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Cryopreservation of *Pistacia vera* embryonic axes – Short Communication

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ABSTRACT: This preliminary study investigated the conservation of *Pistacia vera* genetic resources using seeds and isolated embryonic axes. First, the effect of storing seeds in ambient conditions on embryo viability was evaluated by *in vitro* culture. The germination rate of *P. vera* embryonic axes gradually decreased from 100% to 31% after 30-month storage of seeds. Cryopreservation may thus be necessary for the long-term conservation of embryos. A simple protocol was set up using embryonic axes. It included a single dehydration step with silica gel prior to direct freezing in liquid nitrogen (–196°C). The optimal germination rate was obtained after 60 min dehydration (water content of 0.2 grams of water per gram of dry weight [g·g^{-1} DW]). However, 90 minutes of dehydration (0.14 g·g^{-1} DW) were necessary to obtain seedlings whose qualitative development was equivalent to that of the control embryonic axes.

Keywords: seed; dehydration; water content; liquid nitrogen; germination

Pistachio is a dicotyledonous angiosperm belonging to the family *Anacardiaceae*. According to Zohary (1952), the genus *Pistacia* comprises four sections and 11 species. *Pistacia vera* L. is the only species producing edible seeds (Joley 1979). With its seeds and its high fodder value, the pistachio is a vital source of income for farmers. However, different types of problems can affect the cultivation of this species including orchard ageing, damage due to pests and diseases. The conservation of *Pistacia* genetic resources is consequently essential.

Seed banks are the most common means of preserving plant genetic resources, at least for species which produce orthodox seeds (i.e. seeds that can withstand dehydration, thus ensuring their long-term viability). For example, pea (*Pisum sativum*) seeds were shown to be viable after more than 23 years and maize (*Zea mays*) seeds after 19 years, even though they were stored in ambient conditions (Nagel, Borner 2010). A few 200-years-old seeds of species originating from the Cape floristic region in South Africa were even able to germinate (Daws et al. 2007). In contrast, seeds of other species, called recalcitrant, cannot survive dehydration or storage at low temperatures and consequently cannot be stored for a long period. Most recalcitrant seeds are produced by woody species, shrubs or trees (Li, Pritchard 2009). The influence of conservation on the viability of seeds of *P. vera* L. is almost unknown. This is a limiting factor for the optimal management of seed stocks by seed producers and for the long-term conservation of *P. vera* genetic resources. According to Jacquy (1972), pistachio seeds lose 50% of their viability after one year of storage. Plant tissue culture has been used for *P. vera* L. for *ex situ* conservation (Ozden-Tokatli et al. 2010). The use of *in vitro* culture for the conservation of species with recalcitrant seeds has many advantages, including maintenance under aseptic conditions. However, when used for long periods, it has many disadvantages including the risk of genetic variation and the need for regular sub-culture, which is time and labour consuming (Ozden-Tokatli et al. 2010).

