Chapter 17
In Vitro Conservation of Date Palm Germplasm

S.A. Bekheet

Abstract Date palm (*Phoenix dactylifera* L.) germplasm is difficult to conserve and store in the form of offshoots or in field collections. Tissue culture technologies have had a major impact on the *ex situ* conservation of plant genetic resources. *In vitro* culture techniques supplement date palm conservation efforts and have been applied to germplasm collection, preservation and rapid clonal multiplication. *In vitro* storage methods have been developed for preservation of date palm germplasm and can be used efficiently for international exchange of germplasm because of their obvious advantages over *in vivo* material. Preservation of plant cells, meristems and somatic embryos has become an important tool for long-term storage of germplasm utilizing minimum space and low maintenance. Short- and mid-term storage is achieved by controlling environmental growth conditions and nutrient media composition. Long-term storage has been reported for *in vitro* cryopreservation of date palm cultures. Encapsulation of plant material in alginate beads has been suggested recently as a possible means of date palm germplasm exchange. Knowledge about germplasm diversity and genetic relationships are highly valuable tools in plant conservation strategies. In this regard, a number of molecular biology methods are currently available for analysis of genetic diversity in date palm genotypes. This chapter discusses the general issues and different aspects of plant biotechnology used for management and conservation of date palm cultivars.

Keywords Conservation • Cryopreservation • Encapsulation • *In vitro*

S.A. Bekheet (✉)
Plant Biotechnology Department, National Research Center,
El-Tahrir Str., Dokki 12622, Cairo, Egypt
e-mail: shawky005@yahoo.com

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17.1 Introduction

Soil, water and air have long been considered as the essential natural resources. Recently, germplasm has also been added as a fourth essential resource. In recent years there has been an increasing awareness of the holistic view of biodiversity, including agricultural biodiversity and conservation for sustainable utilization and development (Arora 1997). Today, plant germplasm is commanding attention at high government levels in many nations. Several nations have developed extensive systems to carry out their germplasm activities. Scientists also recognize the need to share genetic resources and to take the responsibility to conserve and preserve plant germplasm for the future (Abd El-Wahab et al. 2004). Plant germplasm is defined as a living tissue from which new plants can be grown. It contains the unique genetic information that gives plants their individual characteristics and links generations of living plants to one another. The genetic diversity of plants generated by evolution, hybridization, natural selection and human manipulation, provides the basis for sustainable food production which supports the world's population. This diversity is threatened by habitat loss, development, the change to cultivation of a small number of advanced lines and other factors such as deforestation, fire and hydroelectric power development. Preservation of the genetic diversity represented in all plant ecosystems of the world has become a major issue of international concern. The importance of plant germplasm is universally accepted as a resource of common heritage to be used freely by all humankind in the maintenance, development and improvement of food, feed, fiber and industrial crops. There is a need to share genetic resources and to assume responsibility to conserve and preserve plant germplasm for the future.

The most widely used method for conserving plant genetic resources depends upon seeds which can be dried to low moisture content and stored at low temperature. It is usually safer, cheaper and more convenient to conserve genetic resources as seeds than by any other method. Currently, more than 80% of the genetic resources of plants are conserved as seeds in gene banks around the world. However, several plant species do not produce seed or produce unorthodox seeds, so their germplasm cannot be stored conveniently in seed banks. In many cases it is convenient to conserve the germplasm by other methods. Each method has advantages and limitations. Therefore, there is a need to develop a complementary conservation strategy.

The most common method used to preserve the genetic resources of these problem species is as whole specimen plants in the field, but there are serious drawbacks with field gene banks. Germplasm conservation in field gene banks involves collecting propagation materials and growing them in orchards or fields in a specific location. Field gene banks traditionally have been used for perennial plants, including species that have a long life cycle, to generate breeding and/or planting material. The main disadvantages of this method are pests, diseases, natural disasters and vandalism. Biotechnology continues to have a key role in the conservation and sustainable utilization of biodiversity. Plant conservation biotechnology is an interdisciplinary subject, to which the tools of modern techniques are applied for plant conservation.

