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Use of Biotechnology for Preserving Rare Fruit Germplasm

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ABSTRACT

The application of recent biotechnological tools for conservation of rare fruit species from developing countries, including *in vitro* and hydroponic culture protocols, improved propagation techniques and molecular marker application, is described. Promising propagation methods include forcing germination of seeds, *in-vitro* protocols well adapted to these rare fruit species that allow the introduction, micropropagation and rooting of plant material, and developing hydroponic culture protocols that allow the early propagation of high-risk genotypes. In addition, the growth of seedlings in controlled environmental conditions in greenhouse and cold chamber provides a useful strategy for obtaining vigorously growing plants from seeds year round. A standard karyotyping protocol has been described working in several species as preliminary tool to start molecular (DNA) studies. In addition, different protocols for DNA isolation and quantification have been assayed in these rare fruit species. Molecular markers based on PCR amplification of the DNA have also become an essential tool for the characterization and conservation of these species. Regarding this PCR amplification of the DNA, two main strategies, RAPD (if the DNA sequence is unknown) and SSR markers (if the DNA sequence is known), have been assayed. These markers have been applied in the genetic characterization of this germplasm, the establishment of genetic relationships between cultivars and species, and the future construction of genetic maps of these rare fruit species. Additional advantages encouraging the utilization of these new technologies in breeding programs include the high levels of synteny between genomes of related species, and a well-established international network of cooperation among researchers.

Keywords: argan, breeding programs, date palm, *in vitro* culture, molecular markers, propagation techniques, *Prunus*, wild almond
Abbreviations: **ABA**, abcisic acid; **BAP**, benzyl amino purine; **DNA**, deoxyribonucleic acid; **GA**; giberellic acid; **H_2O_2**, hydrogen peroxide; **IBA**, indole-3-butyric acid; **L.**, Linneaus; **MES**, 2-(*N*-morpholino) ethanesulonic acid; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **PCR**, polymerase chain reaction; **PPV**, Plum pox virus; **RAPD**, random amplified polymorphic DNA; **SSR**, simple sequence repeat

CONTENTS

RARE FRUIT GERMPLASM AROUND THE WORLD

Human species has used more than 7,000 edible plant species during the around of 8,000 years of history of the agriculture. However, agricultural research is concentrated on only few crops. This narrowing agricultural base is limiting livelihood options for the poor. On the other side we can include the "underutilized plant species", an enormous range of plants that have potential to contribute to food and nutritional security and poverty alleviation. The chronic food shortages present in many developing countries demonstrate clearly the fragility of food security systems based on a few staples. Agrobiodiversity is a necessity for the survival of poor communities and the stabilization of agroecosystems (IPGRI 2006a, 2006b, 2006c). In side this group of "underutilized plant species" we can also include the rare fruit species present in the developing countries. This rare fruit germplasm have a vital role to play not yet fully exploited (IPGRI 1996). Many of these species are particularly useful in marginal lands, where they have been selected to

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Invited Review

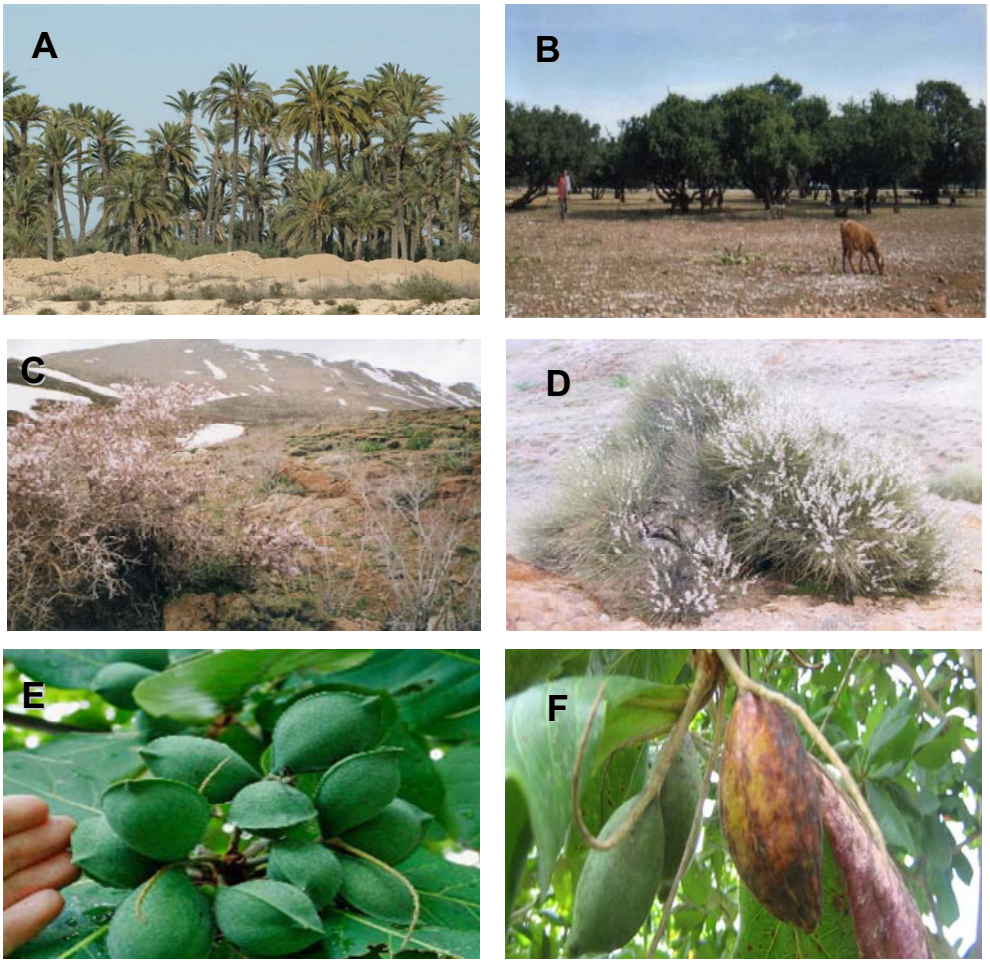

Fig. 1 Rare fruit germplasm from developing countries. Date palm tree in Marrakech (**A**) and Argan tree in the north of Essaouira city (**B**) (Southwest of Morocco). Wild almond species *P. hauskenchtii* (**C**) and *P. scoparia* (**D**) endemic to Irano-Afghan plate and collected in the province of Esfahan (Central Iran). Fruit (**E**) and seed (**D**) of Indian almond.

