Pistachio Germplasm Propagation and Conservation

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Contributor: Esmaeil Nezami, Pedro P. Gallego

The word “pstk” [pistag], used in the ancient Persian language, is the linguistic root from which the current name “pistachio”, used worldwide, derives. The word pistachio is generally used to designate the plants and fruits of a single species: *Pistacia vera* L. Both the plant and its fruits have been used by mankind for thousands of years, specifically the consumption of its fruits by Neanderthals has been dated to about 300,000 years ago. Historically, *Pistacia* spp. germplasms were mainly conserved via conventional macropropagation techniques using in situ (in-site in their native place) habitats or even old orchards, and ex situ (off-site) where material is taken away from their native place to germplasm, botanic gardens, and so on. The strategy is to identify superior genotypes and transfer them to collections as well as to maintain them in the wild. Unconventional biological techniques, including cryopreservation (for the long-term), slow-growth storage conditions and synthetic seeds (for medium-term) and micropropagation (for short-term) have opened new insights for preservation of commercial and endangered *Pistacia* species. It should be noted that although pistachio species are not globally endangered, at least 12 species are currently included in the IUCN Red List of Threatened Species: *P. cucphuongensis* (VU, vulnerable), *P. vera*, *P. aethiopica*, *P. mexicana*, *P. atlantica* (NT, near threatened) and the rest are LN (least concern), so it has been imperative to apply preservation policies to these species.

Keywords: Pistacia vera ; plant tissue culture ; rootstocks

1. Conventional Sexual Propagation

Pistachio reproduces naturally by cross-pollination, so the seeds produced will be as genetically variable as wild genetic populations. It is believed that the first plantings of pistachios in Iran, Turkey, Syria and most of other countries in their native region, were started from selecting and germinating the best seeds of wild *Pistacia* spp. [1]. However, the use of the seed method for pistachio propagations is not really suitable for commercially establishment of pistachio orchards, because nuts of wild *Pistacia* spp. are so sensitive to desiccation, as shown by the low germination rates [2]. Additionally, the resulting trees from seedlings are expected to be genetically variable, although they appear to be quite uniform in the field [3]. Most of the old commercial pistachio orchards currently in use in Iran, Turkey and Syria are the result of two forms of genetic selection: (i) selecting wild pistachios of *Pistacia* sp. and their hybrids capable of bearing fruit in only 5–7 years (normally taking 20–25 years), for hundreds of years, and (ii) using conventional asexual propagation systems, such as grafting selected pistachio tree scions onto rootstocks which are also selected for their agronomic characteristics. This has led to the establishment of orchards in a much shorter time [4][5].

2. Conventional Asexual Propagation: Cuttings and Grafting

Domestication of trees using sexual reproduction, by seeds, present some bottlenecks which naturally delay the process because of the need for two compatible parental trees, in the case of dioecious plants as pistachio, or good conditions for wind-pollination, a long period of time before obtaining the next generation of mature adult tree capable of bearing fruit and, finally, propagation through seed germination is not suitable for establishment of clonally elite orchards since seedlings are not true-to-type trees. Alternatively, asexual or vegetative propagation has been used to improve tolerance/resistance to biotic and abiotic stresses and to accelerate pistachio domestication [6]. Among others, cutting [7] and grafting [8] methods for macropropagation of pistachios have been traditionally used [1][9].

2.1. Propagation by Cuttings

Cuttings are applied to all those woody species in which rooting is not very difficult. In this sense, a range of factors have been reported to impact on the rooting percentage of cuttings from woody species in nurseries: genotype, position on the shoot from which the cutting is made, auxin type and concentration, date of shoot collection and so on, which have been reviewed elsewhere in detail [10][11]. In *Pistacia* spp. cuttings were traditionally used for the clonal propagation of selected rootstocks in order to obtain uniform progeny [12] (and rarely on cultivars [13]). However, attempts to propagate pistachio

rootstocks commercially by soft and hard wood cuttings especially using adult materials have given inconsistent results [13][14].