Biotechnology techniques offer an alternative method for conservation of such plant material. The biotechnology methodologies of germplasm conservation and

utilization fall into three main categories: (1) the use of novel molecular technologies in the assessment of biodiversity and its monitoring; (2) new tools for conservation and the management of collections and (3) use of genetic manipulation to facilitate the widest use of conserved genes (Barlow and Tzotsos 1995). *In vitro* conservation uses tissue culture techniques to store germplasm involving the use of sterile cultures. It is commonly used for vegetative propagated species, recalcitrant-seeded species and species which produce few or no seeds. The tissues conserved include meristems, shoot tips, axillary buds and zygotic embryos. Basically, two methods are currently used – the slow growth method for short- to medium-term conservation of active collections, and cryopreservation for long-term conservation of base collections.

Date palms (*Phoenix dactylifera* L.) have been cultivated and subjected to selection by man since ancient times, and the distinction between wild and cultivated date palms is blurred. The date palm has retained its value for desert dwellers because of its adaptive characteristics to the environment and the wide range of its benefits, aside from its products. It makes a significant contribution toward the creation of equable microclimates within fragile oasis ecosystems, thus enabling sustainable agricultural development in many drought and saline affected regions (Barreveld 1993).

Cultivation of date palm in different ecosystems around the world has resulted in many local cultivars that represent genetic diversity. Currently grown and obsolete cultivars are important genetic resources, and should be preserved for plant breeders to use in the future. Conservation of this genetic diversity is imperative because it is highly heterozygous and has limited importance for conservation by conventional means. Tissue culture and molecular biology techniques have great potential for collection, multiplication and storage of date palm germplasm. Date palm germplasm can be preserved *in vitro* in the form of shoot tips, somatic embryos, and pollen, callus and cell suspension cultures. Miniaturization of explants allows a reduction in space requirements. Disease-free stock is simplifying quarantine procedures for the regional and international exchange of germplasm. Moreover, molecular and biochemical analysis have been used for the characterization of conserved samples. Molecular biology techniques and their use in the conservation, evaluation and utilization of genetic resources can be summarized as: (1) comparisons between collections or populations and measurement of genetic diversity before and after storage and (2) detection and diagnosis of collections. This chapter discusses the general issues and different aspects of biotechnology used for management and operation of *in vitro* conservation of date palm cultivars.

17.2 Need for *In Vitro* Conservation of Date Palm

17.2.1 Threatening Factors

Date palm is a dioecious, perennial monocotyledonous plant successfully cultivated in arid and semiarid regions. The tree has great socioeconomic importance and the fruits nutritional value in different regions of the world, especially in the Middle

East and North Africa. In addition, the date palm tolerates adverse environmental conditions and is important in reducing desertification. There are many factors threatening the genetic diversity and species of date palm including natural and man-made factors. Environmental changes include drought and floods, as well as seasonal fluctuations in temperature and rainfall. Such changes could result in genetic erosion due to crop failure and loss of varieties. Land use changes which include construction and building of roads, factories, canals, dams and new residential areas are other factors. In this respect, in Egypt, date palm tree numbers decreased from 2.5 million to just over 1 million in the Aswan area due to the building of the High Dam (Hussein et al. 1993).

Modern agriculture characterized by the use of improved cultivars in monocropping systems is now taking place, wherein many improved high-yielding varieties are introduced. Expansion in rainfed agriculture and irrigation schemes has been accompanied by changes in the vegetation complexes in rangeland and forest areas and by changing from small-scale agriculture to large-scale modern agriculture. Moreover, biotic factors such as pests and diseases can attack date palms resulting in negative impacts on the genetic variability within species (Bendiab et al. 1993).