withstand stress conditions and where they contribute to sustainable production with few imputs. However, because they have been neglected by researchers in the past not enough is known about their agronomy and yield improvement potential or how to best take advantages of their inherent qualities (IPGRI 2006a; 2006b; 2006c). These rare fruit species from developing countries include date palm (*Phoenix dactylifera* L.) and argan tree [*Argania spinosa* (L.) Skeels (synonyms: *Argania sideroxylon* Roem. & Schult., *Sideroxylon spinosum* L.)] from North of Africa, wild almond (*Prunus*) species from South East of Asia, and white sapote (*Casimora edulis* L.) and Indian almond (*Terminalia catappa* L.) from Central and South America.

Date palm is a perennial dioecious monocotyledon being one of the most important fruit trees in Morocco (**Fig. 1A**). Unfortunately, it suffers from a vascular fusariosis (bayoud disease) that had destroyed more than two thirds of the Moroccan date palm grove. This disease represents the main limiting factor for the growth of this species. All the best Moroccan cultivars of date palm are susceptible to this disease. Searching for new resistant genotypes, with good fruit quality, constitute the main way to fight against bayoud disease (Zaid and Arias-Jemenes 2002). Breeding programs are confronted by several problems including the sex of the progeny, fruit quality and resistance to bayoud disease. All these traits seem to be governed by multigenic traits. However, it is necessary to note the absence of markers related to theses traits. A few studies dealing with isoenzymatic characterisation of dates palm germplasm have been published until now (Baaziz *et al*. 1996; Bendiab *et al*. 1998; Majourhat *et al*. 1999; Azeqour *et al*. 2002; Majourhat *et al*. 2002) but the efficiency was not totally accurate in relation the above cited problems. Other studies on germplasm identification and phylogeny have been published (Sedra *et al*. 1998; Trifi *et al*. 2000; Diaz *et al*. 2003; Billote *et al*. 2004), which could be of great help for cultivar identification, pedigree analysis, germplasm diversity as well as genetic mapping studies. In addition, the development of these markers can open up possibilities to future breeding programs assisted by molecular markers.

On the other hand, argan tree belonging to the *Sideroxyleae* tribe is the only representative species of the tropical family *Sapotaceae* in Morocco (**Fig. 1B**). *Argania* is a monospecific genus with *A. spinosa* as the unique representative (Pennington 1991). *Argan* is an endemic species to Southwest Morocco where it occupies a wide distribution area (800,000 ha). It plays great socio-economical and ecological roles in these arid and semi-arid zones. Indeed, thanks mainly to the prised oil extracted from its kernels and its foliage that feed cheep and goat herds, 90% of the local population incomes depend on an agroforestry system based on this multiuse tree (Benchkroun 1990). However, a great zoo-anthropologic action led to the absence of natural regeneration of this species, threatening it with extinction (Benchekroun and Buttoud 1989). To protect argan groves from present overexploitation, it was classified in 1998 as a "Biosphere reserve" by UNESCO, and many efforts were undertaken by the Moroccan government for its rehabilitation. This species is genetically and cytogenetically poorly known. Data available about these topics are scarce and controversial. Johnson (1991), in his review about the cytology of *Sapotaceae*, reported that the basic chromosome number of tribe *Sideroxyleae*, to which argan tree belongs, is suggested to be x = 11, whereas, the most common chromosome number in *Sapotaceae* family is x = 13 (with 39 counts) followed by x = 12 and 14 (with 27 and 25 counts respectively). This author also reported that polyploidy in this family is rare with only 5 counts mainly occurring in the tribe *Chrysophylaceae*. However, previous work on the cytology of *A. spinosa* failed to establish a common chromosome number. In fact, Miege in 1954 reported a chromosome number of 20 in somatic cells, whereas Humphries *et al*. (1978), in meiotic cells reported 24. Such information is of great interest for the advance of genetic studies and to identify numerical and structural abnormalities during tissue culture protocols and selection programs in this species. In

addition, this information deals with physiological process in plants including plant morphology and fertility (Pegtel 1999; Pagliarini 2000; Kermani *et al*. 2003).

Almond [*P. dulcis* (Mill.) D.A. Webb or *P. amygdalus* (L.) Batsch] is a species of genus *Prunus* subgenus *Amygdalus* (*Rosaceae*, subfamily *Prunoideae*) that is comercially grown world-wide. This species originated in Central Asia was dispersed through cold and xeric environments in the mountainous areas and deserts of western China, Kurdistan, Turkestan, Afghanistan and Iran (Watkins 1976). Different wild *Prunus* species close to almond have been described in these mountainous areas (Kester and Gradziel 1996) (**Fig. 1C, 1D**). These wild almond species play a socio-economical and ecological role. In fact, they have been used for different purpose by native people including direct consumption, grazing of livestock, or oil extraction. On the other hand, the direct utilization of these related *Prunus* species as a rootstock for peach and almond, mainly under non-irrigated native conditions, has been reported by some authors (Grasselly 1976; Denisov 1988). Interspecific crosses (peach x almond, peach x *P. davidiana*, and *P. webbii* x peach) have also been used as peach and almond rootstocks (Kester and Hansen 1966). The introduction of genes from these related species through interspecific hybridization has been used in breeding programs to develop better-adapted cultivars (Gradziel *et al*. 2001).

White sapote is a medium-.sized evergreen tree native to the highlands of Mexico and Central America with medium to large fruits with a thin green –yellow skin and creamy white or yellow fine-textured sweet flesh. Despite the high productivity of the tree and the availability of selected clones, commercial cultivation is limited to small scale populations (Chambers 1984; Morton 1987). In addition, Indian almond is another well known fruit species from South America with very interesting seed properties (**Fig. 1E, 1F**). This species has been grown by natives because of their interesting oil seed (Morton 1985).