Pioneering studies on pistachio propagation has shown that the use of pistachio hardwood cuttings, obtained from adult trees, have exhibited a very low rooting rate (about 5%), which has been attributed to the fact that adult trees lose their rooting capacity with age [15][16]. In fact, the first experiments carried out with *P. vera* and two clonal rootstocks *P. palestina* and *P. atlantica* rooted with IBA at 10 mg L^{-1} was completely unsuccessful [17]. In the 1980s, softwood, instead of hardwood, cuttings of young (one-year-old) *P. chinensis* trees supplied with the rooting phytohormone Indole butyric Acid (IBA) at 5 mg L^{-1} resulted in 92% rooting [18]. In addition, high rooting percentages (78–100%) were obtained from *P. vera* after dipping the softwood cuttings from seedlings into concentrated 500 and 1000 mg L^{-1} IBA, respectively. Moreover, promising high rooting percentages (88%) of softwood cuttings were obtained using a mist system combined with treatments with IBA at 35 mg L^{-1} [13]. However, only a 50% rooting was achieved if older plant material (four-year-old) was used [19]. Finally, Almehdi and co-workers documented the significant effects of genotype, part of the shoot from which the cutting was taken and shoot collection data on successful rooting percentage (40%) of the rootstock 'UCB1' cuttings, but no effect of IBA concentration and shoot lengths was found [20].

2.2. Propagation by Grafting

Grafting has been used in pistachio domestication as far as 3000–4000 years ago [21][22], being the decisive horticultural technique in the spread of pistachio cultivation from Central Asia to the countries of the Mediterranean basin between 1000 B.C. and 1000 A.D. [23][24]. From a biological point of view, grafting can be defined as the fusion into a single organism of a portion of tissue from one plant with a portion of tissue from another plant, which can occur naturally and artificially [25]. From a horticultural point of view, it is a technique of cloning by vegetative (asexual) artificial propagation of plants in which a portion of tissue (called scion) from a selected cultivar (donor) is attached to another (called stock or rootstock) selected plant for its resistance to biotic and abiotic stresses and serves as the root system of the grafted plant, so that both grow as a single and new organism. Finally, from a genetic point of view, grafting implies the formation of a new organism through the fusion of at least two genotypes, each one maintaining its own genetic identity in the new grafted plant [24].

Grafting has been used in the last 3500 years for various biological and horticultural purposes such as vegetative macropropagation, juvenility avoidance, cultivar change, to repair established adult trees, size-controlling grafted plants, physiological studies, biotic and abiotic stress resistances or identification of asymptomatic viruses [8][26][27][28]. In the case of pistachio, it was traditionally used for vegetative macropropagation and genetic improvement using *P. vera* plants as a scion and wild *Pistacia* spp. as rootstocks, as described above.

2.3. Propagation by Budding

Budding is a special type of grafting, in which a small piece of shoot with a single vegetative bud is cut from the wood of the scion and transferred to the rootstock. To improve the efficiency of budding in pistachio species, a number of factors must be taken into account: the selection of a healthy, active rootstock in optimum condition, with a diameter large enough to adequately accommodate the grafted buds, the quality of the scion wood, the use of the most practical technique for budding (e.g., T-budding in the case of pistachio), compatibility between scion and rootstock, the budding season, and proper aftercare [7][29][30][31].

In general, although several improvements based on those traditional techniques of conventional asexual propagation have been reported, low rooting efficiency, lack of producing uniform plants with a high quality at sufficiently large scales were the most important challenges of pistachio nurseries [30][32], and new strategies for conservation using novel unconventional propagation methods based in plant tissue culture were developed for pistachio.

3. Unconventional Asexual Propagation

3.1. Cryopreservation

Cryopreservation can be classified as a long-term conservation technique based on the methods of cooling and storing biological material at very low temperatures in liquid nitrogen for an unlimited time. Specifically, plant cryopreservation refers to the storage of any plant biological material (cells, tissues, or organs) such as seeds, pollen, shoot tips or dormant buds at an ultra-low temperature in order to decrease by a state of non-division and zero metabolism without any genetic alteration or modification in the plant material for an unlimited period of time [33][34][35]. Recently, Sharrock stated the cryopreservation technique as a basic conservation tool for germplasm conservation in the last Global Strategy for Plant

Conservation Report [36]. General procedures for the cryopreservation technique include [33][37]: (1) cold hardening (4 °C generally), (2) pre-culture with suitable cryoprotectant (such as sucrose), (3) physical dehydration, over silica gel, or chemical dehydration by using highly concentrated plant vitrification solutions (i.e., PVS2), (4) freezing by immersion in liquid nitrogen (−196 °C), (5) rapid thawing at 35–40 °C and (6) washing and re-culture into fresh medium.