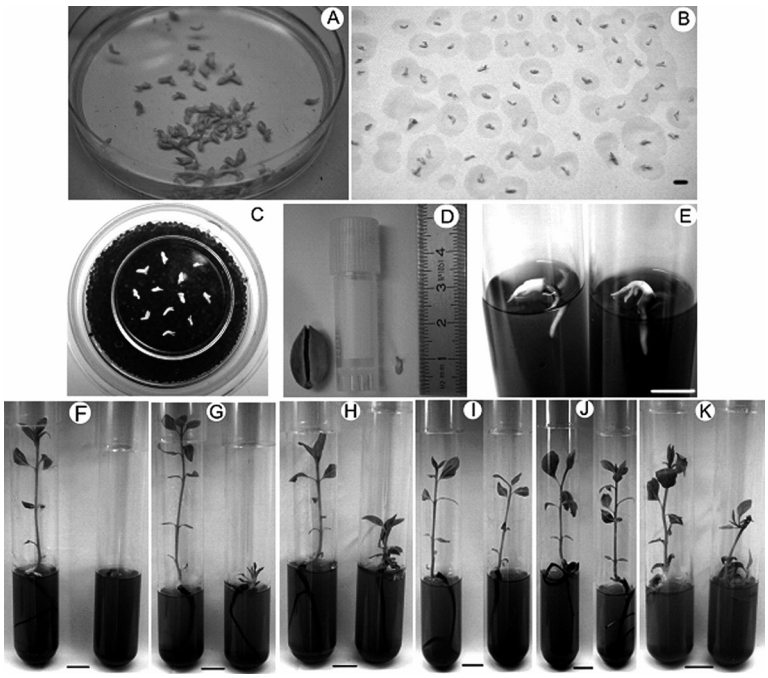

Fig. 1. Cryopreservation of isolated embryonic axes of *Pistacia vera* L.

(a) embryonic axes were placed in sterile distilled water to prevent dehydration during isolation; (b) just before dehydration, embryonic axes were placed for 1 minute on sterile filter paper to allow excess water to be absorbed; (c) dehydration over silica gel; (d) comparative size of a whole seed and an embryonic axis, placed near a cryotube; (e) regrowth of an embryonic axis after 3 days of culture; (f–j) after 4 weeks of culture, seedlings from non-cryopreserved (left tube) and cryopreserved (right tube) embryonic axes, without dehydration (f), after 30 (g), 60 (h), 90 (i) and 120 min (j) of dehydration; (k) abnormal development (no root) of cryopreserved axis not scored as germinated; b_{bar} = 5 mm; e_{bar}–k_{bar} = 10 mm

Cryopreservation, storage at ultra-low temperatures (generally –196°C, the temperature of liquid nitrogen), could be the method of choice to ensure long-term preservation, with a high safety level and reduced cost (Engelmann 2004). Reports on *Pistacia* cryopreservation are scarce. Whole seeds of *P. vera*, *P. terebinthus* and *P. lentiscus* were cryopreserved (Ozden-Tokatli et al. 2007). Cryopreservation of *P. vera* buds with vitrification protocols is currently under development (Ozden-Tokatli et al. 2010).

For species like *Pistacia vera*, which produce voluminous seeds, cryopreservation of zygotic embryonic axes could be the answer: because they are much smaller than whole seeds, dozens of embryonic axes could be preserved in a single cryovial. Some articles have been published on the cryopreservation of isolated embryos of various woody species: *Castania sativa*, *Carya* sp., *Juglans cinerea*, *J. nigra* and *J. regia* (Pence 1990), *Quercus suber* and *Q. ilex* (Gonzalez-Benito et al. 2002). But, to our knowledge, no study has been reported on the cryopreservation of embryonic axes of *Pistacia* species.

This study had two objectives: (*i*) to conduct a preliminary study of the effect of seed storage duration on the viability of *Pistacia vera* embryos, (*ii*) to determine the effect of dehydration on germination and regrowth quality of embryonic axes after cryopreservation.

MATERIALS AND METHODS

Plant material and method of disinfection. *Pistacia vera* seeds were harvested at maturity (late September) in the El Fehoul orchard (Tlemcen, Algeria), released from their epicarp and dried naturally (about 30% moisture content). Then, they were stored in plastic bags, in ambient and dark conditions (temperature between 19 and 25°C).

Seed disinfection consisted of rinsing in 70% ethanol (v/v) for 1 min, then soaking for 10 min in a solution of sodium hypochlorite (NaClO 2.6% available chlorine) containing a few drops of a wetting agent. The seeds were then rinsed three times with sterile distilled water. To facilitate the extraction of embryonic axes, seeds were kept overnight in sterile distilled water (Benmahioul et al. 2009).

***In vitro* germination tests.** Seed lots from three harvest dates were tested in April 2009: 2008

(6 months storage), 2007 (18 months storage) and 2006 (30 months storage). To avoid any genotype effect, seeds were harvested from the same tree. The three experimental lots were disinfected separately. Embryos were aseptically removed by opening the seeds with forceps. The embryonic axes were carefully removed from the cotyledon with a scalpel, and plated on a culture medium. A minimum of 55 seeds was used per year.