In this regard, the principal constraints limiting date palm culture in the Maghreb countries (Morocco, Algeria, Tunisia) are drought, salinity, deserts, development, senescent date palm trees and bayoud disease. The old age of the palm groves is a considerable constraint, since 30% of the date palms in Algeria exceed the most productive age (5–50 years) (Messar 1996). About 45% of the date palm trees in Tunisia are more than 50 years old (Rhouma 1996). An urgent renovation of the plantations is necessary. The loss of date palm diversity was significant due to either wars or indirectly by habitat destruction. Before 1991 Iraq was the largest producer of dates in the world (FAO 2004) and had the most extensive date ‘forest’ in the world, on the Al-Faw Peninsula (MacFarquhar 2003). However, during the Gulf and Iran-Iraq wars, many palm trees were destroyed and more died when the southern marshes of Iraq were drained. For example, these wars destroyed most of the date palm plantations and by 2003 only 3 million date trees survived out of 16 million trees around Basra before the wars started (MacFarquhar 2003) and may well have resulted in genetic erosion of the natural populations.

17.2.2 Germplasm Conservation

Like many other plants, the date palm has been threatened by human intervention and exploitation. Date palm growing countries have a great responsibility to conserve and safe guard date palm genetic diversity to utilize for genetic improvement and development of crop cultivars for domestic and foreign markets. The collected and conserved date palm genetic resources should be readily available to researchers and other interested parties; with detailed characterization, evaluation, and utilization of conserved genetic material as well as proper documentation, including setting up a website for public access.

There are two approaches to conservation of plant genetic resources – *in vivo* and *in vitro*. The *in vivo* conservation approach generally comprises the following methods: seed storage, field gene banks and botanical gardens. Conservation of plant diversity using reserves/protected areas, on-farm and home gardens is considered an *in vivo* conservation approach. Many important varieties of field, horticultural and forestry species are either difficult or impossible to conserve as seeds (i.e. no seeds are formed or if formed, the seeds are recalcitrant) or reproduce vegetatively.

Several techniques to conserve vegetatively propagated species have recently been developed and some are undergoing rigorous testing. For certain species, *in vitro* conservation is the only option available. Although tissue culture offers a great potential for germplasm conservation of vegetatively-propagated material, two factors have been of major technical hindrance to it. First is the genetic instability of the material conserved as tissue culture due to somaclonal variation (Harding 1999). Second, the storage duration of tissue is limited. It is important to emphasize that the two approaches of conservation (*in vitro* and *in vivo*) are complementary.

Conservation of plant gene pool should employ a combination of methods including nature reserves, gene banks, and others, as no single method can conserve all the diversity. The appropriate balance between different methods employed depends on factors such as the biological characteristics of the gene pool, infrastructure and human resources, number of accessions in a given collection and its geographic site and the intended use of the conserved germplasm. The costs of individual conservation methods could be another important factor in developing the complementary conservation strategy.

17.2.3 Constraints of Traditional Preservation

Since date palm is a dioecious and heterozygous fruit tree, and for commercial purposes most often vegetatively propagated through offshoots, its germplasm cannot be stored or handled easily by conventional means. At present, the most common method used to preserve the genetic resources of date palm is *in vivo* as whole plants in the field. There are, however, several problems facing the field gene banks. Collections are exposed to natural disasters and attacks by pests and pathogens and to intrusion by neighboring rural dwellers; moreover, labor cost and requirements for technical personal are very high. In addition, distribution and exchange from a field gene bank is difficult because of the vegetative nature of the material and the greater risk of disease transfer. Vegetatively propagated material, either as whole plants or offshoots, represents the highest risk category involving potential spread of all stages of all types of pests such as insects, mites, fungi, bacteria, nematodes and viruses. Biotechnology can be integrated into all aspects of date palm germplasm conservation such as characterization, acquisition, germplasm exchange and genetic resource management. Future prospects are highly encouraging in terms of the development and application of new techniques and protocols within the context of germplasm conservation. Micropropagation has been successfully achieved either through direct or indirect morphogenesis for different date palm cultivars

(Mater 1986; Taha et al. 2001; Tisserat 1984; Zaid and Tisserat 1983). The procedures developed can also be utilized for conservation by inducing medium- and long-term preservation (Bekheet et al. 2001, 2007).

