puthra and Anil 2002), pistachio (Abousalim and Mantel 1992; Onay et al., 2002; Onay et al., 2007), olea europaea (Revilla et al., 1996), almond (Channuntapipat et al., 2003), *Prunus avium* L.(cherry)

var. (Amiri 2006). Micrografts, developed in the 1970s, involve the grafting of millimeter-size vegetative meristems. Micrografting is mostly used for obtaining virus-free plants, separating viruses in infections, breeding specific genotypic combinations, and for studying graft incompatibility between scions and rootstocks (Burger 1985; Navarro 1988). *In vitro* micrografting may provide several advantages such as elimination of viruses, rejuvenation of mature tissues, year round plant production, enhance compatibility studies and correlative relation between rootstocks and scions, breeding for specific genotypic combinations to increase plant productivity, and extension of ecological limits of a particular plant species (Richardson et al., 1996; Hartmann et al., 1997). Compared to traditional grafting with *in vitro* grafting procedure, micrografting has several potential advantages over the traditional procedure; it is much more rapid, requires much less space, particularly disease-free planting materials and producing genetically uniform plantlets. In *in vitro* micrografting studies, ensuring good contact between the microscion and the rootstock was essential for the graft unions to form successfully. Also, the optimization of culture conditions and media is significant for micrografting studies. The objective of the present study was to investigate influence of BAP for establishment of the microscions cultures and micrografting procedure was to develop for almond cultivar 'Nonpareil'.

Table 1. The effect of BAP on micropropagation of Nonpareil microscions

Concentrations of BAP	Avg No of shoots/ explant (Mean ± SE)	Avg length of shoots (cm) (Mean ± SE)
0.0 mg l ⁻¹ BAP	0.58 ± 0.14 c	0.72 ± 0.15 b
0.5 mg l ⁻¹ BAP	3.41 ± 0.57 b	0.72 ± 0.18 b
1.0 mg l ⁻¹ BAP	6.66 ± 1.27 a	1.95 ± 0.25 a
1.5 mg l ⁻¹ BAP	4.70 ± 1.35 a	1.75 ± 0.38 a
2.0 mg l ⁻¹ BAP	4.00 ± 0.77 ab	0.58 ± 0.10 b
4.0 mg l ⁻¹ BAP	2.91 ± 1.26 bc	0.22 ± 0.07 c

*Data recorded on the 4 weeks; 12 replicates per treatment and repeated twice. Different lowercase letter above any two columns indicate that these two means are statistically different at $p \leq 0.05$ according to Duncan's multiple range test.

Table 2. Effects of BAP and IBA combinations on micropropagation of Nonpareil microscions

Plant Growth Regulators (mg l ⁻¹)		Avg No of shoots/ explant (Mean ± SE)	Avg length of shoots (cm) (Mean ± SE)
BAP	IBA		
0.0	0.2	0.29 ± 0.68 c	1.32 ± 0.49 ab
0.5	0.2	2.41 ± 1.87 a	1.55 ± 0.75 ab
1.0	0.2	2.88 ± 1.83 a	1.63 ± 0.82 a
1.5	0.2	2.64 ± 2.66 a	1.20 ± 0.76 b
0.0	0.4	0.41 ± 1.00 bc	1.35 ± 0.51 ab
0.5	0.4	0.82 ± 0.80 b	1.51 ± 0.65 ab
1.0	0.4	2.00 ± 1.41 a	1.67 ± 0.64 ab
1.5	0.4	1.82 ± 1.28 a	1.47 ± 0.41 ab

*Data recorded on the 4 weeks; 12 replicates per treatment and repeated twice. Different lowercase letter above any two columns indicate that these two means are statistically different at $p \leq 0.05$ according to Duncan's multiple range test.