Cryopreservation. For cryopreservation experiments, embryonic axes were isolated from seeds of the last harvest (2008) and that had been stored for 6 months. During dissection, embryonic axes were placed in a Petri dish containing sterile distilled water to prevent dehydration (Fig. 1a). Just before dehydration, they were placed on sterile filter paper for 1 min to absorb excess water (Fig. 1b).

For dehydration, 12 axes were placed on a 55-mm glass Petri dish and then were enclosed in a 160 ml glass jar containing 40 g of activated silica gel (Fig. 1c). Dehydration was performed in the dark at room temperature (22°C) to test durations: 0, 30, 60, 90 and 120 min. The axes were then transferred to cryotubes (13.5 × 48.3 mm) (6 to 7 axes per tube) and quickly immersed in liquid nitrogen. After two hours of storage at –196°C, the cryotubes were plunged into a water bath at 40°C for 1 min. The embryonic axes were then cultivated. For each dehydration duration, control axes were directly cultivated without cryopreservation. Twenty axes were used per treatment. To determine water content (in grams of water per gram of dry weight [g g^{-1} DW]), 20 embryonic axes per treatment were dried in an oven at 65°C for 24 h.

Nutrient media and culture conditions. The isolated embryonic axes were seeded in glass test tubes (22 × 150 mm) containing 15 ml of MS0, a Murashige and Skoog (1962) medium without growth regulators and supplemented with 2 g·l^{-1} of activated charcoal and 4 g·l^{-1} agar (Benmahioul et al. 2009). Cultures were placed at 22°C, in the dark for the first three days, and then kept at a 16 h photoperiod with irradiance of 40 µmol·m^{-2}·s^{-1}.

Statistical analyses. For these preliminary experiments, no replication was made. Results were evaluated after 30 days of *in vitro* culture. During these experiments, an embryo was considered to have germinated when both the radicle and the shoot had elongated. *In vitro* germination percentages were compared using Pearson's Chi-squared test of independence at a significance level of $P < 0.05$. Seedling data (shoot and root length, number of leaves and nodes per seedling, biomass production of the aerial part and of the root) were tested using the Kruskal-Wallis one-way analysis of variance.

RESULTS AND DISCUSSION

Effect of seed storage on the viability of isolated embryonic axes

Conservation in ambient conditions significantly affected *P. vera* seed viability. The *in vitro* germination of the embryonic axes isolated from seeds stored for 6 months was 100%, whereas it was 87.3% (48/55) for axes isolated from seeds harvested 18 months earlier and 30.9% (21/68) for 30 months previously. This suggests that beyond one year of storage, *P. vera* embryos gradually lose their ability to germinate. This preliminary result is in agreement with the results of Jacquy (1972), who observed a reduction in the *in vivo* germination of *Pistacia vera* stored seeds. Piotto and Di Noi (2003) reported that *P. lentiscus* seeds could lose their viability 'in a comparatively short space of time' but did not provide any information on *P. vera*. Bani-Aameur and Alouani (1999) also highlighted the effect of storage on seed longevity in a species ecologically close to pistachio: the argan tree (*Argania spinosa* L.). These authors reported that the germination percentage of whole kernels stored at room temperature did not exceed 27%. However, storing *Afzelia africana* seeds, with a natural water content of 0.09 g·g^{-1} DW, under ambient conditions for at least 33 months only slightly reduced their germination rate (Bationo et al. 2001) which was 80% instead of 90% for the freshly collected seeds.

Further experiments are needed to identify the conditions which would allow effective and simple medium-term storage of *Pistacia vera* seeds. This would help seed producers to manage their seed stocks optimally.

For long-term conservation, cryopreservation of embryonic axes is often considered for recalcitrant seeds or for seeds that are difficult to store (Li, Pritchard 2009).