17.3 *In Vitro* Conservation of Date Palm

Conservation of date palm genetic resources is particularly important to ensure future access to valuable genes for plant improvement programs. The date palm is a usually vegetatively propagated plant from offshoots because seeds do not ensure true-to-type palms. Tissue culture offers a great potential for the conservation of germplasm of vegetatively-propagated crops. Regeneration and successful propagation of genetically-stable seedlings from cultures are prerequisites for *in vitro* conservation efforts. Significant work is being done on both aspects and for some species (Blake 1983; Khalil et al. 2002) tissue culture maintenance is relevant due to improved techniques resulting in low levels of somaclonal variation. Cryopreservation technique is used for long-term storage of *in vitro* cultures, and is being widely used. *In vitro* methods to propagate the date palm have been developed which may be employed to preserve germplasm (Tisserat 1981).

Tissue culture in combination with molecular biology techniques are of great interest for collecting, multiplication and storage of date palm germplasm. Miniaturization of explants allows reduction in space requirements and consequently labor cost for the maintenance of germplasm collections. The potential of *in vitro* culture methods for the conservation of genetic resources of vegetatively propagated crop species was recently recognized. Two types of *in vitro* gene banks for conservation have been reported: (1) slow growth in cold storage, and (2) cryopreservation (Withers and Williams 1985). In cold storage, temperature varies depending on the origin of stored species. Temperate species may be stored at 4°C, whereas tropical plants are require temperatures in the range of 15–20°C. This technique enables extending the subculture periods from 12 month up to 4 years for many species (Ashmore 1997). Most cold preservation protocols were performed under either low light intensity or complete darkness (Wang and Charles 1991; Zandvoort et al. 1994), yet several plant species were cold preserved under light conditions (Hvoslef-Eide 1992). Otherwise, several types of plant materials including shoot tips, nodal segments and rooted shoots are used for low temperature preservation (Orlikowska 1992). Cryopreservation generally refers to storage between −79°C and −196°C in liquid nitrogen. The major advantage of cryo-storage is that both metabolic processes and biological deterioration are considerably slowed or even halted (Kartha 1981). In addition, it is believed that cryopreserved material remains genetically stable, thus affording an advantage over conventional conservation methods (Withers 1980, 1983). So far, cryopreservation protocols for plant tissues have mainly been based on slow freezing in the presence of cryoprotective mixtures containing DMSO (dimethyl sulphoxide), sugars, glycerol and/or proline. Slow freezing results in freeze-dehydration, leaving less water in the cells to form lethal ice crystals upon

exposure to extreme low temperatures. However, several new cryopreservation procedures such as vitrification, encapsulation-dehydration, preculture-dehydration, and encapsulation/vitrification have been established, which are all based on vitrification (Sakai and Engelmann 2007).

The crucial factor for the successful use of cryo-storage of *in vitro* cultures is plant regeneration, and maintaining their genetic fidelity. Many publications indicate that characteristics such as growth rate, regeneration capability, ploidy levels and RAPD markers are retained after cryogenic storage (Kobayashi et al. 1990; Wang et al. 1994; Ward et al. 1993). Solid regeneration medium is usually used for regrowth of cryopreserved explants (Hirai and Sakki 1999); nevertheless, some results suggest that liquid medium may be better (Pains et al. 2002). Although plant regeneration can be accomplished from adventitious buds and somatic embryos derived from leaf, stem, root or callus, such undifferentiated tissues should be conserved with caution owing to their potential for somaclonal variation. Tissues for *in vitro* conservation should be pre-indexed for pathogens.