In the case of cryopreservation of *Pistacia* germplasm, the former technique was developed successfully for seeds of *P. vera*, *P. lentiscus* and *P. terebinthus* [38] and embryonic axes of *P. vera* [39]; whilst the latter technique was utilized for cryopreservation of the in vitro cultured axillary buds of *P. vera* [40] and [41]. Despite this, problems related to the low efficiency of recovery of cryopreserved plant materials makes the application of long-term conservation for *Pistacia* germplasm conservation costly [42]. Thus, much research remains to be conducted in order to design effective cryopreservation protocols that can be used for a wide range of *Pistacia* species in a more efficient and less costly manner.

3.2. Slow-Growth Storage

Slow-growth storage can be classified as a medium-term conservation technique and consists of maintaining in vitro plant cultures under limited growth conditions, allowing subculture intervals to be extended up to a few months or a few years, without affecting their potential for survival and regeneration after storage [43]. Slow growth protocols require modification of the usual in vitro culture conditions by various means, such as reducing the incubation temperature (but always keeping it above 0 °C) [44], light intensity or even eliminating it completely [45], nutrient concentration in the culture medium and/or supplementation of the culture medium with different osmotic regulators, commonly sugars (sucrose) or alcohols (mannitol or sorbitol) [46] and/or growth retardants (abscisic acid) to minimize the growth of in vitro conserved plants [47][48].

Barghchi, in 1986, pioneered the development of an efficient protocol to increase subcultures interval in pistachio to 18 months by reducing the growth chamber temperature from 26 °C to 4 °C, applying readjustments in photoperiods and light intensity, supplementing the basal culture media with growth retardants such as the phytohormone abscisic acid or the osmotic mannitol at different concentrations [49].

More recently, Kocand co-workers induced cold storage by reducing the temperature of incubation to 4 °C in dark for 2–12 months. They found that during the first 6 months the pistachio plantlets remained green and healthy but after that the occurrence of shoot tip necrosis and death of the shoots in the cold stored plants was found; after 6 months the cold stored plantlets showed more genetic instability than the controls [50]. The studies conducted on slow-growth storage for many plant species are still very limited [51], including *Pistacia* spp. [40], due to almost all species requiring an appropriate conservation strategy.

3.3. Synthetic Seed Techniques

Another unconventional asexual propagation technique considered as medium-term conservation technique is called synthetic seeds or encapsulation, which is a biotechnological tool based on plant tissue culture that has been widely used to propagate, conserve and deliver germplasm of many economically important species [52][53][54]. It consists of artificially constructing synthetic seeds by encapsulating any somatic plant cells, tissue or organs (i.e., somatic embryos, apical or axillary buds, nodal segments, etc.) in gel capsules, forming spherical beads, for conservation [55]. The synthetic seed technology has led to encapsulated, storage and re-growth clonal plant material (alive somatic explants) allowing the possibility of mass production of elite plant species, and even the automatization of this process. Moreover, synthetic seeds show several advantages with respect to conventional propagation systems compared to seeds, cuttings or grafting, because the encapsulated explants upon germination behave like seeds and possess the ability to convert into plantlets under either in vitro or in vivo conditions [56][57]. Although synthetic seeds are a very promising technology, the protocol for the processing of synthetic seeds present several limitations: limited control of somatic embryogenic genesis, developing and maturation and, once encapsulated, the control of seed germination and production of viable explants to be used as plant material. All of these factors need further optimization [58].

In pistachio, the first synthetic seeds were developed using somatic embryos and embryogenic masses in 1996 by Onay and colleagues [59]. Later, pistachio axillary buds were excised from juvenile seeds [41] and shoot tips apices were also encapsulated [40]. Akdemir and co-workers [40] reported that encapsulated shoot apices of *P. vera* cultivars were conserved for up to 12 months at 4 °C in dark conditions with the subsequent recovery of over 90%.

3.4. Micropropagation

The conservation of *Pistacia* spp. germplasm by conventional macropropagation methods has some limitations, such as low rooting efficiency, lack of production of high-quality uniform plants at sufficiently large scales, poor seed viability (after conventional storage), high risk of disease spread, and loss of genetic germplasm, as mentioned above and elsewhere [44][60]. To overcome those limitations, in the last 35 years, several unconventional asexual plant propagation methods based on plant biotechnological advances have been applied for pistachio propagation, including the development of in vitro propagation techniques such as organogenesis [61], somatic embryogenesis [62] and micrografting [19][63][64][65]. These have been widely used for mass propagation and germplasm conservation of *Pistacia* species. There are many good reviews about the principles of in vitro culture techniques; here, only very general definitions and principles, and physiological problems related to micropropagation are summarized.