Cryopreservation of embryonic axes

Effect on in vitro germination

Regrowth of embryonic axes was visible as early as on the third day of culture (Fig. 1e). In living explants, three kinds of development were distinguished: germination leading to complete plantlets, abnormal germination (usually without a root) and callus proliferation. Dehydration, at least up to 0.09 g·g^{-1} DW, did not influence the germination of the embryonic axes (Table 1). Mean germination ranged from 85% to 100%. The tolerance of embryonic axes to liquid nitrogen depended on moisture con-

Table 1. *In vitro* germination of embryonic axes of *Pistacia vera* L. after dehydration or dehydration + cryopreservation (n = 20 embryonic axes per treatment)

Duration of dehydration (min)	IWC	FWC	*In vitro* germination*	
	($g \cdot g^{-1}$ DW)		DH	DH + LN
0	1.05	–	85	0
30	1.06	0.42	95	10
60	1.01	0.20	100	90
90	1.05	0.14	100	85
120	1.06	0.09	95	100

IWC – Initial water content; FWC – Final water content after dehydration; DH – dehydration; LN – liquid nitrogen; *an embryo was considered to have germinated when the radicle and the shoot had elongated

tent. In the absence of dehydration (1.05 $g \cdot g^{-1}$ DW), no embryo germinated after freezing. The optimal germination after cryopreservation (95–100%) was observed with a water content between 0.2 and 0.1 $g \cdot g^{-1}$ DW (Table 1, Fig. 1h–j). It should be noted that in our experiment we did not determine the natural tolerance of the embryonic axes to cryopreservation as our seed sterilization and dissection procedures might have hydrated them.

Our optimal results are slightly better than those reported for whole seeds of *Pistacia vera* (Ozden-Tokatli et al. 2007). In their work, even without dehydration, with a natural water content of 0.25 $g \cdot g^{-1}$ DW, 32% of seeds were able to germinate after cryopreservation. However, the germination was increased with a preliminary dehydration step. Dehydration for 4–12 h over silica gel (corresponding to a water content of 0.14 to 0.13 $g \cdot g^{-1}$ DW) produced the best germination 61–90% (Ozden-Tokatli et al. 2007). In *Citrus suhuiensis* (cv. Limau Langkat), after cryopreservation, the survival of embryonic axes was also higher than that of whole seeds (Makeen et al. 2005).

The interaction between the water content of embryonic axes at freezing and their viability has been demonstrated in other species. Dehydration limits the formation of intracellular ice crystals, which are usually lethal, when the explants are exposed to very low temperatures (–196°C). For *Azadirachta indica* embryonic axes, the best results after cryopreservation were obtained with a water content of 0.23 $g \cdot g^{-1}$ DW (Berjak, Dumet 1996). For *Quercus suber*, the optimal water content of the axes (Gonzalez-Benito et al. 2002) for survival after cryo-