This work offers an overview of the current biotechnological approaches being developed to optimize the conservation of rare fruit species from developing countries including different species from developing countries. These approaches include improved propagation techniques (forcing germination of seeds, *in vitro* culture, hydroponic culture, and artificial cycle of growth) and molecular marker (RAPDs and SSRs) application.

CONSERVATION AND PROPAGATION TECHNIQUES

During the last decades conservation of genetic resources becomes the most important subject in the control of food security problems. Strategies commonly used to preserve plant genetic resources include *in situ* and *ex situ* conservation strategies. The first refers to conservation of a population species on site within the area of natural occurrence, allowing the continuation of the evolution, whereas *ex situ* conservation involves the conservation of a species off-site, in gene banks or botanical gardens providing a higher degree of protection to germplasm. On the other hand, propagation techniques constitute an important component for conservation strategies. Developing adequate protocols for sexual and/or vegetative plant multiplication is also a first prerequisite in conservation programs (IPGRI 1996; Phartyal *et al*. 2002).

Seed banks and forcing germination of seeds in *in vivo* and *in vitro* conditions

Seeds are the most convenient form to store and distribute plant germplasm in herbaceous species and in a less degree also in fruit species. An ideal condition to prolong the seed longevity is mainly dependent on seed moisture content, temperature and type of container used during storage. The optimum stage of seed maturity, seed-lot quality, their processing and harvesting techniques, germination eco-physiology and degree of dormancy too play a crucial role in maintaining seed longevity that need to be considered before large-scale seed storage is initiated. Over the years, techniques for conserving orthodox seeds for several decades have been developed. These involve drying seed to low moisture content (3-7% fresh weight, depending on the species) and storing them, in hermetically-sealed containers, at low temperature, preferably at -18ºC or cooler (IPGRI 1994, 1996). These procedures have been widely adopted by seed banks worldwide. Seed bank constitute a kind of *ex situ* conservation of endangered species. The high-priority stored gene pool collected in genebanks under appropriate condition will be not only used for the large-scale practices of artificial regeneration, but also to the growth of the agroforestry, social forestry, commercial nursery operation, watershed management and restoration of degraded areas.

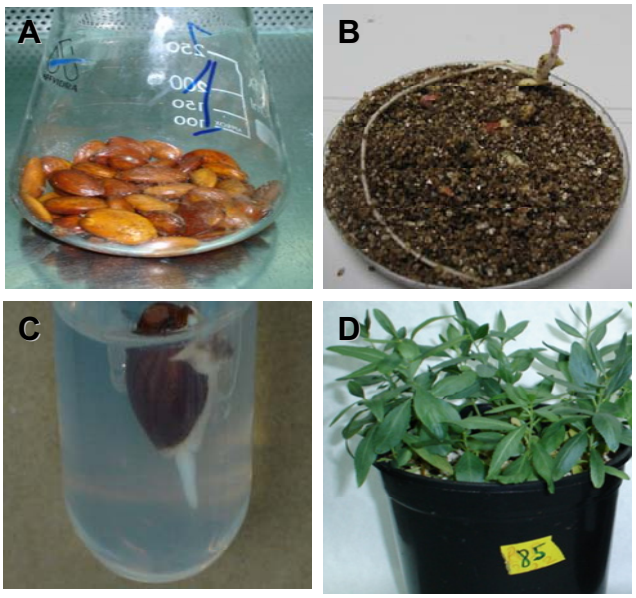

Fig. 2 Forcing germination of seeds from wild almond species. Preparation of seeds (**A**), assays *in vivo* (stratification) (**B**) and *in vitro* (**B**) conditions and the growth of the seedlings after transplantation in pots (**C**).

On the other hand, seed dormancy is an adaptative mechanism well known in temperate fruit species to protect the progeny from freeze damage during the winter (Grigorian 1972; García-Gusano *et al*. 2004). A seed which is dormant will not germinate even when the environment (e.g. water, oxygen, temperature) is adequate for germination (Martínez-Gómez and Dicenta 2001). For example, germination requirements of *Prunus* species vary depending on the species and local environment. Seed dormancy in species of this genera is caused by two mechanisms: an external (physical and hormonal) mechanism which inhibits seed germination and is controlled by the endocarp and the seed coat (maternal tissue) (Mehanna and Martin 1985; Martínez-Gómez and Dicenta 2001), and other internal (genetic and hormonal) mechanism controlled by the embryo (Mehanna and Martin 1985; Martínez-Gómez and Dicenta 2001; García-Gusano *et al*. 2005) which affects later growth of seedlings (Mehanna *et al*. 1985; Seeley *et al*. 1998; García-Gusano *et al*. 2004). The latter mechanism is the most common type of dormancy in plant seeds of temperate regions and it is essentially governed by antagonistic roles of abscisic acid (ABA) and gibberillins (Gas) that are influenced by environmental factors such temperature and light (Hazebroek *et al*. 1993; Debeaujon *et al*. 2000; Koornneef *et al*. 2002). Breaking these mechanisms is necessary for the completion of seed germination. In this sense, a pretreatment of dormant seeds in order to forcing germination in controlled conditions could avoid the prolonged duration of seeds in germination beds in nurseries.

Several treatments are being used to eliminate seed dormancy in *in vivo* conditions (**Fig. 2A**) such as scarification, stratification, gibberillic acid (GA_3) and hydrogen peroxide

(H_2O_2), etc. Stratification has been the method traditionally used to break seed dormancy in *Prunus* species (Zigas and Coombe 1977; Mehanna *et al*. 1985; Frisby and Seeley 1993; Seeley *et al*. 1998). In addition, application in *in vitro* conditions of GAs (**Fig. 2B**) can promote germination of dormant seeds. It suggests that environmental factors that overcome seed dormancy may be mediated by GA biosynthesis during the early phases of germination (Hilhorst and Karssen 1992). Instead, seed from GA-deficient mutants of some plants can not germinate in the absence of exogenous GA (Koornneef and Van Der Veen 1980; Groot and Karssen 1987; Rogis *et al*. 2004; Calvo *et al*. (2004)). Exogenous application of H_2O_2 (concentration between 0.5 and 1%) ameliorated seed germination in plants (Chien and Lin 1994; Fontaine *et al*. 1994). On the other hand, the effect of carbon dioxide in seed germination is opposite to that of oxygen. Many studies stated that increasing the concentration of CO_2 beyond that of surrounding air decrease the seed germination of most plant species (Arya 2005).

