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In vitro regeneration of *Pistacia vera* L. from nodal explants

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ABSTRACT: To enhance the induction of shoots, the excised nodes were cultured on Murashige and Skoog medium containing different cytokinins and various concentrations. The best conditions for shoot induction and growth were with 6-benzyladenine at 1.0 mg·l^{-1}. For axillary shoot proliferation, the excellent result was obtained using 2.0 mg·l^{-1} meta-topoline. Well-developed shoots (more than 2 cm in length) were successfully rooted *ex vitro* at 82% by treatment with commercial rooting powder (2% indole-3-butyric acid; Rhizopon®). Rooted plantlets were acclimatized in the greenhouse and the survival rate of transplantation reached 80%.

Keywords: sterilizing agents; morphogenic response; cytokinin; shoot proliferation; *ex vitro* rooting; tissue culture

The genus *Pistacia* Linnaeus belongs to the family Anacardiaceae, a cosmopolitan family that comprises about 70 genera and over 600 species (Bozorgi et al. 2013). *Pistacia vera* Linnaeus (pistachio) is the only species in this genus which produces edible nuts large enough to be commercially acceptable. The rest of the species produce smaller nuts which are mostly used as rootstocks for *P. vera*.

In current and traditional horticultural practice, the propagation of *P. vera* can be achieved from seed via seedling, or by grafting. Unfortunately, the development of this important species is currently limited due to poor propagation efficiency by conventional methods (Jacquy 1973; Joley 1979).

As with so many fruit and nut tree species, propagation of pistachio from seeds is unsatisfactory. Because the pistachio is an outbreeder, the seed progeny is genetically variable. Also, it is very difficult to identify the gender of the tree until it reaches the reproductive age (5–8 years). Moreover, pistachio seeds lose 50% of their viability after one year of storage (Jacquy 1973; Benmahioul et al. 2015).

Among the greatest weaknesses of using traditional vegetative propagation by grafting and cutting are the low rate of propagation and the high cost of plants (Maggs 1975; Joley 1979; Holtz et al. 1995). In addition, those conventional methods of propagation are too slow to meet the needs of large-scale plantations. To overcome this limitation, *P. vera* can be propagated through *in vitro* propagation techniques (Barghchi, Alderson 1983). These technologies could be a cost-effective means of high volume production of the elite planting material throughout the year, without any seasonal constraints. The tissue culture methods emerge as an important tool to enhance the production of plantlets. Many efforts have focused on establishing *in vitro* propagation and preservation procedures for *P. vera* (Barghchi, Alderson 1983, 1989; Abousalim et al. 1991; Yang, Lüdders 1993; Chatibi et al. 1995; Onay 2000, 2003, 2005; Ozden-Tokatli et al. 2005; Can et al. 2006; Benmahioul et al. 2009, 2012a, b, 2015). Some problems were encountered, particularly those in-

volving contamination, tissue browning and a lack of reaction of the mature plant material (Benmahioul 2009).

The establishment of tissue culture is one of the major difficult stages in the *in vitro* plant propagation. In the present study, the influence of sterilizing agents and cytokinins on *in vitro* introduction and establishment of axillary shoots was analysed. Our specific objectives were (*i*) to establish the best surface sterilization and determine the suitable cytokinin type and concentration for *in vitro* shoot proliferation from nodal explants, (*ii*) to root and acclimatize micropropagated pistachio shoots.

MATERIAL AND METHODS

Plant material and sterilization methods. Mature seeds of *P. vera* were collected from El Fehoul orchards (Tlemcen, Algeria). They were harvested from the same mother tree. The seeds were planted in plastic pots containing a mixture of peat and soil (1:1 v/v) and kept in the greenhouse. The pots were watered (every 2–3 days, on average) with ordinary water. Stem tips from 6-month-old seedlings (Fig. 1a) were used as explants for establishing *in vitro* cultures.