Micropropagation is an in vitro plant tissue culture technique used to multiply a large number of identical selected plants (clones) from a plant stock in a very short time, at a competitive price and with high survival rates. Micropropagation is also known as true-to-type in vitro propagation. It has been used for multiple purposes, such as providing quality plants in large quantities (mass propagation) to meet horticulture, silviculture, genetic conservation and industrial (food, pharmaceutical, nutraceutical and cosmeceutical) needs [66]. Micropropagation has many advantages over conventional propagation systems; among others, the possibility of a massive multiplication of elite genotypes in a very short time, the rescue of those genotypes in danger of extinction and those others difficult to propagate by conventional techniques; all these advantages are easily applicable to the *Pistacia* genus, as described below [67]. The success or failure of micropropagation methods, regardless of whether they are initiated from apical or axillary meristems, adventitious shoots or somatic embryos, depends critically on the effect of a large number of factors, including genotype, culture conditions and, above all, the composition of the culture medium (mineral nutrients of the culture medium, PGRs and vitamins) [68][69][70][71].

In *Pistacia* spp. most of the micropropagation studies have used Murashige and Skoog (MS) [72] as a basal culture medium for the cultivar *P. vera*; [73][74][75][76][77][78] and for several rootstocks such as *P. khinjuk* [2][79][80] *P. atlantica* [19][79], *P. lentiscus* [81], and, less extensively, other basal culture media designed for woody plants such as DKW [82] for *P. vera* [77] or WPM [83] for *P. vera* [61][63][84][85][86]. However, micropropagation of *Pistacia* sp. has been hampered by a low multiplication rate in addition to some physiological disorders such as basal callus (BC), shoot tip necrosis (STN), hyperhydricity (H), Leaf yellowing (LY), or vascular necrosis (VN), when these three basal culture media have been used [61][85][87][88]. In addition, some common problems to micropropagation such as culture browning (CB), caused by phenolic compounds secretions, and contamination (CO) were detected in pistachio micropropagation. **Table 1** summarizes the main in vitro studies carried out on *P. vera* L. cultivars and on the different rootstocks, describing the main problems and common physiological disorders observed and the solutions applied to alleviate them; however, those readers interested in knowing the specific solution to each problem and disorder can consult them in detail in each of the referenced studies.

Table 1. Main studies on *Pistacia* spp. micropropagation indicating basal culture media employed, physiological disorders such as basal callus (BC), shoot tip necrosis (STN), hyperhydricity (H), Leaf yellowing (LY), and vascular necrosis (VN) and some problems such as culture browning (CB) and contamination (CO), and the solutions proposed to alleviate them.

Species	Type of Culture	Basal Media	Problems and Disorders	Solutions Proposed	Reference
			Cultivars		
***P. vera* L.**	**Shoot tip Nodal buds**	**MS**	**BC, CB, STN**	**1. Use seedling 2. Successive subculture 3. Remove auxins**	**[89]**
***P. vera* L.**	**Shoot tip Nodal buds**	**MS**	**BC, CB, STN, H**	**1. Remove apical buds 2. Successive subculture 3. Activated charcoal or polyvinylpyrrolidone**	**[80]**
***P. vera* L.**	**Seedling shoots**	**WPM**	**H, VN**	**1. Use MS free-vitamin 2. Use WPM**	**[84]**
***P. vera* L.**	**Shoot tips and nodal buds**	**MS**	**STN**	**1. Increasecytokinins 2. Eliminate auxins**	**[90]**
***P. vera* cv. ‘mateur’**	**Shoot tips and nodal buds**	**MS**	**STN**	**1. Use of liquid culture 2. Use borone 3. Increase calcium**	**[85]**