The exogenous application of H_2O_2 or stimulation of its synthesis by catalase inhibitors in *in vivo* conditions promote the germination of dormant seeds of many species (Reuzeau and Cavalie 1995; Ogawa and Iwabuchi 2001). Indeed, it was used both as sterilizing and forcing agent to improve the germination of lot of herbaceous and woody species (Buchanan *et al*. 1994; Chien and Lin 1994; Fontaine *et al*. 1994). Stratification and GA_3 treatment was also previously proven to be efficient in breaking dormancy of dormant seed due to its antagonistic effect against ABA (Bhargava 1997; Chung and Pack 2003; Kucera *et al*. 2005). In fact, the treatment with this growth regulator shows an important increase in seed germination percentage of *Tripsacum dactyloides* (Rogis *et al*. 2004). In addition, Calvo *et al*. (2004) observed a drastic increase in *Fagus sylvatica* seed germination when treated with GA_3.

We can conclude that stratification (chilling) treatments *in vivo* have been the most efficient method to break dormancy in the studied wild *Prunus* species in comparison with hormonal treatment in *in vitro* conditions (Mehanna *et al*. 1985; Seeley *et al*. 1998; Martínez-Gómez and Dicenta 2001; García-Gusano *et al*. 2004, 2005). However, recent results indicate that a combination of stratification and a pre-treatment with H_2O_2 *in vivo* reduced the germination time and promoted germination percentage in wild almond species (Zeinalabedini *et al*. subm.) (**Fig. 2A, 2C**). These results have been also observed in other species. For example, a clear effect of H_2O_2 and GA_3 pretreatments was observed in argan seed germination (data not publish). In addition, it was demonstrated that the main obstacle for argan seed germination is physical, and not physiological in nature. In fact, scarifying seed drastically increased their germination percentage and germination speed.

In vitro culture and micropropagation, and tissue cryconservation

Beside sexual and conventional vegetative propagation techniques *in vitro* tissue culture provide complementary protocols for rare fruit species propagation conservation. There are used mainly when problems are encountered with sexual propagation (seeds not available, problemes with germination). Existing meristematic material is the explant of choice for vegetative propagation of rare plant and endangered species. *In vitro* culture can have major advantages over conventional propagation techniques in the management of some of these species. Rapid multiplication under controlled, pathogen-free conditions can be achieved by the inclusion of plant growth regulators or hormones in culture media. The possibility of growing plants and even isolated plant cells in a test-tube under controlled *in vitro* conditions offers unique opportunities for micropropagation and multiplication. Advantages include minimizing environment influences, the potential to handle large numbers of individuals in a very small space, and accelerated growth and development (**Fig. 3A**).

Fig. 3 Propagation techniques assayed. Detail of the *in vitro* culture of different ecotypes of argan tree (**A**). Hydroponic culture of date palm (**B**). Over view of the greenhouse (**C**) for the controlled growth of tree species and the cold chamber (**D**) used in the artificial break dormancy of these species.

For fruit species, tissue culture propagation has progressed rapidly during the last years. The application of tissue culture techniques as alternative propagation methods has been reported as early as the 1960s. Initially, tissue culture has involved micropropagation and somatic embryogenesis. Axillary shoot production (meristem culture) is the system most frequently utilized to regenerate plantlets by micropropagation techniques (Hammerschlag 1986, Bornman 1993). Tissue culture and multiplication has numerous potential uses for temperate fruit and nut tree species, including propagation of rootstocks, own-rooted scion cultivars, virus-free stock plants, and elite genotypes (Negueroles and Jones 1979, Hutchinson, 1987).

In our recent experiments efficient *in-vitro* protocols well adapted to rare fruit germplasm such as argan tree species have been developed allowing the introduction, multiplication and elongation of plant material (Majourhat *et al*. 2007a) (**Table 1**). These results open the possibilities of more efficient conservation strategies for this rare germplasm. The experiments undertaken until now show that argan explants have great plasticity regarding the mineral composition of the media during the multiplication phase. Nevertheless during the elongation phase Murashige and Skoog (1962) MS medium gives better results than the others. At the moment, efforts are oriented to setting up an efficient rooting protocol to complete the cycle of *in vitro* culture and micropropagation of argan species. Preliminary experiments concerning rooting medium shows that NAA at concentrations between 0.1 and 1 mg/l give good results (**Fig. 3A**).

On the other hand, Wenzel and Foroughi-Wehr (1993) reported the utilization in herbaceous species of *in vitro* callus culture for the selection of resistance to environmental stress (freezing tolerance in wheat, salt tolerance in rice)

Table 1 Mediums assayed for *in vitro* argan propagation.

Seed germination		**Introduction**	
MS macro and micronutrients		MS including vitamins	
Sucrose	20 g	Sucrose	30 g
GA_3	0.05 mg	BAP	3 mg
Agar	8 g	NAA	0.100 mg
		MES	0.200 g
		L-glutamine	0.120 g
		Sequestrene	0.010 g
		Agar	8 g

Water qsp 1 litre. pH of media was adjusted to 5.8 before sterilization

Multiplication		**Elongation**	
Quoirin and Lepoivre including vitamins		MS including vitamins	
Sucrose	30 g	Sucrose	30 g
BAP	1 mg	BAP	3 mg
IBA	0.01 mg	GA_3	0.075 mg
Sequestrene	15 mg	L-glutamine	0.120 g
Agar	8 g	Sequestrene	0.010 g
		Agar	8 g

Water qsp 1 litre. pH of media was adjusted to 5.8 before sterilization

and diseases (*Phytophthora* and *Fusarium* spp.), and herbicide tolerance in tobacco. The application of *in vitro* culture techniques for the selection of horticultural characters, however, may be more difficult in the case of fruit species. Applications have been reported in *Prunus* for the evaluation of the compatibility between cultivar and rootstock (Jonard 1986), the resistance to abiotic stress (Datée and Branchard 1986), and the resistance to biotic stress (Martínez-Gómez and Dicenta 2000).

