

Rooting of Micropropagated Transsexual *Pistacia terebinthus* L. Plants from Bulgaria

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Abstract

The *Pistacia terebinthus* L. trees demonstrate exceptional drought resistance of the kind which allows cultivation in small-productive, stony and sliding (pliant to erosion) soils. Biotechnological approaches for preservation, multiplication and inclusion in selection programs of the rare transsexual *P. terebinthus* L. form found in the Rhodopes Mountain (Bulgaria) are under way. The aim of the present research is to improve the rooting of micropropagated plants of this form.

The experiments for optimization of the rooting are made with in vitro propagated plants from a single genotype, obtained from an embryo culture. The plant material is maintained on a solidified DKW nutrient medium supplemented with 2.5 μM BAP, 0.005 μM IBA and 30 g L⁻¹ glucose. The shootlets obtained during multiplication are elongated on hormone-free DKW or WPM nutrient medium for 10 days in glass jars or in polypropylene vessels (PP) with gas-permeating cover. The nutrient media for root induction are based on DKW or WPM salt compositions (50% macro-elements) and contain varying auxin concentrations - 0, 10 or 25 μM IBA, combination of 10 μM IBA and 0.054 μM NAA or 10 μM IBA and 0.5 μM IAA. Agar solidified and liquid media with perlite as supporting material are tested. The percent of rooted plants is evaluated after 25 days. All plants (rooted and non-rooted in vitro) are potted in a peat-perlite mixture for acclimatization. At this stage a lot of non-rooted plants develop roots. Plant's survival is evaluated after 50 days. The highest rooting percent in vitro (76.6%) and the best survival rate after acclimatization (93.3%) is obtained from plants, cultivated on WPM liquid medium with perlite as supporting material, supplemented with 10 μM IBA and 0.5 μM IAA.

INTRODUCTION

Pistacia terebinthus L. is member of the *Pistacia* genus (*Anacardiaceae*) and has significant importance as rootstock for *P. vera* cultivars. It is widely spread in the whole Mediterranean region – Italy, Greece, Turkey, Tunisia, Syria, etc., as a wild species and a rootstock in the plantations. The same species is dioecious tree with thick crown and height up to 8 m. It has long, reddish and smooth shoots which change to an ash colour at maturity. The *P. terebinthus* trees are known by exceptional drought resistance which allow to be cultivated on small-productive and stony soils. *P. terebinthus* crosses easily and spontaneously with *P. vera* and the hybrids have economic significance as pollen donors for pistachio crops (Avanzato and Quatra, 2004). In the summer of 2002 a rare transsexual form of *P. terebinthus* was found in Bulgaria and later the existence of an isolated population of such trees is discovered. (Avanzato, 2003; Avanzato and Quarta, 2004). Until recently, there are only few reports of similar genotypes. A necessary prerequisite for preservation and use of this transsexual form of *P. terebinthus* as a rootstock and eventually as a donor for monoeciousness in the pistachio hybridization program is development of a reliable protocol for in vitro micropropagation.

Regardless of fact that the use of tissue cultures for multiplication of the species from *Pistacia* genus becomes increasingly significant in the last 20 years (Martinelli and Loretto, 1988; Bargchi and Alderson, 1989; Gonzales and Frutos, 1990; Onay et al., 1996, 2004; Onay, 2000; Ghorbani et al., 2002; Mehlenbacher, 2003) there are very few reports on *P. terebinthus*.

Pontikis (1984) applies Anderson medium supplemented with 2.5 mg L⁻¹ BA, 0.1 mg L⁻¹ IBA and 0.1 mg L⁻¹ GA₃ for in vitro propagation of *P. terebinthus* 'Tsikouda' – the most popular pistachio rootstock. There are reports of successful micropropagation experiments of *P. terebinthus* and *P. vera* where the oxidation of the media is controlled by addition of ascorbic acid, and the most successful introduction of culture is achieved at one-month-old seedlings (Gannoun et al., 1995). Sheibani and Villiers (1995) ascertain that the optimum media for propagation of *P. vera*, *P. terebinthus* and *P. mutica* is MS (Murashige and Skoog, 1962) with 5 mg L⁻¹ BAP, and for rooting – MS with 5 mg L⁻¹ IBA.

