Micropropagation of Rare Transsexual Forms of Pistacia (*Pistacia terebinthus* L.) Found in Bulgaria

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Abstract

In summer 2002 in the Rhodopes mountains, (Bulgaria) a rare transsexual form of the normally dioecious *Pistacia terebinthus* was found. Later the existence of a small isolated population of such trees was described. The trees display exceptional drought resistance of the kind which allows cultivation in unirrigated relatively unproductive stony and sliding soils. Tissue culture is a technology that is increasingly being used to propagate species from the *Pistacia* genus using a variety of techniques. In this research, experiments were set up to optimize propagule multiplication and development using micropropagules induced from embryo cultures of a single genotype. Twelve different nutrient media variations (based on Murashige and Skoog and Driver and Kuniyuki) were established. The effect of varying concentrations of macroelements (100% and 50%), BAP (2.5 µM or 5.0 µM), IBA (0.005 or 0.01 µM) and carbohydrates (30 g L^{-1} sucrose and glucose used separately) was established. The highest multiplication rate (3.63 microshoots) occurred in the variant containing 50% MS macroelements, 5.0 µM BAP and 0.01 µM IBA. Experiments for plantlet rooting were made on nutrient media containing 50% MS macroelements and auxin (IBA) at four rates (0 µM, 1.0 µM, 2.5 µM and 5.0 µM) were made. Low percentage of root induction – between 6.25% and 12.5% is reported.

INTRODUCTION

The *Pistacia* genus (*Anacardiaceae*) includes 12 deciduous species, all of which are trees. These species are dioecious and anemophylic. Among them, only *P. vera* is domesticated (Zohary, 1952). In summer 2002 during an expedition to the Rhodopes mountains (Bulgaria), a transsexual form of *P. terebinthus* was found (Avanzato, 2003; Avanzato and Quarta, 2004). In subsequent expeditions, the existence of an isolated population of transsexual trees was discovered (Zhivondov, pers. commun.). Until recently, there were few literature reports of similar genotypes (Ozbek and Ayfer, 1958; Crane, 1974; Kafkas et al., 2000; Kafkas, 2002).

The trees demonstrate exceptional drought resistance of the kind which allows cultivation in unirrigated, stony, sliding and very sloping soils with limited productivity.

The preservation and use of the transsexual form of *P. terebinthus* as a rootstock and eventually as a donor for monoeciousness in the pistachio hybridization programme is perhaps best undertaken through in vitro propagation/culture. The use of this technology to multiply species from the *Pistacia* genus has become increasingly significant within the last twenty years (Martinelli and Loretti, 1988; Bargchi et al., 1989; Gonzales and Frutos, 1990; Mehlenbacher, 2003). Sheibani and Villiers (1995) reported that Murashige and Skoog (1962) medium fortified with either 5.0 mg L^{-1} BAP or 5.0 mg L^{-1} IBA was the optimum one for multiplication and rooting respectively of *P. vera, P. terebinthus* and *P. mutica.* Other authors (Ghorbani et al., 2002) recommended Driver and Kuniyuki (DKW) medium fortified with 2.0 mg L^{-1} IBA and 0.01 mg L^{-1} NAA for in vitro rooting of *P. vera.* Driver and Kuniyuki (1984), Behboodi (2002) examined the influence of different growth regulators on the in vitro propagation of wild species from the genus *Pistacia*. The optimum results were obtained using BAP and NAA. The results

for *P. vera* 'Mateur' were analogous – BAP at (2.0 mg L^{-1}) stimulated adventitious regeneration of shoots (Chatibi et al., 1998a). Ghoraishi (2006) also reported that BAP was the most effective growth regulator and suggested its use at 20 μM in the medium for the micropropagation of *P. mutica*. The author suggested that treatment with 100 μM IBA for five days significantly improved propagule rooting.

The principal limiting factor in the introduction and stabilization of in vitro culture methods for many trees including pistachio is the browning of the explants as a result of phenolic exudation. Tabiyeh et al. (2006) reported a decrease in oxidation and an improvement in explant growth by dipping the base of the explants in 0.1 mM glutathione immediately before subculture. Gannoun et al. (1995) reported the successful micropropagation of *P. terebinthus* and *P. vera* using one-month-old seedlings when ascorbic acid was added to the culture medium to control oxidation.

Special attention is drawn to the optimization of the salt content of the nutrient medium to overcome shoot tip browning, the existence of red pigmentation on the leaves and improvement of the vitality of the plants (Chatibi et al., 1998b), otherwise damage to the plasmalemma, disintegration of the membranes and organelles and cell autolysis can occur. This has been documented by Abousalini and Mantell (1995) using ultrastructure analysis to characterize shoot-tip necrosis in vitro cultivation of *P. vera.*

Onay, (2000) reported a somatic embryogenesis protocol to accelerate in vitro propagation of *P. vera.* He reported that somatic embryos could be induced from leaf explants (Onay, 2000), immature embryos and female flowers (Onay et al., 2004) of *P. vera*. The influence of the species and the concentration of the growth regulators added to the nutrient medium on the formation and growth of somatic embryos and their germination into plants was also reported (Onay et al., 2004).

