Techniques for in vitro seed germination in *Pistacia* species

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A reliable technique for achieving in vitro seed germination in *Pistacia vera* cv. *mateur*, *P. atlantica*, *P. terebinthus* and *P. lentiscus* L. is documented. Both eradication of browning exudate from seeds and germination percentage were affected by the scarification of the seeds with hot water and sulphuric acid. Moreover, in-vitro germination of seeds and elongation of the seedlings were optimal when the seeds were cultured on modified Heller medium (H + 7 mM KH₂PO₄ + 10 μM AgNO₃).

Keywords: *In-vitro*, *Pistacia* species, contamination, browning, AgNO₃, seedlings.

Abbreviations: H₂SO₄ = Sulphuric acid; AgNO₃ = Silver nitrate; H = Heller medium; MS = Murashige and Skoog medium; QL = Quorin and Lepoivre medium; BAP = Benzylaminopurine; NAA = Naphthaleneacetic acid.

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Introduction

*Pistacia* species belong to the Anacardiaceae family and are considered as one of the more important plants in many regions: Magreb, Spain, Turkey, Greece, Iran, Iraq, India. The propagation of these species by seeds is quite difficult as the seeds are sensitive to desiccation and cannot be stored for long periods without a serious loss of viability. Tissue culture techniques may be an alternative methodology for propagation of *Pistacia* species. However, high contamination rates are often observed in plant tissue cultures initiated from explants of *Pistacia* species taken from natural habitats. Explants taken from *Pistacia vera* cv. *mateur*, *P. atlantica* and *P. terebinthus* seedlings and collected from greenhouse were found to be highly contaminated with *Fusarium* species (Mederos et al., unpublished data). For this reason in this study we investigated a method for reducing contamination from these *Pistacia* species. In an earlier study on in vitro shoot formation from seedlings of these species we observed browning exudate to the culture medium. Our results indicated that the browning of *Pistacia* species seeds is related to the inhibition of in vitro germination (Mederos 1991) and for this reason eradication of oxidation of the phenolic exudate was attempted. This study is a part of a project aimed at finding a method to propagate *Pistacia* species in vitro using seedling material.

Material and Methods

Plant material

Seeds of *Pistacia vera* cv. *mateur*, *Pistacia atlantica* Desf., *P. terebinthus* L. and *P. lentiscus* L. were collected between May and June (1993).

Scarification treatment

Seeds from these species were placed separately in a gauze sack and submerged completely in hot water for 15 and 25 min at 33°C followed by immersion in 0.1 N sulphuric acid (H₂SO₄) for 30, 45, 60 and 90 min. At the end of each treatment the seeds were immediately washed repeatedly in running water for 20 min to ensure complete removal of any acid residue.

Surface sterilization

After the scarification treatment, seeds were disinfected with 2 g l⁻¹ Captan (15 min) as fungicide, followed by several treatments:

- **Treatment 1**: Seeds were disinfected with 100 and 150 g l⁻¹ sodium hypochlorite (NaOCl) or 150 and 200 g l⁻¹ calcium hypochlorite [Ca (OCl)₂] for 15, 25 and 45 min, respectively. Five ml of tween 80 was added to all treatments.

![Figure 1](image-url)  Effect of different HgCl₂ treatment regimes on contamination of *Pistacia* species seeds. Data are means and were collected at 17 days of initial culture.
(HgCl₂) for 15 and 25 min respectively. Five ml of tween 80 was added to all treatments.

Treatment 3: The seeds were disinfected with hydrogen peroxide (H₂O₂) for 10 min followed by 1 or 2 g l⁻¹ mercury chloride (HgCl₂) plus tween 80 (5 ml) for 20 and 15 min, respectively.

All seeds were rinsed six times with sterile distilled water. Forty-eight seeds were used for each treatment.