Materials and methods

Establishment of the rootstocks

The seeds from naturally grown wild almond trees found in Diyarbakır Province of southeastern Turkey were used as explants for the establishment of the rootstocks. The explants were surface-sterilized by immersion in a 10% (w/v) commercial bleach solution (NaOCl) for 30 min after pre-sterilisation processes, which included washing with running water for 5–10 minutes and then, rinsing with 70% (w/v) ethanol for 30 seconds. The explants were rinsed with sterilized water 5 times for 5 minutes to remove the NaOCl, then, kernel with embryos were cultured in Magenta GA-7 vessels containing 50 ml MS (Murashige and Skoog 1962) medium including 1.0 mg l⁻¹ BAP (Isikalan et al., 2008).

Preparation of the microscion

Young offshoots were collected during the months of March–April from 7-years-old trees of almond cultivars, Nonpareil growing in the Botanical garden of the University of Harran, Şanlıurfa, Turkey. To evaluate the effect of various cytokinins on shoots development, leaves from offshoots were eliminated, and were cut into 8–12 cm in length pieces, each having an apical shoot. The explants were washed with running water for 5–10 minutes, then they were dipped in 70% ethanol for 30 second, surface-sterilized in 10% (w/v) commercial bleach solution (NaOCl) for 20 min, and rinsed five times with sterile distilled water (5 min per rinse). Sterile apical tips (1.5–2.0 cm in length) were cultured into the MS medium containing 0.5, 1.0, 1.5, 2.0, 4.0 mg l⁻¹ BAP separately. Moreover, aseptic shoots were cultured on media containing different concentrations of BAP

(0.5, 1.0, 1.5 mg l⁻¹) combined with 0.2 and 0.4 mg l⁻¹ IBA separately. *In vitro* regenerated shoots were micropropagated and subcultured every 3 weeks. After 2 or 3 weeks, elite shoot tips were used as microscions for *in vitro* grafting experiments. The results were analyzed in a completely randomized block design. Significance was determined by analysis of variance, with the least significant ($p \leq 0.05$) differences among mean values were estimated using Duncan's New Multiple Range Test.

Media and culture conditions

In this study, all media were supplemented with 3% sucrose (w/v), solidified with 5.45 g l⁻¹ agar (Sigma), and media were adjusted to pH 5.8 prior to autoclaving (120°C for 20 min), and *in vitro* cultures were maintained at 25 ± 2°C with light at 40 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) (“cool white” fluorescent lamps, 75 W) a photoperiod of 16 h.

Grafting procedure and maintenance of micrograft

Micrografting was achieved by grafting the microscions onto the seedlings *in vitro*-germinated. Seedlings (2 weeks-old) germinated under *in vitro* conditions were used as rootstock (Fig. 1A). These were decapitated, and a 2 mm vertical incision was made from the top of each rootstock. Microscions were prepared by cutting the microshoots evenly into 1.5–2.0 cm in length, and each bottom end into a wedge (‘V’) shape. *In vitro* grafting was performed under sterile conditions. The micrografted seedlings were cultured into the MS medium containing 1.0 mg l⁻¹ BAP and IBA with a control group which did not contain any PGRs.

Table 3. Effect of BAP and IBA on development of grafted plants

Treatments	Graft unions rate (%)	Number of roots	Length of roots (cm)	Avg No of shoots / explant (Mean ± SE)
Control	91.6	2.66 ± 2.67 ab	0.48 ± 0.84 b	0.91 ± 1.37 b
1.0 mg l ⁻¹ IBA	100	4.83 ± 4.66 a	1.60 ± 1.48 a	1.25 ± 0.75 b
1.0 mg l ⁻¹ BAP	100	1.41 ± 2.81 b	0.36 ± 0.53 b	2.75 ± 1.35 a

*Data recorded on the 4 weeks; 12 replicates per treatment and repeated twice. Different lowercase letter above any two columns indicate that these two means are statistically different at $p \leq 0.05$ according to Duncan's multiple range test.