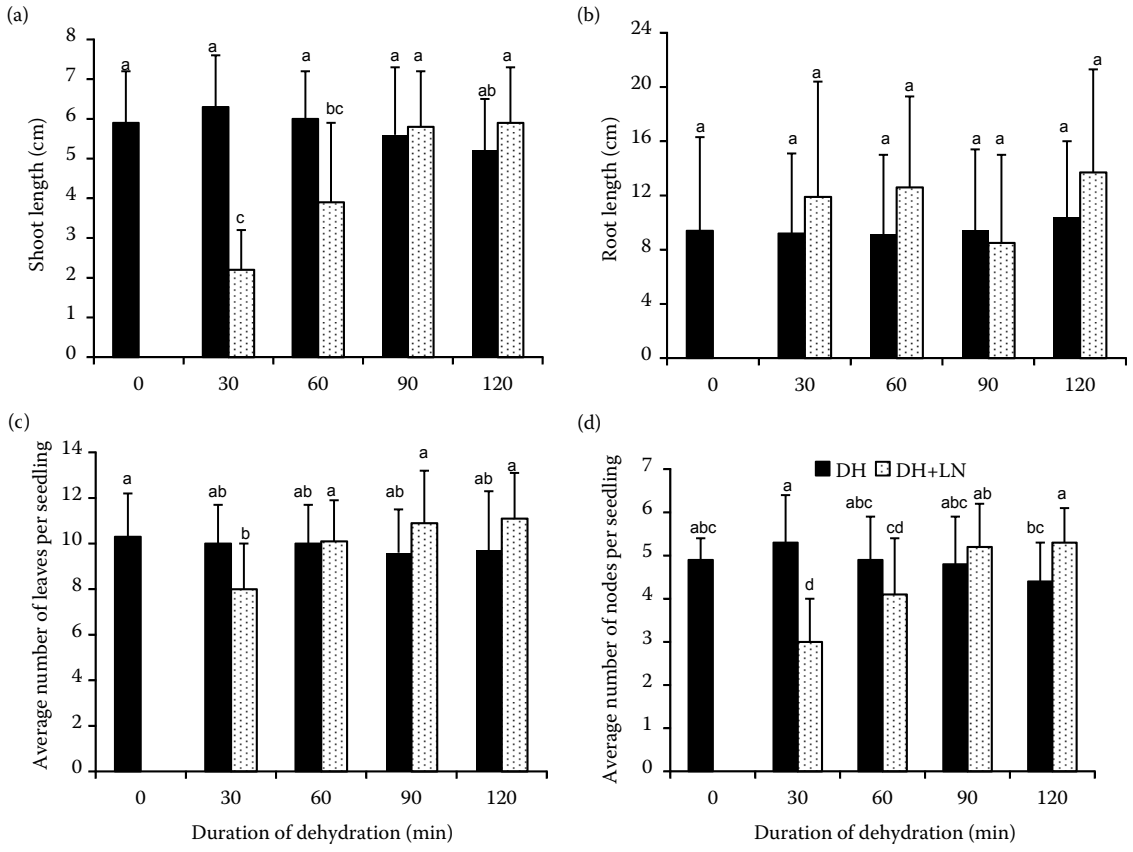

Fig. 2. Effect of the duration of dehydration on the development of *Pistacia vera* seedlings from dehydrated (DH) and dehydrated and cryopreserved (DH + LN) embryonic axes: (a) shoot, (b) root length, (c) average number of leaves, (d) nodes per seedling observations were made after 30 days of culture, bars with the same letter were not significantly different at $P < 0.05$