The increasing availability of other biotechnological techniques (biochemical markers, DNA analysis, transient reporter transgene expression, genetic transformation, etc.) further complements *in vitro* culture opportunities. Research in somatic embryogenesis has recently increased in anticipation of more widespread attempts at genetic transformation (Singh and Sansavini 1998). For fruit species, tissue culture and micropropagation has progressed rapidly during the last years and it has opened new perspectives for genetic transformation (Petri and Burgos 2005). *In vitro* regeneration and transformation systems using mature plant material of woody fruit species have to be achieved as a necessary requirement for the introduction of useful genes into specific cultivars and the rapid evaluation of resulting horticultural traits. Although the commercial production of transgenic annual crops is a reality, commercial genetically-engineered fruit trees are still far from common in wide spread cultivated species and with more reason in the case of rare fruit species from developing countries. In most of these woody fruit species, as argan, transformation and regeneration of commercial cultivars are not investigated.

Finally, *in vitro* cryopreservation of clonal materials at ultra low temperatures (e.g. liquid nitrogen) offers several advantages including indefinite storage life with reduced space requirements, and freedom from labor-intensive subculturing and other maintenance tasks. It consists of suspending all metabolic processes until the revival of the tissue. To ovoid freeze injuries caused by intracellular ice crystals formation different techniques were developed to allow tissue acquiring cold hardiness. The two steps (or slow cooling) method is the must used technique. It consists of a preconditioning of the tissue in presence of low amount of cryoprotectant and a progressive cooling before immersion in liquid nitrogen (Ryynanen and Haggman 2001; Ryynanen *et al*. 2002; Harvengt *et al*. 2004). Other methods are used like vitrification (or rapid cooling) and encapsulation/dehydration (Engelmann 1997; Blakesley *et al*. 1996). The later methods are bases on controlled application of concentrated cryoprotectants (e.g. sorbitol, glycerol, polyethylenglycol, sucrose, etc.) that facilitate tissue desiccation at nonfreezing temperatures, leading to increased viscosity of the cytosol and, upon rapid cooling in liquid nitrogen. Cryoconservation of leaf, petiole, dormant buds, stem pieces, shoot-tips or somatic embryos was investigated as a means for long-term maintenance of germplasm of rare and endangered species. *Grevillea scapigera* an endangered species from Western Australia was conserved liquid nitrogen using four week old *in vitro* shoot tips as explants. The cryopreserved material was able to generate new shoot from callus that was successfully transferred to soil. However the problem encountered here is the low the low survival of thawed explants (Touchell and Dixon 1992).

Hydroponic culture and artificial growth cycle

Hydroponic plant culture offers a precise control of the root-zone environment in plants (**Fig. 3B**). Setting up hydroponic culture includes: the development of a unique nutrient solution recipe for specific crops; the development of procedures to control nutrients in recirculating hydroponic culture; the effect of ammonium/nitrate ratios on growth; the testing of organic buffers to stabilize pH. In our experiment efficient hydroponic culture protocols that allow the early propagation of high-risk genotypes have been developed in date palm (Martínez-Gómez *et al*. 2007a) (**Table 2**). Such a protocol is of great interest in the ecophysiolocal studies like salt and water stress under controlled conditions and could be applied to other rare fruit species as Indian almond or white sapote.

On the other hand, hydroponic culture has been assayed in fruits for commercial purpose in the case of fig. Kamawat *et al*. (2002) concluded that fig tree grown hydroponically could be forced to yield double crops of high quality fruit. In this sense, Sannomaru *et al*. (1998) studied the effects of hydroponic culture and Ca-solution spray to tree on the enzymatic browning of apple fruits in comparison with the conventional soil culture fruit with positive results.

Finally, experimental strategies using hydroponic culture have been also used to understand developmental and biochemical changes resulting from iron deficiency in several deciduous fruit tree species if they are able to originate suitable indicators. Physiological and morphological data observed in an experimental model indicated diverse syndromes and behaviors among the genotypes, probably reflecting proper perception, regulation, and activation of a biological system to iron deficiency and bicarbonate (Cinelli *et al*. 2003).

On the other hand, the growth of woody species from temperate climates requires a periodic endodormancy, which can be artificially achieved through controlled rest periods in cold chambers (Martínez-Gómez *et al*. 2000a). In our experiments using temperate fruits including wild and cultivated *Prunus* species, two cycles of vegetative growth per year can be carried out by employing two 4-month periods of growth in the greenhouse (**Fig. 3C**) and two 2-month periods of rest in cold chambers (**Fig. 3D**).

The size of the pots, the periodic renewal of the soil and the control of mites and fungus are key considerations for the successful management of almond seedlings under these controlled conditions. The cold treatments can also be used to control fungi and mites. Seedlings, properly maintained under these conditions, can provide vigorously growing plant tissue (leaves, root tips, etc.) throughout the year. Artificial cycles of growth have also been used to in-

Table 2 Nutritive solution for hydroponic culture of date palm seedling.

Macronutrients (ml/l)	
KNO_3 (1M)	3
$Ca(NO_3).4H_2O$ (1 M)	2
KH_2PO_4 (1M)	0.5
$MgSO4. 7H_2O$ (1 M)	0.5
Fe-EDTA (186 g/L)	0.5
Micronutrients (g/l)	
KCl (50 mM)	3.786
H_2BO_3 (32 mM)	2.002
$MnSO_4.H_2O$ (18 mM)	3.076
$CuSO_4.5H_2O$ (0.3 mM)	0.078
MoO_4 (NH4)$6.4H_2O$ (75 μM)	0.092

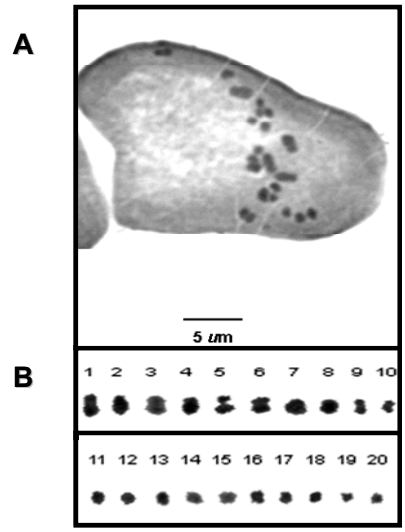

Fig. 4 Karyotypes studies in argan tree. Metaphasic plaque (**A**) and karyotype (**B**) of argan tree.

vigorate weak genetic material in different *Prunus* species (Martínez-Gómez *et al*. 2003a).

