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RESEARCH NOTE

# Micropropagation and ex vitro rooting of pistachio (*Pistacia vera* L.)

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**Abstract** An effective pistachio (*Pistacia vera* L.) micropropagation system was developed involving rapid axillary bud proliferation and ex vitro rooting. The highest shoot proliferation frequency was obtained from nodal explants cultured on Murashige and Skoog (MS) basal salts containing Gamborg ($B_5$) vitamins and supplemented with 4 mg $l^{-1}$ 6-benzyladenine (BA). The addition of 2 mg $l^{-1}$ meta-topolin (*m*T) generated an optimal number of shoots with suitable morphological features, while kinetin (KIN) was found to be unsuitable for pistachio shoot proliferation. Microcuttings were rooted ex vitro after being dipped in rooting powder. The peak ex vitro rooting response was achieved after shoot explants were treated with Rhizopon® 2% indole-3-butyric acid (IBA). Rooted plantlets were transplanted in plastic pots containing a peat–perlite–vermiculite (1:1:1) mixture and then transferred to the greenhouse. After 2 months, 81.5% survival of rooted microshoots was achieved.

**Keywords** *Pistacia vera* L. · *meta*-Topolin · Axillary shoot proliferation · Ex vitro rooting · Acclimatization survival

**Abbreviations**

| | |
|---|---|
| BA | 6-Benzyladenine |
| B5 | Medium described by Gamborg et al. (1968) |
| IBA | Indole-3-butyric acid |
| KIN | Kinetin |
| *m*T | meta-Topolin |
| MS | Murashige and Skoog medium (1962) |
| NAA | Naphthaleneacetic acid |
| PGR | Plant growth regulator |
| SE | Standard error |

Pistachio (*Pistacia vera* L.) is a member of the Anacardiaceae family in the *Pistacia* genus. It is referred to as the "green gold tree" due to its high economic value. *Pistacia vera* L. is mainly cropped in Mediterranean European regions, North Africa, the Middle East and California.

Pistachio trees were introduced in Algeria in the early 1980s. Demonstration orchards were set up in different regions with the aim of promoting this crop. It is now essential to obtain genotypes suitable for cultivation in arid and semi-arid regions under dry and saline soil-climate conditions (Benmahioul et al. 2009a). High quality plant production techniques are also necessary to ensure the successful adoption of this species.

Mass propagation of plant species through in vitro culture is a prime example of the successful commercial application of plant tissue culture technology. In vitro culture is also an effective way to select fairly drought—(Sorkheh et al. 2010) and salt—(He et al. 2009) tolerant

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genotypes that could be grown in harsh environmental conditions, as is the case for pistachio growing in Algeria (Benmahioul et al. 2009a). Despite advances in pistachio micropropagation (Barghchi and Alderson 1989), the results are still not as good as those achieved with other fruit species. Studies on pistachio have shown that the major problem hampering extension of the method concerns the rooting of plantlets (Chatibi et al. 1995; Benmahioul et al. 2009b).

For some species that are not easy to root and acclimatize, especially woody plant species, microshoots have been successfully rooted ex vitro and the plants then survived when planted in the field (Saiju 2005; Annapurna and Rathore 2010; Liu et al. 2010; Huang et al. 2011). The number of roots and the quality of the rooting system of such plants are assumed to be more suitable than those formed in vitro (McClelland et al. 1990). Furthermore, ex vitro rhizogenesis offers an opportunity to improve the efficiency of micropropagation from both biological and economic standpoints (Borkowska 2001).

The present study was conducted to develop an efficient and reproducible standard micropropagation procedure for *Pistacia vera* L. We focused particularly on the effects of a new plant growth regulator (meta-topolin; Escalona et al. 2003) on in vitro propagation. An ex vitro rooting stage was developed for our plant material. To our knowledge, this is the first time that meta-toplin and ex-vitro rooting have been successfully used with pistachio are applied here to pistachio for the first time.