Pollen storage has also been considered as an emerging technology for genetic conservation of date palm. Pollen storage was mainly developed as a tool for controlled pollination of asynchronous flowering genotypes, especially in fruit tree species (Alexander and Ganeshan 1993). Cryopreservation has more potential to succeed in pollen storage as compared to other storage techniques routinely employed for pollen. Pollen can be collected easily and cryopreserved in large quantities in a relatively small space. In addition, exchange of germplasm through pollen poses fewer quarantine problems as compared to seed or other propagules.

Cryopreservation techniques have been developed for pollen of an increasing number of species and pollen cryo-banks of fruit tree species have been established in several countries i.e., India, Indonesia, Italy and South Africa (Barnabas and Kovacs 1997; Bhat and Seetharam 1993). Appropriate molecular markers have been identified to verify true-to-type date palm nursery material; and have great potential of identifying unique value-added individuals for the long-term conservation. The six major steps defined in the conservation are: collection; quarantine; disease indexing and eradication; propagation; characterization; and storage and distribution (Withers 1983). Some problems may arise with the maintenance and repeated transfer of *in vitro* stored date palm germplasm such as danger of contamination during each transfer, which may result in loss of elite plant material and genetic fidelity. Loss of morphogenic potential, and other problems associated with tissue culturing, would be minimized by cryo-storage of tissues in liquid nitrogen (−196°C). Successful cryogenic methods for doing this are now being developed. A brief review of such efforts and their use are described here.

17.3.1 Cold Storage

The main objective of slow growth *in vitro* preservation of germplasm is to limit the number of subcultures and to maintain the genetic diversity of a species under sterile

conditions without endangering plant genetic stability. The slow growth method for an active germplasm collection aims to minimize cell division and growth to increase longevity without genetic changes. Slow growth procedures allow clonal plant material to be held for 1–15 years under tissue culture conditions with periodic sub-culturing, depending on species. Standard culture conditions can be used for short-term storage only with slow growing plant species (coffee and oil palm) However, for most plant species, modified conditions such as environmental conditions and/or the culture medium are necessary for short and medium-term storage; subculturing for 6–12 months, depending on species. The advantage is that the time between transfers is lengthened thereby prolonging storage and reducing maintenance. This is accomplished by using media containing growth retardants and osmotic agents, or low temperature and light intensity (Withers 1991).

Medium selection may be an important factor as not all species can survive in the same medium (Dussert et al. 1997). It is also possible to limit growth by modifying the culture medium, mainly by reducing the sugar and/or mineral element concentrations. Addition of osmotic growth inhibitors (e.g. mannitol) or growth retardants (e.g. absisic acid) is also employed successfully to reduce growth. Osmotic agents are materials that reduce the water potential of cells. Adding osmotic agents to the culture has proven efficient in reducing growth and increasing the storage life of many *in vitro*-grown tissues of different plant species. According to the hypothesis for turgor-driven growth and cell expansion, high levels of osmotic agents in the medium would act against the creation of a critical turgor pressure, which must be established before cell expansion can occur (Zimmermann 1978). This stress condition will inhibit both callus growth and shoot formation (Brown et al. 1979). In this regard, mannitol, sucrose, sorbitol, tributyl-2,4 dichlorbenzylphosphonium chloride (phosphon D), malic hydrazide, succinic acid-2, 2-dimethyl hydrazide (B-995) and ancymidole were reported ideal materials to lengthen the storage life of *in vitro* grown tissues.

The role of sucrose is two-fold, a carbon/energy source and an osmotic agent. High sucrose concentration can be used to reduce plant growth *in vitro*. Mannitol can also be used as an osmotic agent; it is a sugar alcohol produced as a primary photosynthetic product by some plants and can be metabolized by them. Sorbitol is another sugar alcohol that inhibits shoot growth of *in vitro* grown tissue cultures. Shibli et al. (1999) reported that elevated sucrose, sorbitol or mannitol reduced growth of bitter almond micro-shoots significantly and extended the subculture interval to 4 months at room temperature. The type of explant as well as its physiological state at the time of storage can influence the duration of storage. For example, the presence of a root system generally increases the storage capacities. Moreover, organized cultures such as shoots are used for slow growth storage since undifferentiated tissues such as callus are more vulnerable to somaclonal variation. Also, the type of culture vessel, its volume as well as the type of closure of the culture vessel can greatly influence the survival of stored cultures.