This approach has been used in virus resistance studies, as well as to maintain quarantine conditions (e.g. in the study of dangerous viruses such as the *Plum pox virus* (PPV) causing sharka disease; Martínez-Gómez *et al*. 2000b). Artificial cycles of growth have also been used to invigorate weak genetic material such as the aneuploid seedlings of almond (Martínez-Gómez and Gradziel 2003). Similarly, artificial cold treatments have been successfully utilized in studies of frost tolerance in stone fruit flowers (Pedryc *et al*. 1999).

APPLICATION OF KARYOTYPE STUDIES IN THE CYTOLOGICAL CHARACTERIZATION

Chromosome studies are an important prerequisite for fruit genetic and breeding studies (Lespinasse *et al*. 1976; Schuster 1996). These techniques have been used in fruits in the study of interspecific crosses (Salesses and Bonherta 1993), the characterization of germplasm with an unknown ploidy level (Hesse 1971; Toyama 1974), and the establishment of taxonomic and phylogenetic relationships (Singh *et al*. 1984; Soodan *et al*. 1988).

Prunus fruit species are for example characterized by small chromosomes that are difficult to karyotype (Hesse 1971; Oginuma 1987; Salesses and Bonherta 1993; Schuster 1996). In these species, chromosome studies by slide preparation and staining have been reported mainly from apricot, peach and almond (Jelenkovic and Harrington 1972; Kliphuis and Barkoudah 1977; Medeira and Warden 1986; Salesses and Bonherta 1993).

Our results indicated that the following protocol was successfully applied in different fruit species as almond, wild *Prunus* species or argan (Martínez-Gómez *et al*. 2005; Majourhat *et al*. 2007b) and could be applied to other rare fruit species as Indian almond or white sapote. Samples are first placed in cold water for 4 hrs at 0°C, and later treated with 0.2% colchicine for 3 hrs at 5°C; colchicine treatment effectively stopped cell mitosis. Samples are fixed for 24 hrs at 5°C in [methanol, propionic acid, chloroform (6:3:2)] and then stored in 70% ethanol. Samples are then hydrolyzed in 1N HCl at 60°C for 20 min. Longer periods produce an excessive breaking of cells. Finally, staining of samples is performed in acetic acid/orcein 45% for 2 hrs.

In the case of argan, the diploid number determined in the somatic cells from the argan root tips analyzed was of $2n = 20$ (**Fig. 4**). Our result confirms the information reported previously by Miège (1954) but differs from that of Humphries *et al*. (1978). The samples analyzed showed a karyotype constituted for ten pairs of chromosomes and the putative karyotype proposed has been of four sub-metacentric and six metacentric pairs. The four sub-metacentric pairs were the longest with a mean total length between 1.14 and 1.69 µm and the total length of six metacentric pairs were between 0.59 and 1.03 µm (Majourhat *et al*. 2007b).

APPLICATION OF MOLECULAR MARKERS IN THE GENETIC CHARACTERIZATION

One of the goals of rare fruit conservation programmes is to maintain existing level of genetic variation and genetic resources in rare and threatened species. Traditionally, characterization and identification of variability in fruit species and cultivars has been based on morphological and physiological traits. However, such traits are not always available for analysis, and are affected by changing environmental conditions. Molecular marker technology offers several advantages over the sole use of conventional markers and have become an essential tool for the study and conservation of the fruit species. Several types of molecular markers, including isoenzymes, RFLPs, or AFLPs are being employed for the genetic characterization of germplasm, the establishment of genetic relationships between cultivars and species, and the future construction of genetic maps of these rare fruit species. However, the more recent utilization of PCR-based markers has increased the opportunities for molecular characterization and mapping of populations in a wider range of species with unknown DNA sequences (using Random amplified polymorphic DNA, RAPDs), and known DNA sequences (using simple sequence repeat, SSRs, microsatellites).

Studies of germplasm diversity and genetic relationships using molecular markers can be applied to assess the value of these wild species in cultivar development (Martínez-Gómez *et al*. 2007b). Early studies of *Prunus* phylogeny, for example, involved morphological markers (Zhang 1992; Browicz and Zohary 1996), isozymes (Mowrey and Werner 1990) and restriction fragment analysis of chloroplast DNA (Uematsu *et al*. 1991; Badenes and Parfitt 1995). More recent studies analyzed variation in DNA sequences of internal transcribed spacers (ITS) in nuclear or chloroplast DNA (Bortiri *et al*. 2001; Lee and Wen 2001; Potter *et al*. 2002; Bortiri *et al*. 2006). However, more recently, Simple sequence repeat (SSR) markers (microsatellites) generated in different *Prunus* species have also been reported and widely used. Molecular markers developed for fruit also offer a powerful tool to study the evolution of the genome, and for understanding of genome structure and determinants of genetic diversity (Wünsch and Hormaza 2002).

DNA extraction

For developing countries the main obstacle for molecular marker use is the cost of the routinely used products. This situation is the same for the different part of the process, DNA extraction, DNA multiplication (if you are using a PCR based strategy), and amplified DNA electrophoresis.

Looking for a rapid and cheap DNA minipreparation protocol, we assayed several protocols for DNA extraction and quantification (**Fig 5**). A cheap protocol based on CTAB or SDS and using few amount of plants material (40 mg) was optimized.Total genomic DNA was isolated using the CTAB procedure described by Doyle and Doyle (1987) and the DNA isolation procedure described by Gepts and Clegg (1989) with minor variations using SDS extraction buffer.

