



***In vitro* micrografting of pistachio, *pistacia vera* L. var. Siirt, on wild pistachio rootstocks**

Canan Can¹, Mehmet Özaslan^{1*}, Hakan Töremen¹, Kamil Sarpkaya² and Elman Iskender¹

¹ Department of Biology, University of Gaziantep, 27310, Gaziantep, Turkey ² Pistachio Research Institute, 27060, Gaziantep, Turkey (* author for correspondence)

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Abstract

The success of *in vitro* micrografting of shoot tips of pistachio (*Pistacia vera* L. var. Siirt) has been examined. The mature dry nuts of *P. mutica*, *P. terebinthus*, *P. atlantica* and *P. khinjuk* that germinated *in vitro* were used as rootstocks. Different sterilization methods and embryo culture were investigated. Compatibility and growth activity of the shoot tip explants of *P. vera* cv Siirt, taken from *in vivo* grown trees, onto *P. mutica*, *P. terebinthus*, *P. atlantica* and *P. khinjuk* rootstocks were investigated. Current years shoot tips from mature trees of Pistachio micrografted onto *in vitro* juvenile rootstocks never resulted in the restoration of shoot bud proliferation. No compatibility and growth between scion and rootstock were observed.

Key Words: Pistachio, *in vitro*, shoot tip, grafting, rootstocks

***Pistacia vera* L. var. Siirt'in yabani pistacia anaçlarına *in vitro* mikroyaşılaması**

Özet: Bu çalışmada *Pistacia vera* L. var. Siirt'in sürgün uçlarının *in vitro* mikro aşılaması üzerinde çalışmalar yapılmıştır. *In vitro*'da geliştirilen *P. mutica*, *P. terebinthus*, *P. atlantica*, ve *P. khinjuk* bitkileri anaç olarak kullanılmıştır. Farklı sterilizasyon yöntemleri ve embriyo kültürü denenmiştir. *P. vera* cv Siirt'in *in vivo*'da gelişen sürgün uçlarının, *P. mutica*, *P. terebinthus*, *P. atlantica* ve *P. khinjuk* anaçları üzerindeki uyumluluk ve gelişmesi araştırılmıştır. Verim yaşındaki antep fıstığı ağaçlarından alınan sürgün uçlarının *in vitro*'da geliştirilen anaçlar üzerine mikro aşılamasında sürgün gelişimi ve uyumluluk meydana gelmemiştir.

Anahtar Kelimeler: Antepfıstığı, *in vitro*, Sürgün ucu aşılama, Anaç

Introduction

Pistacia vera L. is a dioecious tree species cultivated widely in the Mediterranean regions of Europe and North Africa, Middle East, China and California (Onay et al., 2004). The recent monographical study by Zohary (1952) indicates that genus *Pistacia* include 11 species: *P. atlantica*, *P. cabulica*, *P. chinensis*, *P. falcata*, *P. integerrima*, *P. vera*, *P. kurdica*, *P. mutica*, *P. palestine*, *P. terebinthus* and *P. khinjuk* and 6 of

them grow in Turkey (Davis, 1966). The leading countries producing Pistachio are Iran, Turkey, U.S.A., and Syria (FAO, 2004). *Pistacia khinjuk* (khonjok pistachio tree), a shrub or a small deciduous tree is widely distributed in Egypt, North Iraq, Iran and eastwards to Kashmir (Kawashty et al., 2000) and Southeastern Turkey. *P. terebinthus* (terebinth tree), ranging in size from a shrub to a small tree, is widely distributed in the Middle East and Southern Europe (Aydın and Özcan, 2002) and it is reported to be highly

resistant to *Phytophthora* (Pontikis, 1984). *P. atlantica* and *P. mutica* (Mastic tree) grow in the Mediterranean and Aegean regions of Turkey. *Pistacia mutica* has been selected as a rootstock resistant to root-knot nematodes for edible pistachio tree (*P. vera*) in many parts of Iran (Sheibani, 1987).

The propagation of pistachio is the most difficult among all nut trees (Joley, 1979). Rooting of Pistachio cuttings is rather difficult compared to those of other nut species. Traditional propagation of Pistachio is costly and time consuming so that the propagation has been achieved either by grafting buds from elite clones onto heterozygous rootstocks or by direct germination. It is common to see a male and female scion grafted onto one rootstock since Pistachio is dioecious. Incompatibility between rootstock and scion frequently necessitates intergrafting (Onay et al., 2003). Wild Pistachios are more resistant to many diseases than *P. vera*. Traditional grafting has been used to obtain elite clones by means of early yield and being resistant to diseases.