The aim of the present research is to develop a relevant system for micropropagation of the transsexual form of *P. terebinthus*.

MATERIAL AND METHODS

Establishment of In Vitro Culture

Seed embryos from *P. terebinthus* were cultured in mid August, 2006. The nuts were cleaned from the mesocarp and the seeds excised using a scalpel from their hard shell. The seeds were disinfested using a frequently used procedure: washed under running water for 2 hours, dipped in 95% ethanol for 30 seconds, immersed in 5% calcium hypochlorite $Ca(OCl)_2$ solution for 5 minutes and washed three times with sterile distilled water for 1, 5 and 10 minutes respectively. After disinfection, the embryos were isolated and plated on MS nutrient media with and without the addition of growth regulators.

Multiplication

Nutrient media based on both MS and DKW formulations were used for shoot multiplication. They were enriched with 2.5 μM or 5.0 μM BAP, 0.005 μM or 0.01 μM IBA, 30.0 g L^{-1} sucrose or glucose and 6.5 g L^{-1} agar (Table 1). The pH of the media was adjusted to 5.6.

In two passages of four weeks on the respective nutrient media, the number of newly formed shoots greater than 5 mm in height and the mean length of newly formed shoots (mm) were recorded. The experiments to optimize caulogenesis were undertaken using plants from a single genotype, initially obtained from embryo cultures.

Rooting

The shootlets obtained during multiplication were elongated on hormone-free MS nutrient medium for 10 days. Apical cuttings (approximately 15-20 mm in length) were placed on modified MS rooting media containing 50% macroelements, 20 g L^{-1} sucrose and varying concentrations of IBA (0 μM, 1.0 μM, 2.5 μM and 5.0 μM). Rooting was evaluated after 30 days.

All the plants were cultured in a growth chamber at 22±2°C and a 16 hour photoperiod emitting 40 μmol m^{-2} s^{-1}PPFD.

Acclimatization

The plants were acclimatized in 200 ml plugs containing a 50:50 (peat-perlite) mix and placed in growth chambers at 22±2°C and a 16-hour photoperiod emitting 60 μmol m^{-2} s^{-1} PPFD.

Data Analysis

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

RESULTS AND DISCUSSION

Preliminary experiments with in vitro obtained seedlings of *P. terebinthus* re-confirmed previously reported research of Sheibani and Villiers (1995) that MS basal media formulations were suitable for shoot multiplication. Hence these media formulations were used in this work.

The results obtained showed that a significantly higher coefficient of multiplication (3.63) occurred on the nutrient medium designated MF6, containing 50% macro MS salts (Fig. 1). There was no significant difference in shoot proliferation in the other media irrespective of whether they were MS or DKW based with the exceptions of treatments MF1, MF4 and DF1 which induced the lowest growth rates. The obtained plants were fresh, in good physiological condition and were free from vitrification. Their leaves were green, but with a characteristic anthocyanin nuance.

The plants induced on the medium containing normal strength MS macro nutrients, 2.5μM BAP, 0.005 μM and 30 g L^{-1} (MF1) were significantly taller than all the other treatments including the DKW medium which was enriched with growth regulators, analogous to those in the MS media (Fig. 2). The lowest growth rates were recorded on the half strength MS media except where glucose was the added carbohydrate (Fig. 2).

The length of the plants was similar in both media formulations containing 100% salts with the exception of MF1 (Fig. 2).

When an experiment seeking to ascertain the influence of sucrose or glucose in the nutrient media was set up, the results were ambiguous for both examined indicators – coefficient of multiplication and height of the plants (Figs. 1 and 2).

Nutrient media with two levels of growth regulators - 2.5 μM BAP+0.005 μM IBA and 5.0 μM BAP+0.01 μM IBA were used in our experiments. These concentrations were significantly lower compared to that reported in the literature (Sheibani and Villiers, 1995; Chatibi et al., 1998a; Behboodi, 2002; Ghoraishi, 2006). It was concluded that the lower coefficient of multiplication due to the nutrient media effect was more than compensated for by the good physiological condition of the plants. Furthermore, browning of the shoot-tips as reported by many authors (Abousalim and Mantell, 1995; Chatibi et al., 1998b; Tabiyeh et al., 2006) was not observed.