For the in vitro step, mature seeds of *Pistacia vera* L. were collected from El Fehoul orchards (Tlemcen, Algeria). Aseptic seedlings were obtained as previously described (Benmahioul et al. 2009b). Nodal stem segments excised from 30-day-old aseptic seedlings were subsequently used as explants.

These explants were cultured in vitro as follows. MS medium (Murashige and Skoog 1962) supplemented with $B_5$ vitamins (Gamborg et al. 1968), 100 mg $l^{-1}$ inositol, 500 mg $l^{-1}$ casein hydrolisate (acid hydrolysed, Merck 2245, 500 mg $l^{-1}$), 3% (w/v) sucrose was used throughout this study. Media were adjusted to pH 5.7 with 0.1 N KaOH before sterilization by autoclaving for 20 min at 113°C. All cultures were kept in a culture room at 22 ± 1°C under a 16 h photoperiod with 40 µmol $m^{-2}$ $s^{-1}$ irradiance provided by cool white fluorescent tubes.

For multiple shoot induction, nodal segments (1 cm long) were cultured in flasks (500 ml) containing 100 ml of MS medium along with various cytokinins: 6-benzyladenine (BA), kinetin (KIN) or meta-topolin (*m*T) at 0.5, 1, 2 and 4 mg $l^{-1}$ in order to determine the media requirements for optimal shoot proliferation. There were 10–15 nodal explants per flask. Microcuttings (1–3 cm length) were obtained from elongated shoots derived from inoculated nodal segments and subcultured on the same fresh medium after 30 days. The percentage of explant producing shoots, number of shoots per explant, number of usable shoots (≥1.0 cm long) and shoot length were recorded after the culture period.

After the propagation stage, the microcuttings were transferred onto fresh PGR-deprived medium containing 0.2% activated charcoal. 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.

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 prior to planting. Two different commercial rooting powders were tested: (1) Rhizopon® AA [containing 2% indole-3-butyric acid (IBA)], and (2) Rootone® F [containing 0.065% naphthaleneacetic acid (NAA), 0.056% indole-3-butyric acid (IBA) and 0.013% 2-methyl-1-naphthyl acetamide]. The microshoots were then inserted into Fertiss® 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. Six weeks later the ex vitro rooting treatments were started. The following data were collected: survival percentage, rooting percentage, total number of roots, and root length. We also assessed influence of proliferation medium on ex vitro rooting of microplantlets.

An analysis of variance (ANOVA) of treatment means was performed using Statgraph software, while the mean separation was checked with a Duncan's multiple range test. The significance level was set at ($P < 0.05$). The results are expressed as mean ± standard error (SE).

The proliferation potential of nodal segments was assessed on MS medium supplemented with various cytokinins, and the results are summarized in Table 1. Media supplemented with BA and *m*T were more effective in promoting shoot proliferation than those with kinetin. 4 mg $l^{-1}$ BA generated the highest number of induced shoots per explant (4.5 ± 2.0; Fig. 1a). However, the number of shoots per explant of suitable height and morphological appearance obtained with *m*T was recorded and compared to the BA results. The best results were obtained with 2 mg $l^{-1}$ *m*T (2.5 usable shoots/explant, 2.3 cm shoot height; Table 1, Fig. 1b). Media containing KIN were not beneficial for axillary shoot proliferation and the 4 mg $l^{-1}$ concentration gave particularly poor results (Table 1, Fig. 1c).

We also assessed the effect of subcultures on shoot proliferation in MS medium supplemented with 2 and 4 mg $l^{-1}$ BA or *m*T. Similarly, the highest number of shoot inductions (4.9 ± 2.3) was achieved with 4 mg $l^{-1}$ BA,