Nacheva et al. (2010) set up experiments to establish a method for micropropagation of the transsexual form of *P. terebinthus* from Bulgaria. Twelve different nutrient media variations based on MS (Murashige and Skoog, 1962) and DKW (Driver and Kuniyuki, 1984) were tested. The optimum multiplication rate (3.63 microshoots) occurred on the medium containing 50% MS macroelements, 5.0 µM BAP and 0.01 µM IBA. On the contrary, rooting percentage on the media used in these experiments is very low – between 6.25 and 12.5%.

The aim of the present research is to improve the rooting of micropropagated plants of this form.

MATERIALS AND METHODS

Plant Material

The experiments for rooting optimization are made with in vitro propagated plants from a single genotype, obtained from an embryo culture. The plant material is maintained on a solidified DKW nutrient medium supplemented with 2.5 µM BAP, 0.005 µM IBA, 30 g L⁻¹ glucose and 6.5 g L⁻¹ agar. The pH of the media is adjusted to 5.6. All the plants are cultured in a growth chamber at a temperature 22±2°C, a 16 hour photoperiod (fluorescent tubes OSRAM 40W, 40 µmol m⁻² s⁻¹ PPFD).

Cultivation Plates

- glass jars (600 ml) with glass lids, tightly closed with polyethylene foil ensuring a gas exchange rate - 0.312 GE/day;
- polypropylene vessels (PP) with a gas-permeating cover (Combiness, Belgium, gas exchange rate - 10 GE/day).

Experiment 1

The shootlets obtained during multiplication are elongated on hormone-free DKW nutrient medium for 10 days in glass jars. Apical cuttings (15-20 mm in length) are placed on four rooting media with different combinations of plant growth regulators. All are based on DKW medium with 50% macro salts, 100% micro salts, 100% vitamins, 20 g L⁻¹ glucose, pH 5.6. D0 - growth regulators free (control); D2 – 10 µM IBA; D5 – 25 µM IBA; D2N – 10 µM IBA and 0.054 µM NAA.

All media are prepared in polypropylene vessels in two modifications:

- agar solidified (6.5 g L⁻¹);
- liquid with perlite as supporting material.

Rooting is evaluated after 25 days.

Experimental Design of Experiment 1

- Elongation on DKW in glass jars → rooting in PP vessels on four DKW agar solidified media;
- Elongation on DKW in glass jars → rooting in PP vessels on four DKW liquid media with perlite

Experiment 2

Microplants are elongated on hormone-free WPM nutrient medium for 10 days in glass jars or in PP vessels. Apical cuttings (15-20 mm in length) are placed on four

rooting media based on WPM medium with 50% macro salts, 100% micro salts, 100% vitamins, 20 g L⁻¹ glucose, pH 5.6 and different combinations of plant growth regulators. W0 - growth regulators free; W1 - 10 µM IBA; WA - 10 µM IBA and 0.5 µM IAA; WN - 10 µM IBA and 0.054 µM NAA. Media are prepared in PP vessels in two modifications: agar solidified medium (6.5 g L⁻¹) and liquid medium with perlite as supporting material. Rooting is evaluated after 25 days.

Experimental Design of Experiment 2

- Elongation in glass jars → rooting in PP vessels on four WPM agar solidified media;
- Elongation in PP vessels → rooting in PP vessels on four WPM agar solidified media;
- Elongation in glass jars → rooting in PP vessels on four WPM liquid media with perlite.