Our results indicated that DNA average yield, range from 1 to 1.5 µg/mg of fresh leaf tissue and 260/280 ratio range from 1.61 to 1.85. Even without using liquid nitrogen this extraction protocol gave rise to high yield and quality DNA from different species of great economical and ecological interest. DNA extracted with this protocol was useful for RAPD-PCR reaction (**Fig. 6**). However, using Qiagen Kit, we were not able to extract DNA with high molecular weight and quality from wild *Prunus* and *Argania* species

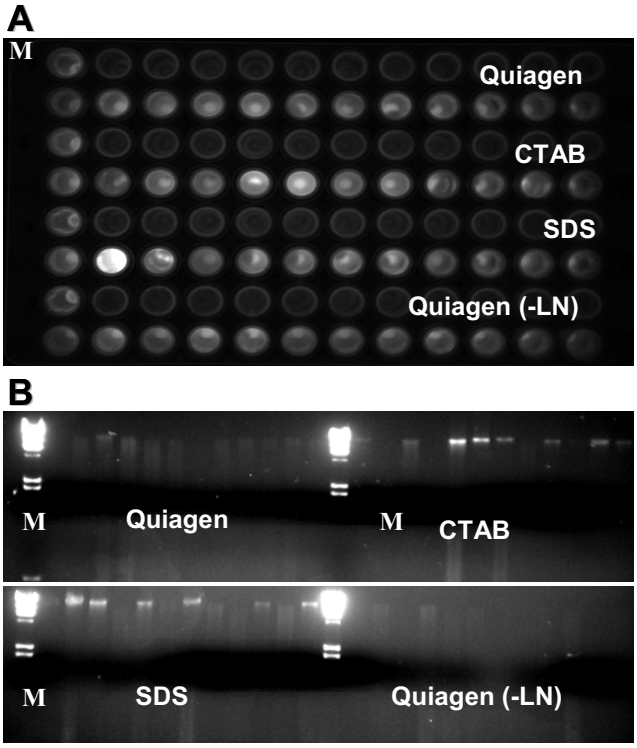

Fig. 5 DNA extraction and quantification. Protocols assayed for DNA extraction (CTAB, SDS, Quiagen membranes withouh liquid nitrogen and Quiagen membranes with liquid nitrogen) and quantification of argan DNA using ethidium bromide in microplates (**A**) and agarose gel electrophoresis (1.5%) (**B**). M = molecular marker *λ Hind* III.

(**Fig. 5**) and could be applied to other rare fruit species as Indian almond or white sapote.

Application of RAPD markers

Random amplified polymorphic DNA (RAPD) markers are based on the PCR amplification of random locations in the genome (Welsh and McClelland 1990). RAPDs are characterized by using arbitrary primers and permit the quick construction of genetic maps and the saturation of specific genomic regions with molecular markers. A single oligonucleotide is utilized for the amplification of genomic DNA. In contrast to isoenzymes and RFLPs, RAPDs are dominant markers. These markers are especially used in rare species including Indian almond or white sapote where there are not known sequences.

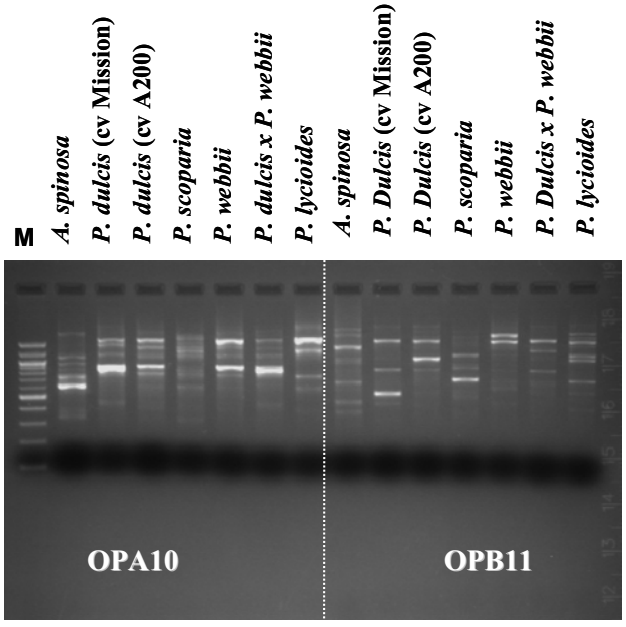

Fig. 6 Molecular characterization of fruit germplasm using RAPDs markers. Agarose gel (2%) showing the allelic segregation of the OPA10, and OPB11 RAPD markers in different species including argan (*A. spinosa*), almond (*P. dulcis* cv. 'Mission' and 'A200'), the wild almond species *P. webii*, *P. lycioides*, and *P. scoparia*, and the interspecific hybrid *P. dulcis* x *P. webbii*. M = 1Kb Plus DNA Ladder.

At the moment, these markers are also being used in the molecular characterization of argan tree species a fruit species which is poorly known from the molecular point of view. Our results also indicated the polyvalent use of these kinds of markers in different species (**Fig. 6**). RAPD techniques have been also successfully used in cultivated and wild *Prunus* for identifying cultivars (Lu *et al*. 1996), estimating genetic diversity and assessing possible origins for selected genotypes (Warburton *et al*. 1996; Bartolozzi *et al*. 1998; Martins *et al*. 2003; reviewed in Teixeira da Silva *et al*. 2005), and construction of maps.

Problems with DNA quality and a general sensitivity to changes in the reaction conditions can hamper the routine utilization of RAPD markers. This feature as well as the codominant nature, the variable degree of repeatability and the problems in transferring across populations, limits their utilization primarily to map construction. These difficulties can be overcome by converting RAPDs to sequence-characterized amplified regions or SCARs (Paran and Michelmore 1993). In contrast to RAPD and AFLP methods, SCAR is a PCR-based method that employs specific primers. These primers amplify single bands corresponding to genetically defined loci. SCARs can potentially be converted into codominant markers and are less sensitive to reaction conditions. Different SCAR markers are being evaluated for marker-assisted selection in *Prunus*, including identification of the *Ma1* root-knot nematode resistance gene in Myrobalan plum (Lecouls *et al*. 1999) and the identification of the *Ff* (flesh adhesion) gene in peach (Jun *et al*. 2002).