**Fig. 1** Micropropagation and ex vitro rooting of pistachio (*P. vera* L.). Multiple axillary shoots obtained after 30 days of culture on MS medium containing 4 mg $l^{-1}$ BA (**a**) or 2 mg $l^{-1}$ *m*T (**b**). Nodal explant showing no differentiation of axillary buds on MS with 4 mg $l^{-1}$ KIN, after 30 days of culture (**c**). Shoot multiplication obtained after two successive subculture passages on MS supplemented with 2 mg $l^{-1}$ *m*T (**d**). Vigorous shoots obtained after 1 month on hormone-free MS containing 0.2% activated charcoal (**e**). Ex vitro rooting (note roots emerging out the Fertiss® plugs) (**f**). Ex vitro rooting of *P. vera* L. after 6-weeks: Rhizopon (**g**), Rootone F (**h**) and control (**i**). Ex vitro rooting of microcuttings propagated on media contaning *m*T (**j**) or BA (**k**). Greenhouse-grown micropropagated plant after 2 months under ex vitro conditions (**l**). *Bar* = 1.0 cm

but the best mean number of usable shoots and the best stem lengths were obtained on media enriched with 2 mg $l^{-1}$ *m*T (Table 2). Approximately 3–4 good quality shoots per explant were produced. They were 1.5–2.0 cm-long, with well developed leaves (1–2 cm) after 30 days (Fig. 1d). Shoots produced with 4 mg $l^{-1}$ BA were smaller as compared to those produced with *m*T. Meta-topolin at 2 mg $l^{-1}$ concentration can thus be recommended for multiplication of *Pistacia vera* L.

Hormone-free MS basal medium containing 0.2% activated charcoal proved interesting for vigorous lignification (Fig. 1e).

**Table 1** Effect of various concentrations of three cytokinins (BA, KIN, *m*T) on shoot proliferation from nodal explants after 30 days of culture ($n = 50$ explants per treatment)

| Cytokinin (mg/l) | | | Explants developing shoots (%) | Number of regenerated shoots/explant | Number of usable shoots | Usable shoot length (cm) |
|---|---|---|---|---|---|---|
| BA | KIN | *m*T | | | | |
| 0.5 | – | – | 96[a, b] | 1.8 ± 1.2[e] | 0.9 ± 0.7[d, e] | 1.7 ± 0.8[b, c] |
| 1 | – | – | 96[a, b] | 2.2 ± 1.2[d, e] | 1.1 ± 0.8[d] | 1.6 ± 0.5[c] |
| 2 | – | – | 98[a, b] | 3.0 ± 1.4[b, c] | 1.0 ± 0.9[d] | 1.4 ± 0.4[c] |
| 4 | – | – | 98[a, b] | 4.5 ± 2.0[a] | 1.6 ± 1.0[c] | 1.5 ± 0.4[c] |
| – | 0.5 | – | 70[c] | 0.9 ± 0.7[f, g] | 0.5 ± 0.6[f] | 2.1 ± 1.0[a, b] |
| – | 1 | – | 52[c, d] | 0.7 ± 0.7[f, g] | 0.1 ± 0.3[g] | 1.5 ± 0.5[c] |
| – | 2 | – | 90[b] | 1.1 ± 0.6[f] | 0.6 ± 0.5[e, f] | 2.2 ± 0.8[a] |
| – | 4 | – | 44[d] | 0.6 ± 0.7[g] | 0.1 ± 0.4[g] | 1.7 ± 0.4[b, c] |
| – | – | 0.5 | 100[a] | 2.3 ± 0.9[d, e] | 1.1 ± 0.7[d] | 2.2 ± 0.9[a] |
| – | – | 1 | 100[a] | 2.7 ± 1.3[c, d] | 1.6 ± 0.8[c] | 2.1 ± 0.8[a] |
| – | – | 2 | 100[a] | 3.4 ± 1.6[b] | 2.5 ± 1.3[a] | 2.3 ± 0.7[a] |
| – | – | 4 | 100[a] | 3.0 ± 1.7[b, c] | 2.0 ± 1.2[b] | 2.3 ± 0.8[a] |