The shoots were prepared for a sterilization procedure by removing all expanded leaves and they were washed under running water. After division, the explants were rinsed by soaking them in 70% ethanol (v/v H_2O) for 1 min and then disinfected according to two methods tested:

(*i*) The first lot from stem segments was disinfected for 10 min in an aqueous mercury chloride ($HgCl_2$) solution at 0.1% (w/v). Two 10 min rinses in a calcium chloride ($CaCl_2$) solution at 0.3% (w/v) were carried out in order to reduce toxicity;

(*ii*) The second lot from stem segments was disinfected for 10 min in a solution of sodium hypochlorite (NaOCl 2.6% available chlorine) containing a few drops of a wetting agent.

Afterwards, for each lot, the stem segments were washed thoroughly 3–4 times with sterile distilled water, and inoculated vertically onto a shoot induction medium.

Culture media and conditions. All the cultures were grown in a culture room at 22 ± 1°C, 16 h photoperiod with light at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (photosynthetic

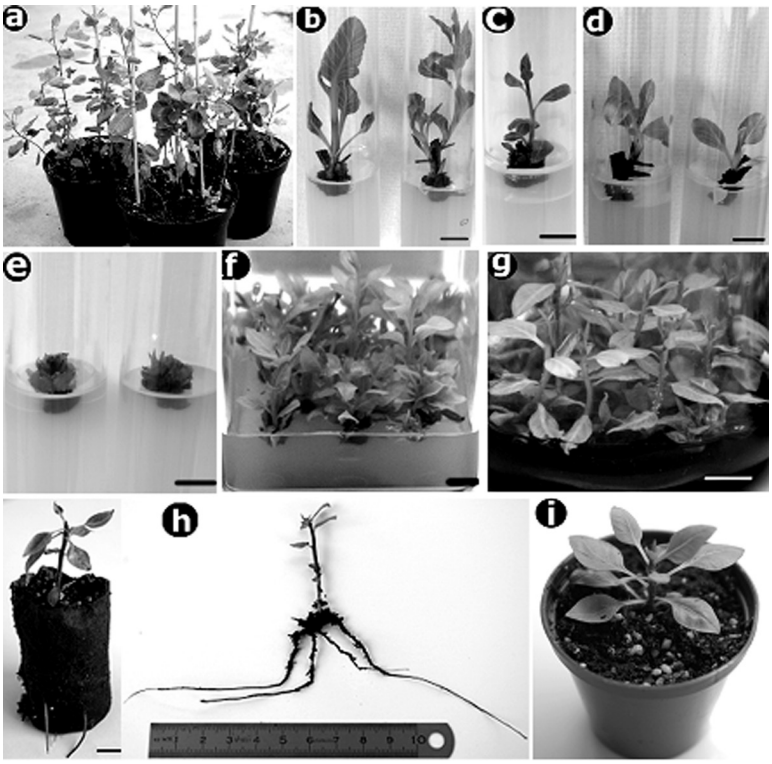

Fig. 1. *In vitro* regeneration of *Pistacia vera* from nodal explants (bar = 10 mm): using greenhouse-grown mother plants as a source of nodal stem segments (a), shoot development from nodal segment explants after 30 days of culture on Murashige and Skoog (MS) medium containing 1 $mg \cdot l^{-1}$ 6-benzyladenine (b), 0.5 $mg \cdot l^{-1}$ zeatin (c), 2 $mg \cdot l^{-1}$ kinetin (d), callus induction from nodal segments on MS medium supplemented with thidiazuron (e), development of multiple shoots after 1 month of culture on the MS medium fortified with 2 $mg \cdot l^{-1}$ meta-topolin (f), vigorous shoots obtained after 30 days on hormone-free MS medium containing 0.2% activated charcoal (g), *ex vitro* rooting of microcuttings treated with 2% indole-3-butyric acid (Rhizopon®, Hazerswoude-Rijndijk, Netherlands) (h), acclimatized microplant in the greenhouse after 2 months under *ex vitro* conditions (i)