Plant material can be stored for longer periods by increasing the size of the storage containers. Moreover, replacing cotton plugs with polypropylene caps, thus reducing the evaporation of the culture medium, increased the survival rate during storage (Sharma and Chandel 1992; Withers 1992). Alternative techniques include modifications of the gaseous environment of cultures, and desiccation and/or

encapsulation of explants. Growth reduction can be achieved by reducing the quantity of oxygen available to cultures. The simplest method consists of covering the explants with parafilm, mineral oil or liquid medium. Reduction of the quantity of oxygen can also be achieved by decreasing the atmospheric pressure within the culture chamber or by using a controlled atmosphere. In this respect, oil palm polyembrogenic cultures were conserved for 4 months at room temperature in a controlled atmosphere with 1% oxygen (Engelmann 1990).

In most cases, a low temperature, often in combination with low light intensity or even darkness, is used to limit the growth. Temperatures in the range of 0–5°C are employed with cold tolerant species (Monette 1986), but for certain tropical species, which are generally cold sensitive, temperatures of 15–20°C, are used (Banerjee and De Langhe 1985; Corbineau et al. 1990). Storage at reduced temperatures, for example, has been used with strawberry (Reed 1992), grape vines (Galzy and Compan 1988), potato (Sarkar and Naik 1998), grasses (Dale 1980), legumes (Mandal 1995). However, despite the apparent success in the application of slow growth techniques, a number of concerns remain. A main concern is the problem of genetic stability (Harding 1991, 1994). In this respect, *in vitro* conservation by slow growth has thus been developed to resolve some of these problems by prolonging the subculture interval (Yu-Jin and Xiu-Xin 2003).

17.3.2 Short and Mid-Term Storage

The objectives of slow growth in date palm are: (1) to devise an effective and reproducible protocol for minimizing growth and development of plant material, (2) to avoid induction of abnormal growth and development during storage and (3) to preserve viability of stored plant population as *in vitro* cultures. Slow growth is achieved in date palm for short (3–6 months) and mid-term conservation (9–12 months). In this respect, Bekheet et al. (2001) described a method for preservation of Egyptian date palm (Zagloul cv.) tissue cultures by slow growth. Shoot buds and callus cultures were successfully stored for 12 months at 5°C in the dark. Uniform proliferated shoot buds and equal pieces of callus cultures were cultured on standard growth media [MS-hormone-free for shoot buds and MS supplemented with 10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3 mg/l dimethylaminopurine (2ip) for callus] and incubated at 5°C and complete dark conditions. One hundred percent of survival and healthy shoot bud cultures with no vitrification were obtained after 3 months of incubation. However, 70% of bud cultures survived for 12 months of storage (Fig. 17.1a). However, callus cultures remained fully viable with slight browning after 12 months of storage at 5°C in the dark. The role of sorbitol as an osmotic stress agent in the *in vitro* storage of date palm cultures was examined. Under normal growth conditions, healthy shoot bud cultures were obtained after 6 months of storage in a medium containing 40 mg/l sorbitol. However, the period was extended to 9 months in the case of callus cultures (Fig. 17.1b). In this regard, 18 date palm cultivars produced through tissue culture from different laboratories around the world were transplanted into the date palm germplasm bank at King Faisal University, Saudi Arabia (Al-Ghamdi 2001).