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

**Table 2** Evaluation of the morphogenetic potential of shoot cultures obtained on media containing BA and *m*T after being tested in two successive subcultures on the same medium ($n = 40$ explants per treatment)

| Cytokinin (mg $l^{-1}$) | BA | | *m*T | |
|---|---|---|---|---|
| | 2 | 4 | 2 | 4 |
| Explants producing shoots (%) | 97.5 ± 5.0[a] | 100 ± 0.0[a] | 100 ± 0.0[a] | 100 ± 0.0[a] |
| Number of regenerated shoots/explant | 3.1 ± 1.7[b] | 4.9 ± 2.3[a] | 4.5 ± 2.4[a] | 4.5 ± 2.1[a] |
| Number of usable shoots | 1.5 ± 1.1[bc] | 1.2 ± 1.0[c] | 3.2 ± 1.7[a] | 2.0 ± 1.0[b] |
| Usable shoot length (cm) | 1.6 ± 0.5[b] | 1.7 ± 0.4[ab] | 1.8 ± 0.5[a] | 1.5 ± 0.4[b] |

Means ± SE followed by the same letter within rows are not significantly different at $P < 0.05$

After approximately 2–3 weeks under ex vitro conditions, the first adventitious roots began to emerge out of Fertiss plugs (Fig. 1f). In the first rooting experiment, microshoots of *P. vera* L. issued from *m*T-proliferation medium responded differently to each rooting powder treatment as indicated by their survival rate, rooting percentage and total number of roots (Table 3). Microshoot survival after 6 weeks was highest (100, 92.9 and 87.1%) for control, Rootone® F and Rhizopon® treatments, respectively. However, control shoots yielded significantly fewer rooted plants (47.5%) than Rhizopon®-treated (78.6%) and Rootone® F-treated (74.3%) shoots. The best treatments with respect to root production on microshoots was the Rhizopon® treatment, with a twofold greater number of primary roots produced (3.8 ± 2.7) over control (1.7 ± 0.9; Table 3, Fig. 1g–i). The average root length in the different treatments showed significant variation. The mean root length (5.9 cm) was highest for the control, followed by the Rootone® F treatment (3.5 cm).

In the second rooting experiment, our results showed that the *ex vitro* rooting of *Pistacia vera* L. did not depend upon the previous BA or *m*T culture of microshoots. No difference was noted between the two types of microcuttings rooted using Rhizopon® (Table 4). The rooting percentages were high for shoots propagated on media containing *m*T and BA (82.5 and 75%, respectively). The number of roots per plantlet ranged from 3 to 4 roots (Table 4, Fig. 1j, k).

**Table 3** Percentage rooting, number of roots and length of roots produced ex vitro by micropropaged shoots issued from *m*T-proliferation medium and treated with two rooting powders (Rhizopon AA or Rootone F)

| | Control | Rootone F | Rhizopon AA |
|---|---|---|---|
| Survival (%) | 100 ± 0.0[a] | 92.9 ± 6.0[ab] | 87.1 ± 6.1[b] |
| Rooting (%) | 47.5 ± 10.6[b] | 74.3 ± 8.1[a] | 78.6 ± 6.1[a] |
| Number of roots/plantlet | 1.7 ± 0.9[b] | 2.6 ± 1.6[b] | 3.8 ± 2.7[a] |
| Root length (cm) | 5.9 ± 4.5[a] | 3.5 ± 3.1[b] | 1.9 ± 1.6[c] |

Means followed by the same letter within rows are not significantly different at $P < 0.05$

Shoots rooted by this ex vitro method were transplanted into pots. From 189 ex vitro rooted shoots, 154 (81.5%) plantlets survived in the greenhouse (Fig. 1l).

Pistachio microshoot proliferation is promoted by BA (Abousalim et al. 1991). In our experiments, maximum shoot induction was achieved in MS medium supplemented

**Table 4** Effect of the proliferation-medium (MS containing BA or *m*T) on survival and rooting percentages of microplantlets treated with Rhizopon® 2% IBA (40 shoots per treatment)

| | BA | *m*T |
|---|---|---|
| Survival (%) | 82.5 ± 3.5[a] | 92.5 ± 3.5[a] |
| Rooting (%) | 75 ± 7.1[a] | 82.5 ± 10.6[a] |
| Number of roots/plantlet | 3.1 ± 2.4[a] | 3.8 ± 2.3[a] |
| Root length (cm) | 2.3 ± 1.9[a] | 1.9 ± 1.6[a] |