Acclimatization

All plants (rooted and non rooted) are potted in 200 ml plugs containing a 70:30 (peat-perlite) mixture and placed in growth chambers at a temperature 22±2°C and a 16-h photoperiod (fluorescent tubes OSRAM 40 W, 60 µmol m⁻² s⁻¹ PPFD) for acclimatization. Plant's survival is evaluated after 50 days.

Data Analysis

Thirty shoots for each variant of nutrient media are established. The experiment is replicated three times. Data are analyzed using analysis of variance (ANOVA) and the means separated using Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Our experiments with in vitro obtained seedlings of *P. terebinthus* confirm the reports (Sheibani and Villiers, 1995) that MS basal medium is suitable for shoot multiplication. However, the level of rooting on MS-basal media is unsatisfactory (Nacheva et al., 2010).

Thus other basal media formulations (DKW and WPM) are tested in the present study. These basal media are developed and successfully applied in micropropagation of different tree species. Ghorbani et al. (2002) recommend DKW media with addition of 2 mg L⁻¹ IBA and 0.01 mg L⁻¹ NAA for in vitro rooting of *P. vera*.

The results obtained in our experiments show that nutrient media, based on WPM salt composition, enhance the rooting efficiency of *Pistacia terebinthus* plantlets in vitro up to 90% after acclimatization and are superior compared to media based on DKW salt composition (Figs. 1 and 2).

The experiments are set in a way to ascertain the influence of the supporting material (agar or perlite) on the rooting efficiency. On DKW-based media the results are ambiguous for both rooting of in vitro plants and for plants, survived after acclimatization (Fig. 1). However, the application of WPM-based liquid media with perlite as supporting material results in higher rooting efficiency of pistacia plantlets in vitro than agar solidified WPM media (Fig. 2).

In the present study (experiment 2) we pay a lot of attention to the effect of in vitro culture conditions of the source plants on the rooting efficiency. The plants are elongated in two type cultural vessels – glass jars and PP vessels. The elongation of source microplants in PP vessels results in higher rooting percent in vitro on agar solidified media (Fig. 2) and in the highest number of roots per plant in vitro (Fig. 3). It is concluded that as far as the different gas exchange rate of the cultural vessels affects the development of microplants, leaf structure, rate of photosynthesis in vitro and the endogenous levels of plant growth regulators it can influence and their rooting ability (Nacheva and Ivanova, 1998, 2006). However, there are no significant differences in the root length of the two types of source plants on agar solidified media (Fig. 4).

The results obtained confirm our previous observations (Nacheva et al., 2010) that even for non-rooted in vitro plants the auxin level in the media is sufficient for development of roots at the time of acclimatization. Thus the number of rooted in vivo

plants is 2 to 10 times higher than rooted in vitro plants and the above discussed effects due to the nutrient media and cultural conditions are more than compensated (Figs. 1 and 2).

In conclusion as a result of the present study a reliable method for rooting of *P. terebinthus* is described. The highest rooting percent in vitro (76.6%) and the best survival rate after acclimatization (93.3%) is obtained from plants, cultivated on WPM liquid medium with perlite as supporting material, supplemented with 10 μ M IBA and 0.5 μ M IAA. Most of the non-rooted in vitro *Pistacia* plants readily develop roots ex vitro and successfully acclimatize. Thus the number of plants obtained can be considerably increased.

ACKNOWLEDGEMENTS

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Tables

Table 1. Nutrient media for rooting.