Micrografting that was developed in 1980s (Burger, 1984; Jonard, 1986) is an aseptically placement technique of shoot tip (varying in size from 0,1 to 0,8 mm) onto a decapitated rootstocks (Baydar and Çelik, 1999). The technique that is routinely used to obtain virus-free plants in citrus (Paiva et al., 1993; Koç et al., 1988), almond (Juárez et al., 1992), and peach (Navarro et al., 1983), improvement programs has also been applied in rejuvenation and/or reinvigoration of other tree species (Revilla et al., 1996; Estrada-Luna et al., 2002). It is unnecessary to wait for the maturation of rootstock, due to plant tissue culture techniques. Any attempts to rejuvenate mature materials by *in vitro* micrografting of mature scion shoot tips onto juvenile wild pistachio rootstocks have never been reported up to now. The aim of this study

was to provide the micrografted plants using wild pistachio rootstocks in a short period of time. Hence, it will be able to provide more micrografted seedling on a little area in a short time. Furthermore, the technique may resolve the seasonal-dependence for grafting.

Materials and methods

Dry nuts of *P. khinjuk*, *P. terebinthus*, *P. atlantica*, *P. mutica* were used to grow *in vitro* seedlings that served as rootstocks. They were collected from the orchards in Gaziantep, Mersin and Siirt provinces of Turkey. All nuts were incubated at +4 °C to break dormancy. *In vivo* grown axillary and lateral shoot tips of *P. vera* cv Siirt were used as scion sources obtained from the University of Gaziantep campus and Pistachio Research Institutes experimental orchards.

The nuts were cultured in the tubes containing 15ml MS medium (Murashige and Skoog, 1962) with 1 g l⁻¹ of activated charcoal, 5 mg l⁻¹ Benzyl amino purine (BAP), 250 mg l⁻¹ malt extract (MA), 250 mg L⁻¹ casein hydrolysate (CH), 30 gr l⁻¹ sucrose.

Sterilization methods

Modified sterilization methods as described by Onay (1996) were used. Mature kernels, from which the outer pericarp and shells had been removed, were sterilized by 5 different procedures (Table I), because of high contamination. They were presterilised by agitating in water for 5 minutes. Then, it was agitated in the water containing detergent and tween-20 for 5 minutes after rinsing in water for 5 minutes. The nuts were then sterilized by following the procedures given in Table I.

Table 1. Procedures applied for surface sterilization of dry nuts of wild Pistachio spp (x: washing times).

Procedure I	Procedure II	Procedure III	Procedure IV	Procedure V
- %70 Ethanol (2 min)	- 70% Ethanol (4 min)	- 70% Ethanol (2 min)	- 70% Ethanol (2 min)	- 70% Ethanol (5 min)
- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (2x)	- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (1x)
- 5% NaOCl (5 min)	- 10% NaOCl (10 min)	- 20% H ₂ O ₂ (10 min)	- 5% NaOCl (5 min)	- 15% NaOCl (15 min)
- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (2x)	- Wash in sdH ₂ O (1x)	- Wash in sdH ₂ O (1x)
- 15% NaOCl (10 min)	- 5% NaOCl (5 min)	- 10% NaOCl (5 min)	- 20% H ₂ O ₂ (15 min)	- 70% Ethanol (2 min)
- Wash in sdH ₂ O (3x)	- Wash in sdH ₂ O (2x)	- Wash in sdH ₂ O (3x)	- Wash in sdH ₂ O (3x)	- Wash in sdH ₂ O (3x)
	- 5% H ₂ O ₂ (5 min)			
	- Wash in sdH ₂ O (3x)			

Embryo culture

Mature kernels, from which the outer pericarp and shells had been removed, were presterilised by agitating in water for 5 minutes. Then, it is soaked in solution containing 10% NaOCl and tween-20 for 5 minutes, followed by 70% alcohol application for 2 minutes. The kernels were then washed three times with sdH₂O and seed coat was removed. Following partial trimming of the cotyledons, the embryos were cultured singly in flasks containing MS media.