Means followed by the same letter within rows are not significantly different at $P < 0.05$

with 4 mg $l^{-1}$ BA. However, the number of usable shoots and vigorous microplants was higher with *m*T than with BA. Explants on BA produced few long shoots, while those on medium containing meta-topolin produced a greater number of elongated shoots, in line with the results reported by Kaminek et al. (1987). These latter authors found that *m*T was nearly twice as effective as BA in the induction of shoot growth of cuttings. The effectiveness of *m*T in shoot bud differentiation has been widely reported (Kubalakova and Strnad 1992; Baroja-Fernandez et al. 2002; Escalona et al. 2003; Bairu et al. 2008; Koetle et al. 2010). Meta-topolin, which was first isolated from poplar leaves, differs from isoprenoid cytokinins with respect to its biochemistry, receptors and biological activity (Strnad et al. 1997). This aromatic cytokinin could be a new source of cytokinins with high morphogenetic activity. Bairu et al. (2009) recently indicated that *m*T reduced in vitro shoot tip necrosis in *Harpagophytum*. Interestingly, for Barghchi and Alderson (1989), this abnormality hindered micropropagation of pistachio. According to our results, *m*T was a good replacement for BA in *P. vera* L tissue culture.

The practical prospects of mass micropropagation are largely dependent on the rooting percentage and survival rate of the plantlets in field conditions. Ex vitro rooting was used to simplify the procedure and reduce production costs. The main advantages were that no rooting step under sterile conditions was required, and the fact that rooting and acclimatization took place simultaneously. This method has been successfully applied with numerous species: *Quercus robur* (Meier-Dinkel et al. 1993), *Fraxinus* spp. (Kim et al. 1998), *Stackhousia tryonii* (Bhatia et al. 2002), *Ceratonia siliqua* (Romano et al. 2002), *Rotula aquatica* and *Eupatorium triplinerve* (Martin 2003a, b).

Ex vitro rooting has never been described for *P. vera* L. The in vitro regenerated plantlets were rooted and acclimatized ex vitro under a plastic tray with high relative humidity (~90%) in a standard culture room. The use of Rhizopon® (2% IBA) gave the best results in terms of rooting percentage and number of roots per microplant. The positive effects of IBA on ex vitro root induction have been reported for many woody species: *Corylus* sp. (Yu and Reed 1995), *Fraxinus pensylvanica* (Kim et al. 1998) and *Malus zumi* (Xu et al. 2008).

Pruski et al. (2000) reported that the best ex vitro rooting of *Prunus virginiana* and *P. pensylvanica* was obtained with an IBA/NAA combination (average 84%). They also mentioned that shoots treated with Rootone® F showed 75% rooting but did not significantly differ from those treated with IBA alone (71%). In our experiment, Rootone® F was also effective for rooting (52 rooted out of 70 on average, or 74.3%). However, Bhatia et al. (2002) demonstrated that the mixing of two or more auxins markedly reduced the rooting percentage, likely due to antagonistic effects.

Shoot rooting was not influenced by the proliferation medium. However, microplantlets produced with *m*T had peak ex vitro rooting (82.5%) as compared to those treated with BA (75%). Bairu et al. (2007) reported that plants treated with *m*T showed faster growth and superior rooting compared to BA. In addition to its effect on rooting, *m*T also enhanced micropropagated plantlet acclimatization. Werbrouck et al. (1995) demonstrated that the accumulation of the derivative [9G] BA at the base of plants could result in slow BA release during acclimatization, which can cause different problems such as growth heterogeneity and rooting inhibition.

The results of our study demonstrated that *m*T was suitable for effective in vitro proliferation of axillary shoots. In vitro shoots can be rooted easily ex vitro in a humidity chamber with commercial rooting powder (Rhizopon® 2% IBA). Plants obtained by ex vitro rhizogenesis have a well developed root system and better subsequent growth and development. Results could now be applied *P. vera* L. genotypes in vitro selected for their tolerance to salt (Benmahioul et al. 2009a) which are potentially valuable candidates for the valorisation of saline edaphic conditions.

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