Acclimatization

Successfully grafted plantlets were removed from the culture vessels, and rinsed with tap water to remove remaining agar from the root system. Subsequently, they were transplanted into individual commercial plastic pots filled with an autoclaved mix of sand, perlite and soil [1:1:1 (v/v)], covered with a beher glass to maintain a 90±5% relative humidity. During the first 2 weeks, the beher glass were gradually punctured to allow air exchange for plant acclimatization. Grafted plantlets were maintained at 25 ± 2°C with light at 40 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) ("cool white" fluorescent lamps, 75 W) a photoperiod of 16 h.

Results

Effect of BAP on shoot multiplication of microscions

For micropropagation of microscions, apical tips were cultured on MS medium (Fig. 1B) which was supplemented with different concentrations of BAP. From the results presented in Table 1, it appears that the number of shoots changed depending on different concentrations of BAP. When the number of shoots was compared, there were statistically significant differences among concentrations tested. Also, it is clear that the addition of BAP (1.0 mg l⁻¹) to the medium increased the shoot number and enhanced them vigorously. The average number of shoots per explant decreased significantly ($P < 0.05$) whereas, increasing BAP concentration from 1.0 mg l⁻¹ to the high concentrations (2.0 or 4.0 mg l⁻¹). The lowest shoot formation was observed on media supplemented with 4.0 mg l⁻¹BAP (2.91±1.26). The maximum number of shoots (6.66±1.27) was obtained from MS medium with 1.0 mg l⁻¹ BAP (Fig 1C), followed by 4.70±1.35 shoots from 1.5 mg l⁻¹ BAP. At the same time, maximum shoot length (1.95±0.25) was observed in the presence of 1.0 mg l⁻¹ BAP (Table 1). In the absence of growth regulators, it was observed as 0.58±0.14 new shoot formation. The results showed that using BAP for the new shoot formation was absolutely necessary.

Effects combination of BAP and IBA on shoot multiplication of microscions

Results in Table 2 showed that using of IBA alone lead to decrease at length and in number of shoots. However, the length and number of shoots are determined to increase when used together with BAP and IBA. For development of microscions, the best result was obtained from MS medium supplemented with 1.0 mg l⁻¹ BAP + 0.2 mg l⁻¹ IBA (Table 2). However, only the use of BAP proved to be more beneficial than the combination of IBA and BAP for development of microscions. Consequently, for multiplication and development of scions, the best plant growth regular was determined as 1.0 mg l⁻¹ BAP (6.66 ± 1.27 - 1.95±0.25), when Table 1 and Table 2 were compared.

Effects of BAP and IBA on micrograft development

Grafted plantlets under *in vitro* conditions were cultured on MS medium containing 1.0 mg l⁻¹ BAP and 1.0 mg l⁻¹ IBA with a control group, separately (Fig. 1D). At all treatments tested, initial traces of graft unions were observed between scions and rootstocks (2 weekly) within 7- 10 days after graft procedure. After 2 weeks, cultured graft unions were 90% on medium with BAP and IBA (1.0 mg l⁻¹) except for the control group. It was also noted that there was a color change in plantlets on the hormone-free medium and leaves became light-green, white or brown, while healthy shoots were obtained on medium supplemented with 1.0 mg l⁻¹ BAP.

After 25 days of culture, new shoot formation was observed from the medium supplemented with 1.0 mg l⁻¹ BAP, while the best response for root formation and elongation was obtained from the medium with 1.0 mg l⁻¹ IBA (Table 3). At the same time, it was determined that there were no significant differences between IBA and BAP for graft union rate. For shoot number, the best result was obtained on the medium supplemented with 1.0 mg l⁻¹ of BAP and an average of 2.75 shoots was formed from each explant. Although similar results were obtained on graft union rate at all tested group, the medium supplemented with 1.0 mg l⁻¹ BAP was determined as favorable one for development grafted plantlets (Fig. 1E-a,b). *In vitro* micrografted plantlets were successfully acclimatized and no problems were encountered with the establishment of micrografted plants *in vivo* (Fig. 1F).