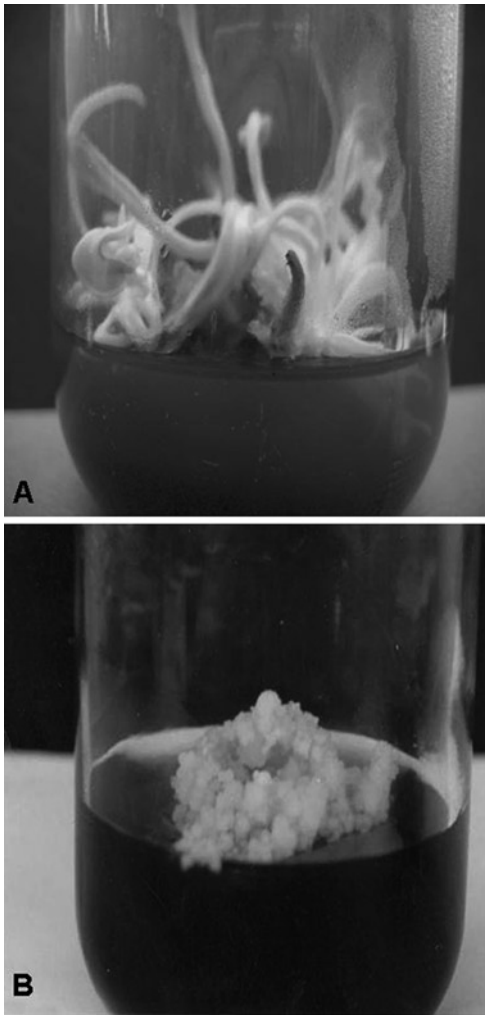

Fig. 17.1 (**a**) Shoot buds of date palm stored for 12 months at 5°C in the dark. (**b**) Callus culture of date palm stored for 9 months on medium contained 40 mg/L sorbitol at normal growth conditions

17.3.3 Cryopreservation

17.3.3.1 Advantages of Cryopreservation

Cryo-storage is a long-term *in vitro* conservation method, which is carried at between −79°C and −196°C. At these temperatures, cell division and metabolic and biochemical processes are arrested and thus the cells retain their properties unchanged for an indefinite period of time (Nimo et al. 1992; Paul et al. 2000). Also, cryopreservation offers maximum stability of phenotypic and genotypic characteristics of stored germplasm. Ultra-low temperature effectively suspends or reduces metabolic activities of tissues to near zero thereby allowing, in theory, near infinite

conservation without genetic alteration. The main advantage of cryopreservation is the limited need for viability indexing or subculturing, besides being relatively cheap and manageable. Moreover, cryopreservation is the only available method for long-term conservation of vegetatively-propagated plant germplasm.

In vitro conservation of a wide array of tissues ranging from shoot tips, buds, embryos to protoplast has been successfully cryopreserved in liquid nitrogen (LN). However, desiccation of sensitive tissue such as vegetative plant parts including meristems, shoot tips, and axillary buds, are easily injured even with slight desiccation. Long-term storage using cryopreservation offers an alternative to slow growth. It is attractive because of the potential reduction in maintenance costs and reduced exposure of germplasm to contamination and genetic change. In addition, it is not demanding of space in that a very large collection can occupy a relatively small area. The technology for cryopreservation has advanced significantly in the past decade, especially for tropical crops. Techniques have now been developed for more than 200 different plant species cultured in various forms, including cell suspensions, calli, apices, somatic and zygotic embryos (Reed 2008).

17.3.3.2 Cryopreservation Procedures

In vitro collections of date palm plantlets have been established by the Groupe de Recherche Français sur le Palmier Dattier, France. Management of large-scale *in vitro* collections poses numerous practical problems. Moreover, risks of contamination and of somaclonal variation increase over time. It is therefore essential to develop long-term conservation techniques to conserve elite date palm cultivars. This technique is being optimized by several research groups in order to apply to a wide range of genotypes, and maintain genetic integrity. For successful cryopreservation, it is essential to avoid lethal intracellular freezing which occurs during rapid cooling in LN. Recently, new approaches have been developed for cryopreservation of plant apices by directly plunging them into LN such as is used in vitrification. This is a physical process that can be defined as the transition of aqueous solution from liquid into amorphous glass at the glass transition temperature, while avoiding ice crystallization. The vitrification procedure requires a highly concentrated solution, which sufficiently dehydrates cytosols without causing injury so that they turn into a stable glass when plunged into LN. Vitrification-based procedures involve removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. These techniques are more appropriate for complex organs like embryos and shoot apices; they are also less complex and do not require a programmable freezer, hence are suited for use in any laboratory with basic facilities for tissue culture (Charoensub et al. 1999; Niino et al. 1992a; Scocchi et al. 2004).