Germination of rootstocks

P. mutica and *P. terebinthus* were cultured in five different media to obtain the best germination. The nuts of rootstocks were germinated in the dark at 22±2 °C. The media used were as follows;

- Hormone-free media consisting of MS (control group)
- MS+0.5mg l⁻¹ BAP+100 mg l⁻¹ CH+100 mg l⁻¹ MA
- MS+0.5mg l⁻¹ BAP+250 mg l⁻¹ CH+100 mg l⁻¹ MA
- MS+0.5mg l⁻¹ BAP+500 mg l⁻¹ CH+100 mg l⁻¹ MA
- MS+0.5mg l⁻¹ BAP+250 mg l⁻¹ CH+250 mg l⁻¹ MA

Micrografting

The nuts sterilized by the procedure V (Table 1), were germinated on MS media supplemented with 0.5 mg l⁻¹ BAP, 250 mg l⁻¹ CH, and 250 mg l⁻¹ MA. *In vivo* grown shoot tips were sterilized into two stages to avoid tissue death. They sterilized by immersion in a 5% (v/v) commercial bleach solution for 5 min followed by rinsing in 70% ethyl alcohol for 2 min. They were then washed with sdH₂O twice, before rinsing in 70% ethyl alcohol for 30 seconds. At this stage, the shoots were trimmed and taken for second stage sterilization that were 70% ethyl alcohol application for 30 sec, followed by three wash in sdH₂O. Finally the shoots were further sterilized in 3% (v/v) commercial bleach solution for 2 min and rinsed in sdH₂O.

Sterilized scions of 0.2-0.3 mm in diameter were micrografted onto 20-22 days-old *in vitro* seedlings

which had been decapitated above the cotyledons and vertically slitted by razor blade. Micrografted seedlings were cultured on liquid MS supplemented with 1% activated charcoal, 250 mg l⁻¹ casein hydrolysate, 250 mg l⁻¹ malt extract, and 0.5 mg l⁻¹ BAP. The cultures were placed in dark at 26–28 °C. The evaluation of *in vitro* micrografting was carried out 6–8 days after micrografting.

Results

Sterilization studies

The major obstacle of this study was the high contamination rate occurred during *in vitro* culturing of dry nuts for rootstock use Procedure V resulted nearly the best results for four species (Table 2). The *in vitro* germination rates of the species were 41.6% (n=120) for *P. mutica*, 35.2% (n=102) for *P. khinjuk*, 20.4% (n=88) for *P. terebinthus*, 24.3% (n=123) for *P. atlantica*.

Embryo culture

No lateral roots were observed in main roots of plantlets developed through embryo culture (Table 3, Figure 1).

Germination of nuts

It was observed that *P. mutica* had thicker root structure, produced more lateral roots, and it had higher ratio of germination than those of *P. terebinthus* (Table 4). Germination time varied between 8-20 days after culturing.

Micrografting

There was no compatibility between scion and rootstock. Browning and scion death occurred 5-6 days following micrografting (Figure 2).

Discussion

P. vera grown widely in the Southeastern part of Turkey has a great importance for the regions economy. Traditional production techniques are applied in the region for this nut tree, and the demand is never covered. Therefore there is a need for grafted

Table 2. Effect of different surface sterilization methods on seed germination and contamination.

Procedures	Species*							
	<i>P. mutica</i>		<i>P. atlantica</i>		<i>P. khinjuk</i>		<i>P. terebinthus</i>	
	C	G	C	G	C	G	C	G
I	66.6	12.5	0	33	100	0	54.2	16.6
II	87.5	0.4	19	42.8	33	44	58.3	33.3
III	35.2	37	0	22	77.7	22.2	37.5	25
IV	70.8	0.8	11.1	40.7	55.5	44.4	42	12.5
V	12.9	41.6	35.2	24.3	17.8	35.2	53.5	20.4

*C: Contamination percentage of *in vitro* cultured nuts, G: Germination percentage of *in vitro* cultured nuts

Table 3. The germination and contamination rates (in %) of embryo cultures of wild Pistachio species.

	<i>P. khinjuk</i>	<i>P. atlantica</i>	<i>P. terebinthus</i>	<i>P. mutica</i>
Contamination rate	100	100	70	60
Germination rate	60	0	40	33

Table 4. Effect of five different media on germination rate (in %) of *P. mutica* and *P. terebinthus*.

Media	<i>P. mutica</i>	<i>P. terebinthus</i>
MS (Control)	22.2	21.8
MS+5mg/l BAP+100 mg/l CH+100 mg/l MA	40.0	18.1
MS+5mg/l BAP+250 mg/l CH+100 mg/l MA	46.6	16.6
MS+5mg/l BAP+500 mg/l CH+100 mg/l MA	44.4	18.1
MS+5mg/l BAP+250 mg/l CH+250 mg/l MA	41.6	20.4

seedlings of Pistachio in the region. The aim of this study was to establish an *in vitro* method for clonally propagation of grafted Pistachio seedlings.

Embryo culture that is troublesome process has low germination proportion for the nut of wild pistachio. The individuals germinated were weak in appearance and did not have roots. Therefore, in this study embryo culture was not used for the subsequent propagation of wild species for use in micrografting.