Discussion

Micrografting is a technique that potentially can combine the advantages of rapid *in vitro* multiplication with the increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach 1988). In general, micropropagation is difficult from mature tissues or organs of all woody trees. However, previous publications indicate that almond is difficult to micropropagate efficiently from mature tissues also (Caboni 1994; Akbas et al., 2009). Each plant species that was propagated *in vitro* has different plant growth regulator requirements and concentrations. Due to these reasons, optimization of culture conditions and media were important for micropropagation studies. In this research, MS medium containing 1.0 mg l⁻¹ BAP was chosen as the optimum medium for multiplication and development shoot tips of Nonpareil. Gürel and Gülşen (1998) also reported that BA was absolutely necessary for *in vitro* propagation of shoot tips of almond (Nonpareil and Texas) during culture initiation and proliferation stages. On the contrary, Channuntapipat et al. (2003) reported that AP medium containing 0.049 μM IBA and 3 μM BA was effective for propagation shoot tips of Nonpareil 15-1. Gürel and Gülşen (1998) reported that the best result for shoot development and growth of almond was obtained from the combination of 0.1 mg l⁻¹ IBA and 1.0 mg l⁻¹ BAP. In contrast, our results showed that the use of only BAP on medium proved to be more beneficial than the

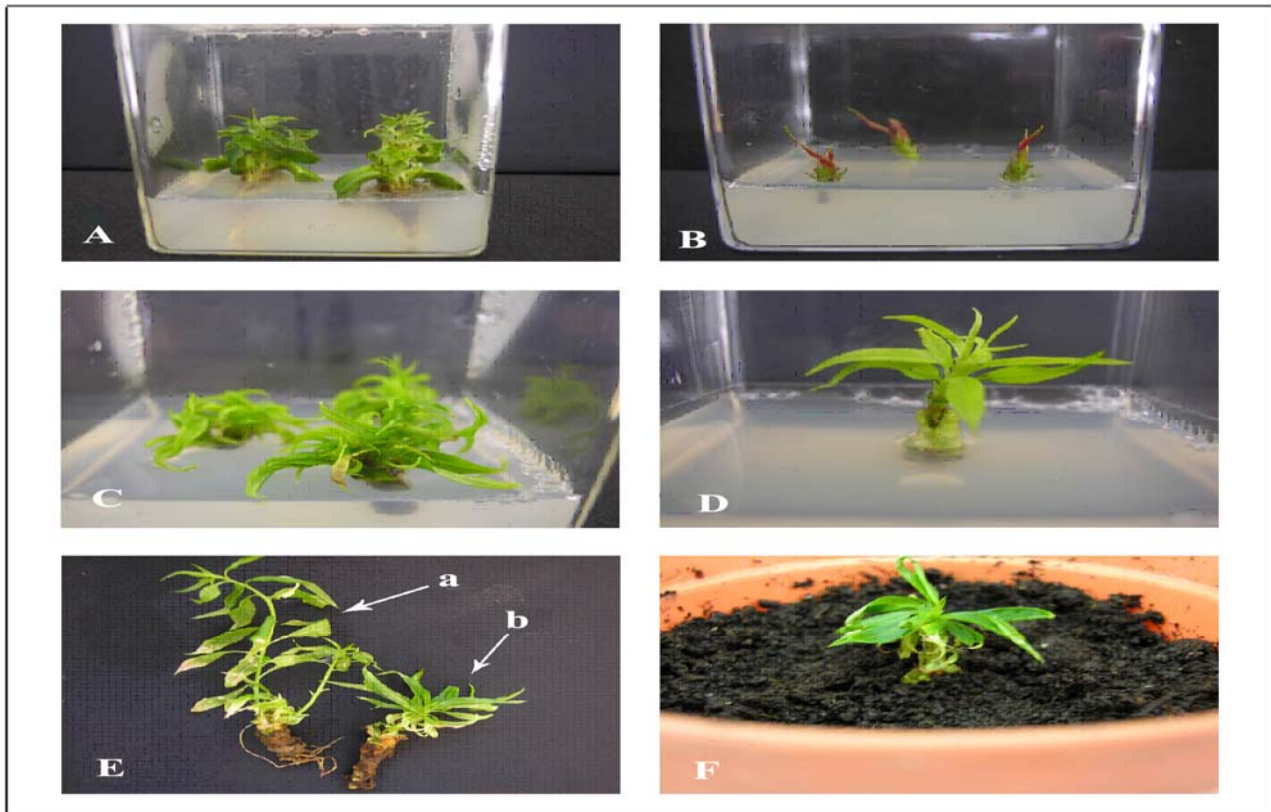


Fig 1. Micrografting of Almond (*Amygdalus communis*) Cultivar 'Nonpareil'
(A) Seedlings (2 weeks-old) were germinated from mature seeds of Almond.
(B) Aspect of shoot tips cultured on MS medium.
(C) Aspect of multiple shoots on MS medium supplemented with 1.0 mg l⁻¹ BAP.
(D) Aspect of shoots micrografted on MS medium.
(E) **(a)** Aspect of micrografted plantlet on MS medium supplemented with 1.0 mg l⁻¹ BAP.
(b) Aspect of micrografted plantlet on MS medium hormone-free