photon flux density) (cool white fluorescent tubes) at the culture level. The vessels used in the experiments were applicable for culture initiation after surface disinfection, the nodal segments were put into the glass tubes (25 × 150 mm), each containing 15 ml of nutrient media, and for a multiplication step the shoots were transferred in flasks containing 50 ml of the medium. The basal Murashige and Skoog (MS) medium (Murashige, Skoog 1962) supplemented with vitamins B_5 (Gamborg et al. 1968), 100 mg·l^{-1} inositol, 500 mg·l^{-1} casein hydrolysate and 3% (w/v) sucrose was used throughout this study. Media were adjusted to pH 5.7 with 0.1N KOH and supplemented with 0.7% (w/v) Difco Bacto agar (before sterilization by autoclaving at 113°C for 20 min).

Shoot induction and multiplication. Shoot tips were trimmed aseptically to 8–10 mm long segments and inoculated onto the basal MS medium supplemented with various cytokinins: 6-benzyladenine (BA), kinetin (KIN) or zeatin (ZT) at 0.5, 1, 2 or 4 mg·l^{-1} and thidiazuron (TDZ) at lower concentrations (0.1, 0.2, 0.4 or 1 mg·l^{-1}). After 30 days of culture, the following parameters were monitored: percentage of contamination, percentage of regeneration, shoot length and number of leaves per shoot.

Sprouted axillary shoots (1 cm or larger) were excised from the mother nodal explants after 30 days and subjected to further proliferation on MS medium amended with 2 mg·l^{-1} meta-topolin (mT) (Benmahioul et al. 2012a). The shoots longer than 20 mm were isolated from proliferating cultures and transferred onto a fresh PGR-free medium containing 0.2% (w/v) activated charcoal (Benmahioul et al. 2012b).

***Ex vitro* root induction and acclimatization**. For *ex vitro* rooting, plantlets were rinsed in water to remove the culture medium. The basal ends of the shoots were dipped in commercial rooting powder (2% indole-3-butyric acid; Rhizopon®, Hazerswoude-Rijndijk, Netherlands) prior to planting (Benmahioul et al. 2012a). The microshoots were then inserted into Fertis® plugs (4 × 6 cm) containing a peat-perlite-vermiculite (80/15/5%) mixture, placed in covered plastic trays and maintained under high relative humidity conditions. The culture room conditions were the same as for the *in vitro* proliferation tests.

Regenerated plantlets were transplanted to pots containing peat/perlite/vermiculite (1:1:1 v/v/v) and then transferred to the greenhouse.

Statistical analyses. An analysis of variance of treatment means was performed using the Statgraph software (Manugistics, Inc., Rockville, USA), while the mean separation was checked by Duncan's multiple range test. The significance level was set at $P < 0.05$. The results are expressed as mean ± standard error.

RESULTS AND DISCUSSION

Surface sterilization

Results of sterilization procedures and development of shoots from initial explants are documented in Table 1. Seven days after incubation on an establishment medium, the axillary buds started to burst. The sterilization procedure was successful and 95.7% of the inoculated explants were free of contamination. Sodium hypochlorite was more conducive to rosette development (75%) but did not significantly differ from mercuric chloride (66.7%).

The establishment of tissue culture is one of the most difficult stages in the *in vitro* plant propagation (Riffaud, Cornu 1981; Meynier 1985; Nin et al. 1994; Mascarello et al. 2007). Two major problems are associated with this phase: the right choice of explants and the sterilization procedure. Various methods have been applied to surface sterilize explants but these depended on the explant type and species used.

There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ (Sujatha, Reddy 1998; Husain, Anis 2009; Wie et al. 2015). However, a long period of exposure to $HgCl_2$ leads to browning and death of explants (Danso et al. 2011; Sen et al. 2013).