Variants	Macroelements	IBA (μM)	NAA (μM)	IAA (μM)
Experiment 1				
D0	50% DKW	0	0	0
D2	50% DKW	10	0	0
D5	50% DKW	25	0	0
D2N	50% DKW	10	0.054	0
Experiment 2				
W0	50%WPM	0	0	0
W1	50%WPM	10	0	0
WN	50%WPM	10	0.054	0
WA	50%WPM	10	0	0.5

Figures

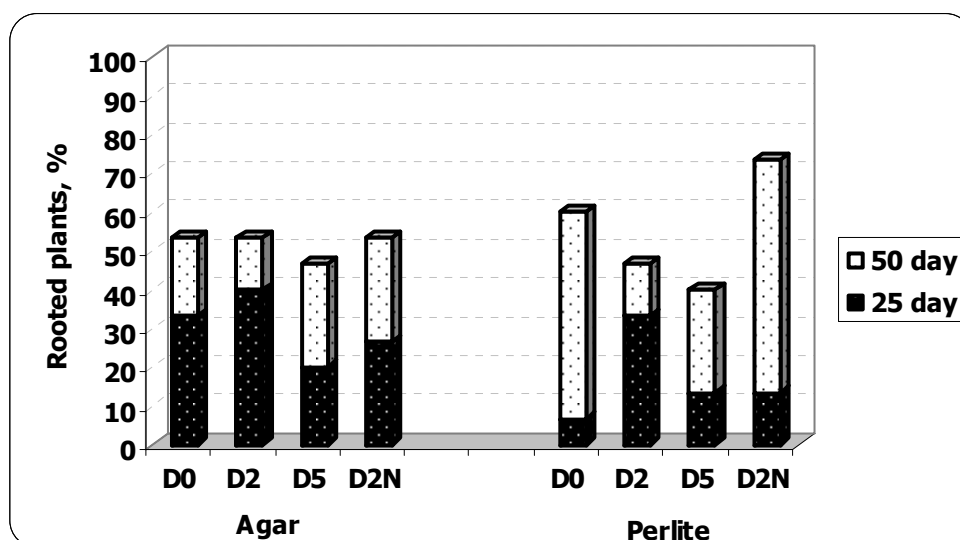


Fig. 1. Effect of culture conditions on the rooting efficiency (%) of *Pistacia terebinthus* L. plantlets in vitro (25 days) and after acclimatization (50 days) (Experiment 1).

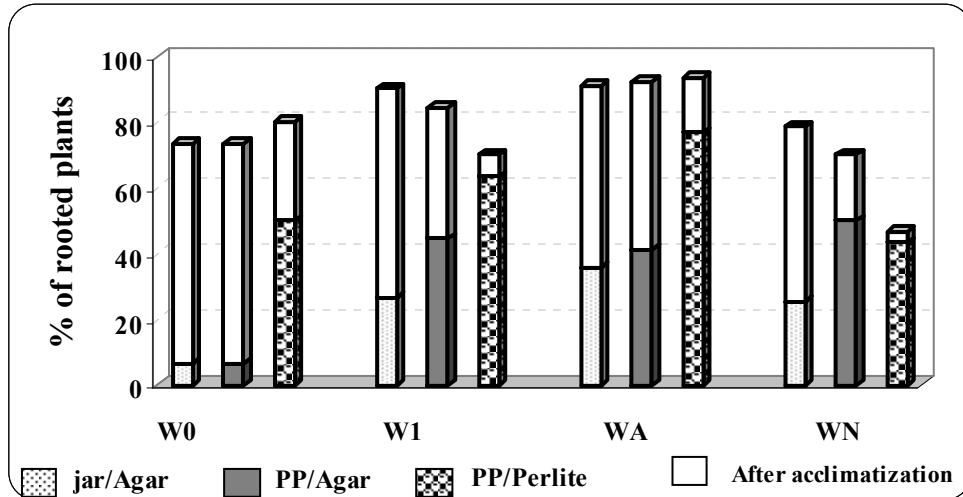


Fig. 2. Effect of in vitro culture conditions on the rooting efficiency (%) of *Pistacia terebinthus* L. plantlets.