combination of IBA and BAP for shoot development. The difficulty in the rooting of almond occurs both *in vivo* and *in vitro* especially when shoots come from mature trees. Although several trials were performed on almond in order to overcome this problem by using different auxin types, concentrations, basal salt composition and phenolic compounds, the rooting frequency especially of paper shell almond cultivars, still needs to be improved as rooting levels up to 75% were achieved in hard shell cultivars (Caboni et al., 1997). Thus, development of a suitable micrografting technique can provide a solution to overcome rooting difficulties of plant species like *in vitro* proliferated almond tissues and explants. The technique of micrografting was used in this study because this procedure is a safe and an alternative method for producing genetically uniform, disease-free planting materials and micropropagation of fruit trees. In the present study, an effective micrografting technique was developed and 90% success was obtained for Nonpareil. Channuntapipat et al. (2003) obtained 50–65% success from micrografting of Nonpareil 15–1 and Ne Plus Ultra. Ghorbel et al. (1998) micrografted apical buds from *in vitro* shoots of the almond cultivar Achak on to rootstocks derived from zygotic embryos of the same cultivars with 60–80% success. In this research, an efficient procedure was developed for micrografting of Nonpareil and 90% success was obtained for graft union rate. The MS medium

supplemented with 1.0 mg l⁻¹ BAP was determined as optimum medium for development grafted plantlets. This procedure provided more micrografted seedling on a little area in a short time. Furthermore, this technique can be adopted in breeding of specific genotypes or other species that are difficult to root. Likewise, when considering the application of genetic transformation to the almond, it is also important to develop protocols for efficient regeneration of plants from adult trees.

References

- Abousalim A, Mantell SH (1992) Micrografting of pistachio (*Pistacia vera* L. cv. 'Mateur'). *Plant Cell, Tissue and Organ Culture*. 29, 231–234.
- Akbaş F, Işıkalan Ç, Namlı S, Ak BE (2009) Effect of plant growth regulators on *in vitro* shoot multiplication of *Amygdalus communis* L. cv. Yaltsinki. *African Journal of Biotechnology*. 8 (22), 6168–6174.
- Ainsley PJ, Hammerschlag FA, Bertozzi T, Collins GG, Sedgley M (2001) Regeneration of almond from immature seed cotyledons. *Plant Cell Tissue and Organ Culture*. 67: 221–226.