The use of sodium hypochlorite for sterilization of plant explants from different sources has been reported. Kilinç et al. (2015) sterilized the mature lentisk seeds using sodium hypochlorite (20%) for 20 min. Also, Ahmed et al. (2011) reported that the best results were obtained with 1% NaOCl for

Table 1. Influence of sterilizing agents on decontamination and regeneration of pistachio shoots

Sterilizing agents	Number of explants cultured	Contamination (%)	Morphogenic response (%)	Rosettes (%)
$HgCl_2$	45	4.4[a]	86.7[a]	66.7[a]
NaOCl	48	4.2[a]	83.3[a]	75[a]
–	93	4.3	84.9	71

percentages followed by the same letter within each column are not significantly different at $P < 0.05$

2 min sterilization. The hydrogen peroxide (10% (w/v) solution for 5–10 min was also a successful surface sterilization method (Ozden-Tokatli et al. 2005).

Benmahioul (2009) reported the effect of the season on percent response of explants and frequency of contamination. Indeed, all explants collected during autumn (dormant axillary bud) from adult trees were contaminated after a few days. However, the best results were recorded with nodal segments taken in full growth (spring). The contamination percentage of 84.7 and 38.7% was recorded with sodium hypochlorite and mercuric chloride, respectively.

The main problem of *in vitro* propagation of the mature material of pistachio is the endogenous contamination of initial explants and phenolic compound exudation. However, in pistachio like in many other woody plants it was found that juvenile explants are far more responsive and are comparatively easy to establish. In the present study, the two types of tested sterilizing agents showed good results. No difference was noted between $HgCl_2$ and NaOCl. Nevertheless, the best morphological appearance of shoots was obtained in explants treated with sodium hypochlorite. Using explants from juvenile material (seedling) was better for sterilization and gave more than 95% of sterile explants.

Shoot induction and multiplication

Nodes with axillary buds were placed onto MS medium amended with different cytokinins (BA, KIN, ZT and TDZ). The induction of shoots varied with the type and concentration of these cytokinins tested. The mean percentages of shoot induction were 34.4, 58.3, 62.5 and 75% for TDZ, KIN, BA and ZT, respectively (Table 2).

Concerning the concentration of the cytokinin employed, BA at 1 mg·l^{-1}, KIN at 2 mg·l^{-1} and ZT at 4 mg·l^{-1} gave the highest percentage of burst axillary buds (91.7%). However, differences in shoot height and foliar organogenesis were noted. In comparison with the other three cytokinins tested, MS medium containing BA (1 mg·l^{-1}) was found to be most favourable for shoot elongation (2.0 cm) and produced 6–8 leaves per shoot (Fig. 1b). These results were similar to the findings of Xu et al. (2008), who pointed out that BA was very effective in the shoot proliferation of *Malus zumi* (Matsumura) Rehder. In our experiment, the media containing ZT gave also the best results but shoot elongation was restricted (Fig. 1c). Kinetin was found less suitable for shoot growth (only 0.6 cm) in comparison with BA (Table 2, Fig. 1d). In return, TDZ induced undesirable callus formation on the explants. The callus was generally limited to the developing shoot apical meristem (Fig. 1e).

Cytokinins are known to induce both axillary and adventitious shoot formation from meristematic explants (George 1993). The most efficient and commonly used cytokinin in plant tissue culture is BA (Bonga, Von Aderkas 1992). 6-Benzyladenine, its riboside and nucleotide are naturally occurring cytokinins in plant tissues and are relatively stable in comparison with other cytokinins (Letham, Palni 1983); this may be the possible explanation for the enhanced morphogenic response as obtained with BA. However, TDZ is reported to stimulate the formation of callus, adventitious shoots, or somatic embryos at concentrations higher than 1 mM (Huetteman, Preece 1993).