On all nutrient media, the plants arose from dark brown to black coloured callus produced at the base of the stalk. Similar coloured callus was observed on the leaves that were in contact with the nutrient media. Oxidation of the media, which is a typical problem in the in vitro cultivation of species from the *Pistacia* genus (Gannoun et al., 1995) was observed after the first week. The best results from the experiments of the examined media for multiplication were received on media MF6, which demonstrated the best multiplication rate 3.63 and good though significantly inferior propagule length (8 mm).

The experiments for rooting were carried out on nutrient media containing different levels of IBA. A low percentage of rooting was observed and ranged from 6.25 to 12.5% (Fig. 3). This level of rooting was unsatisfactory and will require further investigation. For instance, higher hormonal concentrations, analogous to that reported by

Sheibani and Villiers (1995); Ghorbani et al. (2002); Ghoraishi (2006) will be used in an effort to improve this characteristic. It was observed that adventitious roots formed on leaves that had contact with the nutrient media.

In conclusion, the study of the methods for in vitro propagation of the rare transsexual form of *P. terebinthus* will ensure its preservation. The optimum multiplication rate (3.63) occurred on the medium with 50% macro MS salts, supplemented with 5.0 μM BAP+0.01 μM IBA and 30.0 g L^{-1} sucrose. This shows that cultures of the plant are very sensitive to media with a high salt content while simultaneously require high BAP concentrations. On the contrary, rooting percentage was very low. It is probable that the salt concentration in the medium even at the 50% concentration was too strong. It is possible that autotrophic rooting systems could better facilitate rhizogenesis. The rooted plants obtained readily acclimatized and will be used as motherstock in successive experiments and eventually as a donor for monoeciousness in the selection programmes of pistachio.

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Tables

Table 1. Nutrient media for multiplication.

Variants	Macroelements	BAP (µM)	IBA (µM)	Sucrose (g L^{-1})	Glucose (g L^{-1})
MF 1	100%MS	2.5	0.005	30	
MF 2	100%MS	5	0.01	30	
MF 3	100%MS	2.5	0.005		30
MF 4	100%MS	5	0.01		30
MF 5	50%MS	2.5	0.005	30	
MF 6	50%MS	5	0.01	30	
MF 7	50%MS	2.5	0.005		30
MF 8	50%MS	5	0.01		30
DF1	100%DKW	2.5	0.005	30	
DF2	100%DKW	5	0.01	30	
DF3	100%DKW	2.5	0.005		30
DF4	100%DKW	5	0.01		30

Figures

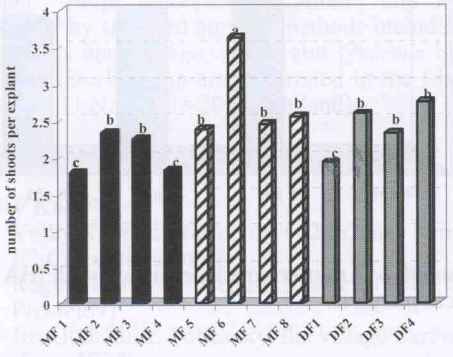

Fig. 1. Effect of different MS and DKW nutrient media formulations on propagule multiplication of *Pistacia* plantlets. Different letters indicates significant difference between treatments ($P<0.05$) by DMRT.

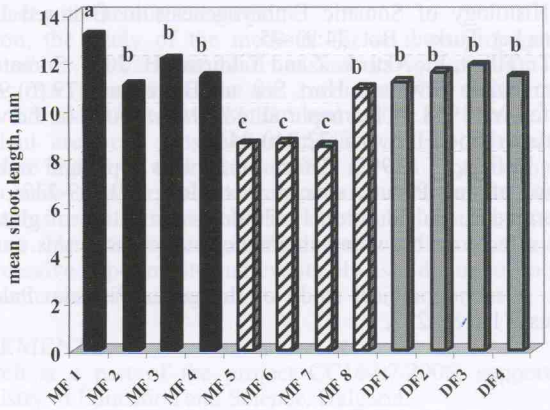

Fig. 2. Effect of different MS and DKW nutrient media formulations on shoot growth of *Pistacia*. Different letters indicate significant difference between treatments ($P<0.05$) by DMRT.

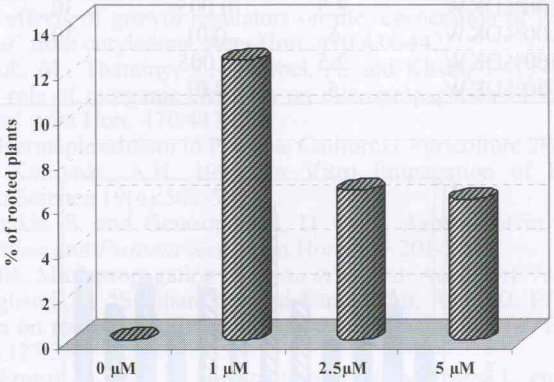

Fig. 3. Effect of half strength MS nutrient media enriched with IBA at four concentrations on rooting *Pistacia* plantlets.