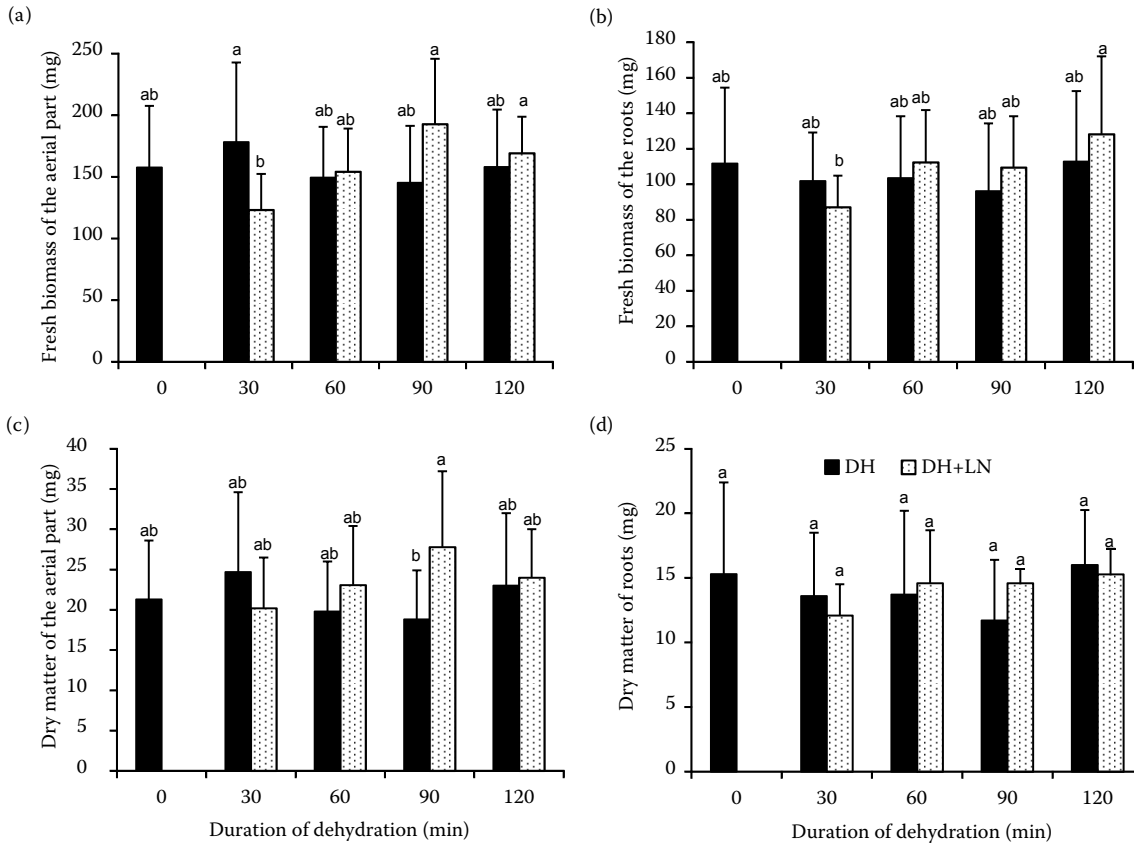

Fig. 3. Effect of the duration of dehydration on biomass production by *Pistacia vera* seedlings grown from dehydrated (DH) and dehydrated and cryopreserved (DH + LN) embryonic axes: (a) fresh biomass of the aerial part, (c) of the root, (b) dry matter of the aerial part, (d) of the root

measurements were made after 30 days of culture; bars with the same letter were not significantly different at $P < 0.05$

preservation was 0.34 g·g^{-1} DW. But the germination rate was low. Cryopreservation of embryonic axes of some woody species is difficult. For butternut (*Juglans cinerea*), 36% of embryos isolated with approximately 3 mm of cotyledonary tissue germinated after exposure to –196°C (Beardmore, Wendy 1998). In some cases, the use of cryoprotective media significantly improved survival after freezing. De Boucaud et al. (1991) showed that a cryoprotective solution containing 5 M 1,2-propanediol and 20% sucrose improved the results obtained in the common walnut (*Juglans regia* L.). Pre-culture in an enriched sugar solution increased the survival of cryopreserved excised embryos of *Zizania texana* (Walters et al. 2002). However, our preliminary work indicated that no such cryoprotective treatments would be necessary to obtain a high germination rate for embryonic axes of *Pistacia vera.*

Effect on in vitro development

In vitro seedlings that developed from embryonic axes dehydrated for up to 120 min (0.09 g·g^{-1} DW water content) were similar to the control: after 30 days of culture, no significant differences were observed either in shoot and root length (Fig. 2) or in biomass production (Fig. 3). However, in the case of cryopreserved embryonic axes, the duration of dehydration affected the seedling development. Shoots were significantly shorter (Fig. 2a) when the embryonic axes were dehydrated for less than 90 min (i.e. water content > 0.14 g·g^{-1} DW). But 60 min of dehydration were sufficient to obtain shoots with a similar number of nodes (and leaves) to the control (Fig. 2c–d). Concerning roots, the shortest dehydration duration tested (30 min) was sufficient to obtain the same growth as that of control embryonic axes (Fig. 2b). Seedlings obtained from cryopreserved axes produced a volume of biomass (fresh and dry matter) equivalent to that produced by controls, whatever the duration of dehydration (Fig. 3).

The quality of the development of cryopreserved *Aesculus hippocastanum* L. embryonic axes was also affected by their water content (Wesley-Smith et al. 2001). Germination was obtained with a water content of less than 1 g·g^{-1} DW, but the highest proportion of normal germination was with a water content between 0.5 and 0.25 g·g^{-1} DW.

CONCLUSIONS

The exact conditions that will enable *Pistacia vera* seeds to remain viable are not yet known. Their degrees of tolerance to high desiccation (water content ≤ 0.05 $g \cdot g^{-1}$ DW) and to storage at low temperatures require further investigation.

Cryopreservation is currently the best technique to ensure the long-term conservation of species with intermediate and recalcitrant seeds. For pistachio, a very simple method, comprising two hours of dehydration followed by direct immersion in liquid nitrogen, was developed for embryonic axes. It ensured 100% recovery with a good qualitative appearance of the cryopreserved plantlets. The replication of this method on several harvests and a range of genotypes should indicate if it is possible to consider the establishment of a cryobank for Algerian *P. vera* L. resources.

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