The axillary shoots (1 cm or longer) were separated from mother nodal explants and transferred for proliferation on MS medium amended with 2 mg·l^{-1} mT

Table 2. Effect of the type and concentration of cytokinin on regeneration, shoot length and average leaf number per rosette after 30 days of culture (n = 24 explants per treatment)

Cytokinin (mg·l^{-1})	Regeneration (%)	Shoot length (cm)*	Number of leaves per shoot*
BA			
0.5	62.5	0.6 ± 0.1	3.4 ± 1.0
1	91.7	2.0 ± 1.4	6.9 ± 1.3
2	41.7	0.4 ± 0.1	2.3 ± 1.0
4	54.2	0.2 ± 0.1	3.0 ± 1.0
Mean	62.5^{ab}	1.2 ± 1.2^{a}	4.8 ± 2.3^{b}
KIN			
0,5	58.3	0.4 ± 0.1	2.6 ± 1.0
1	50.0	0.7 ± 0.3	3.6 ± 0.7
2	91.7	0.6 ± 0.3	3.9 ± 1.2
4	33.3	0.6 ± 0.3	4.0 ± 1.4
Mean	58.3^{b}	0.6 ± 0.3^{b}	3.6 ± 1.2^{c}
ZT			
0.5	45.8	1.6 ± 1.0	6.9 ± 3.6
1	83.3	1.3 ± 0.7	5.8 ± 2.3
2	79.2	1.3 ± 0.7	6.5 ± 2.2
4	91.7	1.1 ± 0.6	4.8 ± 2.0
Mean	75.0^{a}	1.2 ± 0.7^{a}	5.7 ± 2.3^{a}
TDZ			
0.1	50.0	–	–
0.2	41.7	–	–
0.4	33.3	–	–
1	12.5	–	–
Mean	34.4^{c}	–	–

*means ± standard error followed by the same letter within each column are not significantly different at $P < 0.05$, BA – 6-benzyladenine, KIN – kinetin, ZT – zeatin, TDZ – thidiazuron

(Benmahioul et al. 2012a). The highest number of shoot inductions (on average, six shoots per explants) and faster growth and better appearance of shoots were achieved with this aromatic cytokinin (Fig. 1f). Meta-topolin could be a new source of cytokinins with high morphogenetic activity. Meta-topolin is becoming increasingly popular with plant tissue culturists because of its positive effects on several parameters of tissue culture, such as high rate of shoot multiplication and better rooting and acclimatization (Benmahioul et al. 2012a).

After the propagation stage, the shoots longer than 20 mm were isolated from proliferating cultures and transferred onto a fresh PGR-free medium containing 0.2% (w/v) of activated charcoal (Fig. 1g). The aim was to enhance the elongation and strength of plantlets so that they would be suitable for subsequent *ex vitro* rooting and acclimatization experiments.

Ex vitro rooting and acclimatization

The success and cost effectiveness of *in vitro* propagation rely on the rooting percentage and survival of plantlets in field conditions (Martin 2003). In our study, the microplants (approximately 2–3 cm in length) were excised and the cut ends were pulse treated by dipping in commercial rooting powder (Rhizopon®) and then inserted into Fertis® plugs. Thereafter, they were placed in covered plastic trays and maintained under high relative humidity conditions. This method gave the best results in terms of rooting percentage (82%) and number of roots per microplant (3–4 long roots) (Fig. 1h). Approximately 80% of the plantlets transferred to *ex vitro* conditions in a greenhouse were acclimatized correctly (Fig. 1i).

CONCLUSIONS

Maintenance of an aseptic condition is a prerequisite for successful *in vitro* shoot induction and proliferation. This study indicates that the use of explants from juvenile material (seedling) was better for sterilization and gave more 95% of sterile explants. The contamination percentage was low whatever the sterilizing agents tested ($HgCl_2$ or NaOCl) and the *in vitro* morphogenic response was very high. On the other hand, the best induction and elongation of shoots in this experiment were achieved using MS medium supplemented with 1.0 mg·l^{-1} BA which gave the highest percentage of shoot initiation and the good morphological appearance of shoots. For axillary shoot proliferation, the best result was obtained using 2.0 mg·l^{-1} meta-topolin. *In vitro* shoots can be rooted easily *ex vitro* in a humidity chamber with commercial rooting powder (2% indole-3-butyric acid; Rhizopon®). This efficient protocol for rapid micropropagation recommended by Benmahioul et al. (2012a) can be utilized for large-scale mass propagation of *P. vera*.

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