Tissue culture
Forty-eight seeds were placed on different culture media. The media used were Heller (H) (1953), modified Heller (H + 7 mM K₁₂PO₄), Murashige and Skoog (MS) (1962), modified MS (MS + 7 mM K₁₂PO₄), Quoirin and Lepoivre (QL) (1977), modified QL (QL + 7 mM K₁₂PO₄) basal macroelement (Table 1). All media were supplemented with 10 μM AgNO₃, Fe-EDTA and the macroelement formula described by Murashige and Skoog (1962) plus 0.2 mg l⁻¹ thiamine-HCl, 0.2 mg l⁻¹ pyridoxine-HCl, 100 mg l⁻¹ myo-inositol, 30 g l⁻¹ sucrose (Merck) and 6 g l⁻¹ Difeo Bacto agar, supplemented with BAP (1 μM), alone or combined with NAA (0.5 μM). The pH was adjusted to 5.6 in all the culture media which were sterilized under 0.5 atmosphere at 115°C for 20 min. The seeds were incubated in a growth chamber at 75% relative humidity and at 24°C with 16 hour photoperiod using cool white fluorescent lamps (33 μE m⁻² s⁻¹ photosynthetically active radiation).

Statistical analysis
Results (recorded on different days depending on the growth responses being measured) were analysed by Duncan Multiple Range Test (DMRT) using the statistical graphic system of the Statistical Graphics Corporation computer programme and p < 0.05 constituted a significant difference.

Results and Discussion
Preliminary results indicated that pre-soaking of the seeds with Captan as fungicide was necessary. On the other hand, the use of Captan or Benlate at the same concentration (2 g l⁻¹) and immersion time (15 min) showed no significant differences between these fungicides (p < 0.05) (data not shown), although the effectiveness of Captan and Benlate as fungicides in eliminating seed fungi is well recognized (Watts et al. 1993; George 1996). An early attempt to achieve in vitro and in situ seed germination using imbibed seeds with or without testa and soaking intact seeds without scarification did not stimulate any germination. Scarification was effective with testa removal (Mederos et al. unpublished data). Moreover, hot water treatment for 15 min at 33°C, and 0.1 N sulphuric acid for 45 min, prior to disinfection, was effective for these Pistacia species and similar results with hot water have been previously reported from other species (Hol & van der Linde 1992). After surface disinfection with treatment one, seeds of P. vera cv. mateur, P. atlantica, P. lentiscus and P. terebinthus were still highly contaminated (83–100%) and browning exudates were considered and dismissed. In preliminary experiments, exhaustive washing of the seeds in ascorbic acid solution (0.1 and 0.5 μM) for 30 and 45 min, respectively after treatment one or two could not remove the contamination and/or browning from the seeds, in direct contrast to the results obtained in a recalcitrant Richmus species (Mederos & Schöbert 1995).

The results obtained in treatment two showed a significant effect on decontaminating seeds (Figure 1). When seeds were treated with 1 g l⁻¹ HgCl₂ for 15 and 25 min between 42 and 83%
Table 2 Effect of different culture medium containing 10 μM AgNO₃ on percentage of germinated seeds and elongation of seedlings. Dates are means and standard error (S.E.). When AgNO₃ was omitted from the media, germination was between 17 and 23%. All data were recorded at 25 and 43 days of culture

| Pistacia species | Modified culture medium | Germination seeds (%) | Elongation seedlings (mm) |
|------------------|--------------------------|-----------------------|--------------------------|
|                  |                          | 15        | 23        | 25        | 43        |
| P. vera cv. mateur | Heller                   | 83        | 92        | 37 ± 0.21e | 41 ± 0.30e |
|                  | Murashige & Skoog        | 58        | 67        | 25 ± 0.30h | 27 ± 0.30h |
|                  | Quuirin & Lepoiivre      | 25        | 42        | 17 ± 0.30h | 21 ± 0.39h |
| P. atlantica     | Heller                   | 75        | 92        | 40 ± 0.58e | 44 ± 0.33e |
|                  | Murashige & Skoog        | 25        | 25        | 18 ± 0.30h | 23 ± 0.30h |
|                  | Quuirin & Lepoiivre      | 50        | 67        | 23 ± 0.39h | 27 ± 0.37h |
| P. terebinthus   | Heller                   | 58        | 83        | 33 ± 0.25e | 37 ± 0.37e |
|                  | Murashige & Skoog        | 42        | 50        | 19 ± 0.39h | 24 ± 0.30h |
|                  | Quuirin & Lepoiivre      | 25        | 25        | 16 ± 0.39h | 19 ± 0.33h |
| P. lentiscus     | Heller                   | 50        | 75        | 25 ± 0.48h | 29 ± 0.46h |
|                  | Murashige & Skoog        | 25        | 33        | 21 ± 0.25h | 23 ± 0.25h |
|                  | Quuirin & Lepoiivre      | 17        | 17        | 14 ± 0.25h | 17 ± 0.37h |

Means in columns followed by the same letter are not significantly different at 0.05 confidence level (Duncan’s test).