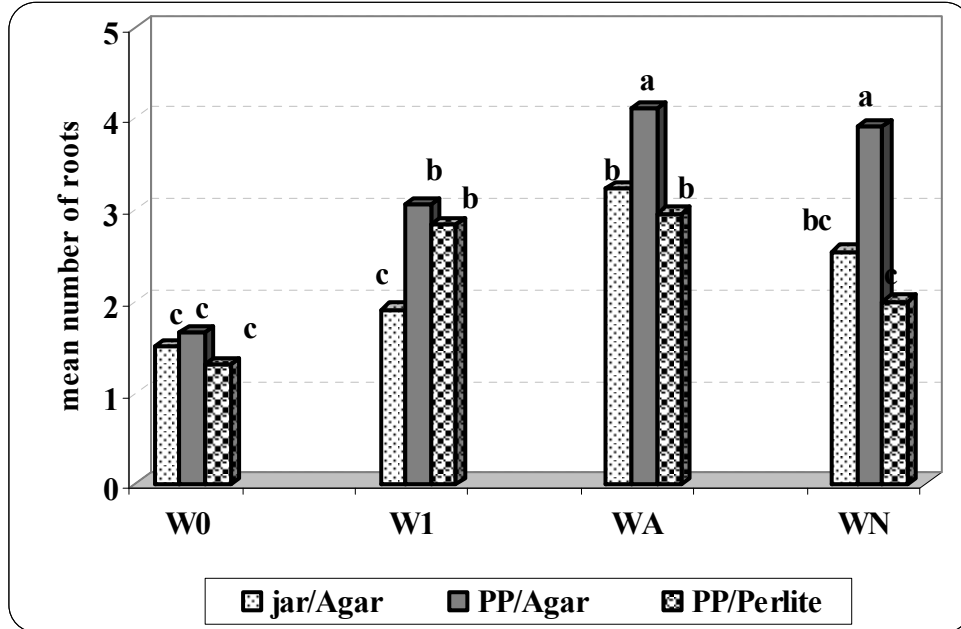


Fig. 3. Effect of in vitro culture conditions on the mean number of roots per plantlets.

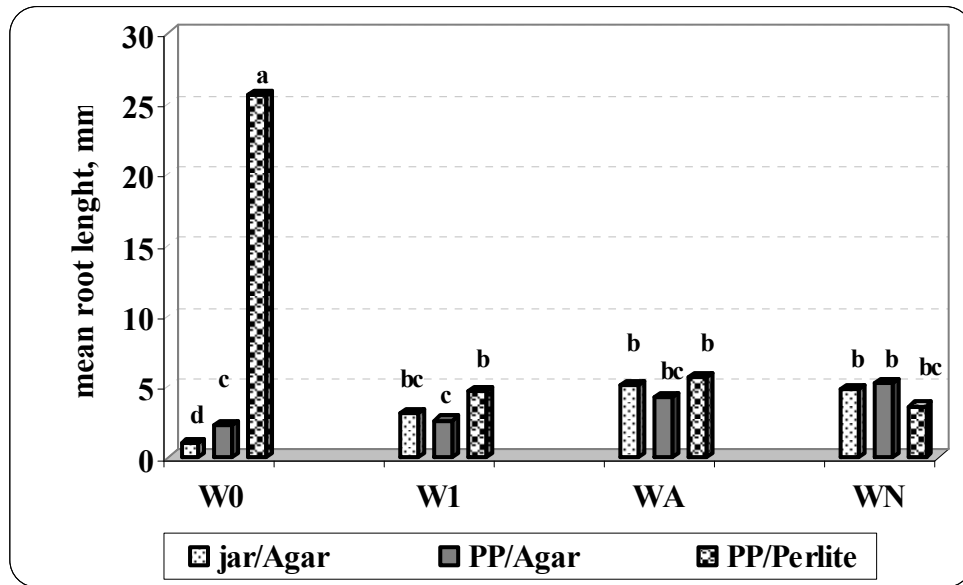


Fig. 4. Effect of in vitro culture conditions on the mean root length (mm) of *Pistacia terebinthus* L. plantlets.