- Amiri ME (2006) *In vitro* techniques to study the shoot-tip grafting of *Prunus avium* L. (cherry) var. Seeyahe Mashad. J. Food Agric. Environ. 4 (1), 151–154.
- Burger DW (1985) Micrografting: a tool for the plant propagator. Comb Proc Int Plant Prop Soc 34: 244–248.
- Caboni E, Damiano C (1994) Rooting in two almond genotypes. Plant Sci. 96, 163–165.
- Caboni E, Tonelli MG, Laur P, Lacovacc P, Kevers C, Damiano C, Gaspar T (1997) Biochemical aspects of almond microcuttings related to *in vitro* rooting ability. Biol. Plant. 39, 91–97.
- Channuntapipat C, Sedgley M, Collins G (2003) Micropropagation of almond cultivars Nonpareil and Ne Plus Ultra and the hybrid rootstock Titan X Nemaguard. Scientia Horticulturae. 98, 473–484.
- Gebhardt K, Goldbach H (1988) Establishment, graft union characteristics and growth of *Prunus* micrografts. Physiol. Plant. 72, 153–159.
- Ghorbel A, Chatibi A, Thaminy S, Kchouk ML (1998) Micrografting of almond [*Prunus dulcis* (Miller) D.A.Webb] cv. Achak. Acta. Hort. 470, 429–433.
- Gürel S, Gülşen Y (1998) The effects of IBA and BAP on *in vitro* shoot production of almond (*Amygdalus communis* L.). Turk Journal of Botany. 22, 375–379.
- Hartmann HT, Kester DE, Daveis FT Jr, Geneve RL (1997) Plant propagation-Principales and Practices, 6th ed. Prentice-Hall, Englewood Cliffs, NJ.
- Işıkalan Ç, Akbaş F, Namlı S, Tilkat E, Başaran D (2008) *In vitro* micropropagation of almond (*Amygdalus communis* L. cv. Nonpareil). Afr. J. Biotechnol. 7(12), 1875–1880.
- Miguel CM, Druart P, Oliveira MM (1996) Shoot regeneration from adventitious buds induced on juvenile and adult almond (*Prunus dulcis* Mill.) explants. In Vitro Cell Dev. Biol. Plant. 32, 148–153.
- Mnoney EE, Mantell SH (2001) *In vitro* micrografting of cashew. Plant Cell Tiss Org Cult. 66, 49–58.
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–479.
- Navarro L (1988). Application of shoot-tip grafting *in vitro* to woody species. Acta Hort. 227, 43–55.
- Onay A, Pirinç V, Adıyaman F, Işıkalan Ç, Tilkat E, Başaran D (2002) *In vivo* and *in vitro* micrografting of pistachio, *Pistacia vera* L.cv. ‘Siirt’ Turk. Journal Biol. 27, 95–100.
- Onay A, Tilkat E, Işıkalan Ç, Namlı S (2007) Micrografting of Pistachio (*Pistacia vera* cv. Siirt). In: Mohan Jain S, Häggman H (ed) Protocols for micropropagation of woody trees and fruits, Springer, the Netherland, pp. 289–298.
- Ramanayake SMSD, Kovoov A (1999) *In vitro* micrografting of cashew (*Anacardium occidentale* L.). J Hort Sci Biotech. 74 (2), 265–268.
- Revilla MA, Jose P, Abelardo C, Roberto R (1996) *In vitro* reinvigoration of mature olive trees (*Olea europaea* L.) through micrografting. In Vitro Cell Dev Biol- Plant. 32, 257–261.
- Richardson FVM, Mac Ant Saoir S, Harvey BMR (1996) A study of the graft union in *in vitro* micrografted apple. Plant Growth Regul. 20, 17–23.
- Sriskandarajah S, Goodwin PB, Speirs P (1994) Genetic transformation of the apple scion cultivar Delicious via *Agrobacterium tumefaciens*. Plant Cell Tiss. Org. Cult. 36: 317–329.
- Thimmappaiah Puthra GT, Anil SR (2002) *In vitro* grafting of cashew (*Anacardium occidentale* L.). Sci Hort.92, 177–182.
- Yıldırım H, Onay A, Süzerer V, Tilkat E, Özden-Tokatlı Y, Akdemir H (2010) Micrografting of almond (*Prunus dulcis* Mill.) cultivars ‘Ferragnes’ and ‘Ferraduel’. Scientia Horticulturae. 125:361–367.