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contamination was observed (Figure 1A). However, between 17% and 50% of seeds of these woody plants remained contaminated with 2 g l⁻¹ HgCl₂ and 15 or 25 min immersion time. Moreover, in the treatments with 1 and 2 g l⁻¹ HgCl₂ seeds of these Pistacia species showed necrosis (55% and 58%, respectively). The overall effect of disinfecting the seeds with H₂O₂ plus HgCl₂ depends on the concentration of the latter compound and the immersion time as shown in experiment three (Figure 2). Satisfactory decontamination percentages were obtained with treatment 3 (Figure 2A and B). The most satisfactory results were obtained in H₂O₂ for 10 min plus 1 g l⁻¹ HgCl₂ for 20 min for P. vera cv. mateur and P. terebinthus (Figure 2A). However, sterilising seeds with H₂O₂ for 10 min plus 2 g l⁻¹ HgCl₂ for 15 minutes proved best for P. atlantica and P. lentiscus (Figure 2B). We recommended hydrogen peroxide treatment prior to mercuric chloride application to minimise tissue damage as a result of the surface sterilization process. Moreover, due to the toxicity of HgCl₂, Pistacia seeds must be rinsed thoroughly at least six times with sterile distilled water prior to culture (Mederos 1991).

Germination percentages were significantly lower when seeds were cultured in the normal Heller, QL, MS culture media (between 17% and 23%) than with the rest of the treatments. Under our preliminary experimental conditions the seeds excreted browning substances to the culture media and we observed very necrotic brown seeds after twenty five days of culture. In these media, with AgNO₃, browning of the culture media was eradicated and the percentage of germination was stimulated. Similar results are reported in organogenesis from juvenile shoot explants of Pistacia atlantica Desf. (Mederos & Trujillo 1998). As shown by the results presented in Table 2, the most satisfactory results were obtained in the modified culture media supplemented with AgNO₃ where the seeds did not excrete substances into any of these media. As far as the percentage of germinating seeds and elongation of the seedlings were concerned, growth on the modified Heller medium (H + 7 mM KH₂PO₄) was discernibly different if we compared the same parameters measured for seedlings on the modified QL and MS media supplemented with 10 μM AgNO₃ (Table 2). AgNO₃ has a very slight positive effect on organogenesis and/or anti-browning effect from different material plant (Housti et al. 1992; George
Best seedling growth was obtained on the modified Heller medium for all the *Pistacia* species used (Table 2). Moreover, the elongation of the seedlings had stopped on day 43 of culture. The results of the comparison between these *Pistacia* species established that modified Heller solution gave consistently better results than any other macroelement solution using solidified media. On the other hand, we found that a short pre-culture period using normal or modified Heller (H + 7 mM K$_2$HPO$_4$ + 10 μM AgNO$_3$) liquid medium for 15 to 17 days caused browning and necrosis of the *Pistacia* seeds (83–87%) and lower seed germination (7–13%) (Mederos et al. unpublished data).

The present results indicate that *in vitro* germination of seeds from *Pistacia* species is possible in the same culture medium but under different conditions. However, other investigations show that there are differences in explant viability of *Pistacia* species seedlings cultured on the same micropropagation medium (Gonzalez & Frutos 1990; Mederos et al. 1994a; b; 1997a; b; Mederos & Trujillo 1998). Different responses from different types of explants (embryo, seed, apical and/or axillary shoot) may be due to the different genotypes being cultured on the explant themselves. However, the results of the present study are encouraging, as the recalcitrant seeds of these *Pistacia* species fail to germinate *in situ* and/or *in vitro* after drying.

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