Application of SSR markers

Simple sequence repeat (SSR) markers (microsatellites), also based on the PCR technique, are currently becoming the markers of choice for genetic fingerprinting studies for a wide range of plants. Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic variability within crop species and closely related species (Gupta *et al*. 1996). In the case of *Prunus*, SSR markers covering the almost whole genome have been obtained in different species including peach, apricot, Japanese plum, almond and cherry (Aranzana *et al*. 2003). They are being successfully used for the molecular characterization and identification of almond cultivars and related *Prunus* species (**Fig. 7**).

Simple sequence repeat markers generated in different *Prunus* species include highly informative markers which have been applied in the molecular characterization of almond and related wild and cultivated species (Martínez-Gómez *et al*. 2003b, 2003c). In addition, these transferable markers were used in several genetic and taxonomic studies (Gupta 1996; Dirlewanger *et al*. 2002; Martínez-Gómez *et al*. 2003b). Finally, chloroplast microsatellites have been also found and shown to be highly useful markers for phylogenetic and molecular genetic studies in *Prunus* (Ohta *et al*. 2005).

In agreement with previous results (Gupta *et al*. 1996), nuclear SSR markers are the most polymorphic and abundant markers with codominant inheritance and very transferable across closely related species. These molecular markers are ideal for assessing genetic variability in related species and understanding the genetic relationships among them. However in the case of chloroplast SSR the polymorphism observed is much lesser in agreement with higher level of conservation of the chloroplast DNA (uniparental and mostly maternal) in comparison with the nuclear DNA (zygotic) (Zeinalabedini *et al*. subm.) (**Fig. 7**). Nuclear SSR may thus be used to detect natural hybridization between these species as already has been reported for *Amygdalus*

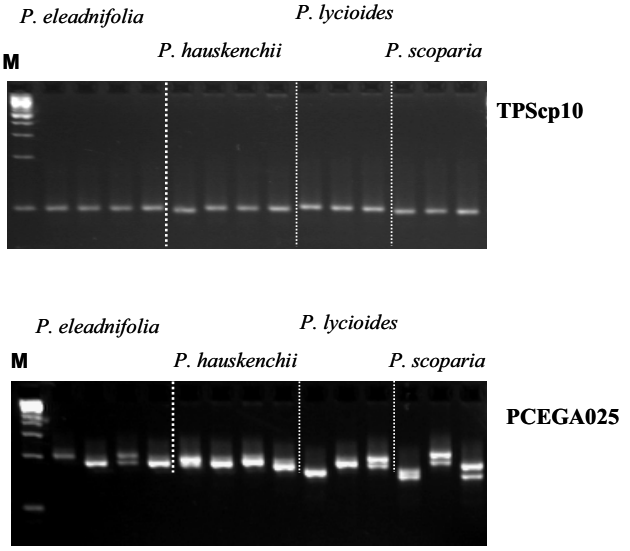

Fig. 7 Molecular characterization of fruit germplasm using SSRs markers. Metaphor® agarose gel (3%) showing the allelic segregation of the TPScp10 chloroplast SSR marker and the PCEGA025 nuclear SSR marker assayed on the wild almond species *P. eleagnifolia*, *P. hauskenchtii*, *P. lycioides*, and *P. scoparia*. M = 1 Kb Plus DNA Ladder.

subgenus (Browicz and Zohary 1996).

Amplification of SSRs is usually successful in a wide range of cultivated and their wild relative species. These results agree with reports by Cipriani *et al*. (1999) and Martínez-Gómez *et al*. (2003b) of the successful utilization of these markers in a wide range species from the same genus. Results obtained showed a high degree of homology for the SSR loci between *Prunus* species (Martínez-Gómez *et al*. 2003a) and support a close evolutionary distance between these species as suggested by Watkins (1976) and Browicz and Zohary (1996). The level of polymorphism in the wild almond species was similar to that reported by different authors in peach and cherry (Cipriani *et al*. 1999; Testolin *et al*. 2000; Cantini *et al*. 2001; Aranzana *et al*. 2002; Dirlewanger *et al*. 2002). Chloroplast microsatellite primer sets amplified the PCR products with the same size and number of alleles as those predicted in *P. salicina* by Ohta *et al*. (2005) and generated discrete bands for all used *Prunus* species. The chloroplast microsatellites obtained will be used as good markers to assess genetic diversity and to clarify phylogenetic relationships in *Prunus* (**Fig. 7**).

Phylogenetic relationships performed using nuclear and chloroplast SSRs indicated a more diffuse clustering between wild almond species (Zeinalabedini *et al*. subm.). This fact could be due to the natural introgression of genes (nuclear and chloroplast DNA) reported in this wild almond species (see review by Kester and Gradziel 1996). These authors also suggest that the sweet kernel trait, in addition to being a natural variant within *P. dulcis*, may have been independently transferred to *P. dulcis* from other wild almond species. Recent results also illustrate the important differences among marker types in taxonomic studies of different *Prunus* species and interspecific hybrids. For example, nuclear SSR may be used to detect natural hybridization between these species as already has been reported for *Amygdalus* subgenus. Thus it is important to choose the right marker for the particular scale and question being evaluated (Zeinalabedini *et al*. subm.).

CONCLUDING REMARKS

In the case of rare fruit germplasm the typical long generation time along with the extensive space requirements and other limitations of these fruit tree crop species have frustrated the development and testing of these species in their regions of origin in developing countries and in other regions. These limitations, however, allow biotechnological tools (including *in vitro* and hydroponic culture protocols, improved propagation techniques and molecular marker application) to improve conservation, particularly applicable to these tree crops. Some of these fruit species include a large number of intercompatible species which provide an enormous gene pool available for breeding of the cultivated species. Little use has been made of this variability because the slowness of classical breeding methods. However, genomic methodologies, including the development of quick gene sequencing and cloning tools, may make it possible to rapidly discover and incorporate genes of interest from this exotic material. Additional advantages encouraging the utilization of these new biotechnologies to tree crop conservation and improvement include high levels of synteny between genomes and a well-established international network of cooperation among researchers.

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