Firm contact between rootstock and scion is extremely important at the graft junction to get fusing between them and the formation of callus. Different sterilization procedures were used to prevent the high contamination. The best germination and sterilization results for all species (except; *P. atlantica*) were obtained with procedure V, which is the best among 5 procedures for *P. khinjuk* having big contamination

problem. Using of unique sterilization technique is too practical and is necessary to evaluate results at the end of micrografting. Hence, the plants were exposed to same sterilization procedure, growth and germination performance of which may be tested.

Penicilium, Aspergillus, yeast that caused the contamination throughout the study, produces aflatoxins are secondary mould metabolites known to be highly toxic and potential carcinogens (Abdulkadar et al., 2000). Wild nut is consumed in some Middle East countries such as; Iran and Turkey, which is potentially important for public health.

As seen in Table 4, the excessive differences in germination were not observed among media. However the media containing BAP, CH, and MA exhibited better development of shoots and roots for *P. mutica*.

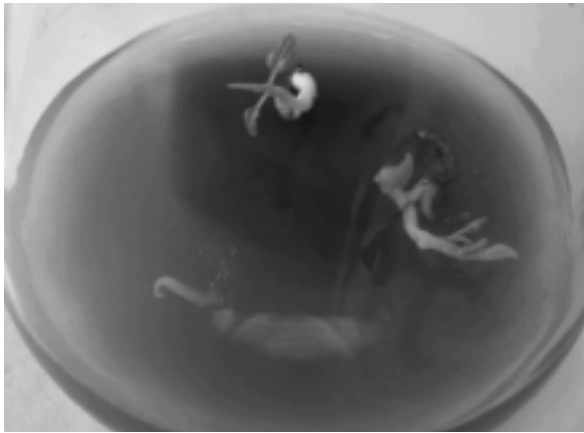


Figure 1. Embryo culture of *Pistacia khinjuk*.

P. terebinthus showed undesired results in terms of contamination, germination and development. Our results showed that very thin seedling of *P. terebinthus* was not suitable for micrografting. *P. khinjuk* is the most germinated and developed species among four species, mature dry nuts of which from the Gaziantep and Siirt provinces were used. The germination and development ratio of the nuts from different regions differ from each other.

Necessity of disinfection of *in vivo* shoot tips and the effect of their picking season on success (Deogratias et al., 1991) are other disadvantages. Equilibrium between phenolic compounds and hormones quite affects the studies on plant tissue culture. If it is lost, it is known that it causes rapid browning or blackening of affected tissues and this can result in tissue necrosis and explant death in the most extreme cases. At the micrografts using *in vivo* scions from the seasons, where polyphenol oxydase and peroxidase are high proportions, the withering and death of scions are observed in a short time, due to enzymatic oxidation (Poessel et al., 1980). It was proved that the success of micrografting was dependent on peroxidase activity in peach (Jonard, 1986) and apricot (Deogratias et al., 1991).

It is estimated that another reason for failure may be tissue incompatibility. Occurring of *in vitro* shoot tip grafting between the variety of *P. vera* and wild pistachios may draw this conclusion. Estrada-Luna et al. (2002) reported that homografts (grafts between same species) of species of prickly pear cactus (*Opuntia* spp.) grew significantly faster than the heterografts (grafts between different species) after 90



Figure 2. Micrografting of *Pistacia vera* L. var. Siirt. (a) Shoot tips of *P. vera* micrografted onto *P. atlantica*, (b) Shoot tips of *P. vera* micrografted onto *P. mutica*. Photographs were taken 5 days after micrografting, (c) Shoot tips of *P. vera* micrografted onto *P. atlantica*. Photographs were taken 15 days after micrografting.

days of *ex vitro* transfer.

Rootstock diameter must be enough thick for shoot buds or cuttings for success of micrografting because the bud of pistachio is fairly bigger than bud of many fruit trees (Joley, 1979; Woodroof, 1979). The *P. vera* buds remain dormant in a long time. Graft unions were able to fail due to the dormancy so that this necessitates detailed studies.

Micrografting of *P. vera* have previously been reported (Onay et al., 2004), and successful micrografted seedlings were obtained. In our study we have used wild Pistachio species as rootstocks that is demanded from farmers. Further research has to be done by using *in vitro* grown shoot tips, shoot tips from *in vitro* grown cuttings, *in vivo* grown shoots of different parts of the tree at different seasons, different cultivars and ages of pistachio as scion sources and different wild pistachio species or hybrids as rootstocks.

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