Species	Type of Culture	Basal Media	Problems and Disorders	Solutions Proposed	Reference
P. vera cv. 'mateur'	Shoot tips of grafted seedling	MS	CB, STN, LY, H	1.Successive subcultures 2. Decrease subculture period 3. Decrease BAP	[86]
P. vera cv. 'Antep'	Immature kernels	MS	STN, BC, H	1. Change BAP concentrations 2. Increase Ca^{2+} or BO_3^-	[61]
P. vera L.	In vitro shoots	MS	STN	1. Increase Ca^{2+} (24 mM)	[88]
P. vera cv. mateur	Shoot tips and nodal buds	MS	CB	1. Apply L-cystein 2. Activated charcoal or $AgNO_3$	[91]
P. vera L.	Nodal buds	MS	BC	-	[92]
P. vera L.			STN	1. Increase B or Ca	[93]
P. vera L.	Nodal buds		STN, CB	1. Use of ascorbic, citric acid 2. Increase subcultures 3. Substitute BAP for Metatobolin and Kinetin	[87]
P. vera L.	Shoot tips Nodal buds	MS	STN, H	1. Optimize RITA®® 2. Decrease cytokinin	[79]
			Rootstocks:		
UCB1	Shoot tips of seedlings	DKW	STN, LY, CO	1. Use 2% CO_2 2. Increase light intensity 3. Eliminate carbon 4. Increase level of BO_3 and $Zn(NO_3)_2$	[94]
P. atlantica	Shoot tips and nodal buds	MS	CB	1. Apply L-cystein 2. Activated charcoal or $AgNO_3$	[91]
P. atlantica	Shoot tips and nodal buds	MS	CB	1. Apply L-cystein 2. Activated charcoal or $AgNO_3$	[91]
P. khinjuk	Shoot tips of seedlings	MS	BC	-	[2]
P. khinjuk *P. atlantica*	Shoot tips Nodal buds	MS	STN; H	1. Optimize RITA®® 2. Decrease cytokinins	[79]
UCB1	Shoot tips Nodal buds	MS	BC, STN	1. Readjust iron salts	[95]
UCB1	Shoot tips Nodal buds	MS, POM	BC, STN, LY	1. Readjust mineral nutrients and vitamins	[96]

Recent studies on pistachio in vitro propagation growth response to mineral nutrients of culture media have revealed the key roles of some ion interactions (SO_4^{2-} × Cl^-, K^+ × SO_4^{2-} × $EDTA^-$, and Fe^{2+} × Cu^{2+} × NO_3^-) for shoot quality, proliferation rate, and shoot length, whilst physiological disorders (BC and STN) were found to have been significantly impacted by independent ions as Fe^{2+} and $EDTA^-$, respectively [95]. To alleviate STN of *P. vera* L., some authors have recommended an increase in both the MS boron concentration (up to 100–1000 µM) and calcium concentration (15 mM calcium gluconate [85] or 12–24 mM $CaCl_2{\cdot}2H_2O$ [88]). On the other hand, depletion of MS NH_4NO_3 and $CaCl_2$ (10.33 mM and 1 mM, respectively) and enhancing $FeSO_4{\cdot}7H_2O$ and $Na_2EDTA{\cdot}2H_2O$ (both 0.15 mM) has been recommended to control STN and LY (chlorosis) in *P. vera* cv. 'mateur' [86].

Early studies on the effect of up to 48 µM $AgNO_3$ have demonstrated improved shoot regeneration and growth and strong anti-browning effect on culture media and explants in some *Pistacia* species [91][97]. However later on, media supplemented with $AgNO_3$ at 24 µM caused some deteriorative effects on the proliferation rate of *P. vera* rootstock, although BC was prevented. The most recent studies have revealed the negative effect on some growth parameters of NO_3^-, Mg^{2+}, Ag^+ and $gluconate^-$ at high concentrations [98].

To improve pistachio shoot multiplication, standard culture media vitamins have been replaced with other vitamins [79] or a mixture of them [86][91]. In some pistachio studies [78][79][92], MS-vitamin has been replaced by Gamborg B5-vitamin mixture [99]. Some amino acids, such as L-valine and L-Cystein have been employed by some authors as alternative vitamins during pistachio micropropagation [81][91]. Interestingly, among standard MS-, DKW- or B5-vitamin mixtures, nicotinic-acid

and pyridoxine-HCl were characterized as having a positive and significant effect, improving shoot length and the total healthy fresh weight of pistachio rootstock [98].

Recently, the application of RITA®® as an alternative approach to semi-solid media has enabled researchers to develop efficient protocols for mass-propagation of economically important plant species. In recent years, micropropagation of pistachio buds was improved using RITA®® through immersion of nodal and apical explants for 24 min every 16 h in MS medium containing 4 mg L^{-1} BA and 0.1 mg L^{-1} GA_3 while reducing hyperhydricity and STN symptoms [79]. Therefore, RITA®® could be considered for the mass propagation of pistachio and its rootstocks.

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