Engelmann (2000) described seven vitrification-based procedures in use for cryopreservation: (1) encapsulation-dehydration, (2) vitrification, (3) encapsulation-vitrification, (4) desiccation, (5) pregrowth, (6) pregrowth-desiccation and (7) droplet

freezing. With the advent of these new cryogenic procedures, especially vitrification, encapsulation-vitrification and encapsulation-dehydration, the number of cryopreserved species has increased significantly in recent years.

Encapsulation and dehydration cryopreservation methods are based on embedding plant material in alginate beads which protect them from dehydration and freezing damage. This method has been successfully applied to coconut plumules. Apical domes excised from embryos were used as plant material; pretreatment duration, sugar concentration, dehydration period and freezing were tested as key factors. A good survival level around 60% was obtained and 20% of leaf shoots were developed from the cryopreserved explants (N'Nan et al. 2008). Even though cryopreservation is suitable for vegetatively-propagated species, it seems unsuitable for seed propagated species, which is due to high sensitivity to desiccation, structural complexity and heterogeneity in terms of developmental stage and water content at maturity.

17.3.3.3 Development of Date Palm Cryopreservation

For long-term conservation of date palm, cryopreservation in LN appears to be a logical choice, since all metabolic process and all cell division are arrested at those temperatures. Cryopreservation is recognized as having the distinct advantage of allowing long-term conservation with minimum space and maintenance. In this regard, Finkle et al. (1979) investigated the possibility of conservation of a tropical palm and the date palm using cryopreservation. Tisserat et al. (1981) reported that embryogenic callus cultures of date palm were subjected to 0°C, −15°C, −23°C, −30°C and −196°C for up 3 months in the presence of protective mixture. Revived cultures developed callus upon recovery and development of thawed callus from all frozen temperatures was rapid after a quiescent period of 2–4 weeks. Thawed callus cultures were nodular and yellow-brown in color during the log phase. The nodules enlarged and differentiated into embryos as new growth. Plantlets were produced from the callus within 3 months.

Ulrich et al. (1982) stated that embryogenic date palm (Medjool cv.) callus cultures were treated with a cryoprotective mixture of polyethylene glycol (Carbowax 6000), glucose, and dimethyl sulfoxide (10%/8%/10%, w/v); treated with the mixture, frozen to −196°C, and then thawed; or left untreated. Growth subsequent to treatment was measured as fresh weight increase and the number of embryos produced during 18 weeks of culture. The addition of a cryoprotective mixture to the unfrozen controls at 0°C did not adversely affect growth and morphogenesis of callus cultures of date palm, but treatment at −196°C decreased the subsequent rate of culture growth, compared to the control treatments. This inhibitory effect was probably directly related to the number of cells injured or killed by the freeze-thawing process. Later, Tisserat et al. (1985) reported that cryopreserved pollen of date palm (Deglet Noor cv.) was dusted on freshly opened spathes of 10-year-old Deglet Noor female trees; fruit yield and developments were similar using either frozen or non-frozen pollen.

Date palm embryos can continue normal growth and development after cryopreservation provided they are pretreated with a cryoprotectant mixture of glycrol and sucrose and then dried to a water content of 0.4–0.7 g/g. In this respect, Mater (1987) reported that date palm callus was treated with a cryoprotective mixture and frozen to −25°C for 4 months. Freezing did not affect the potential of embryogenesis induction from callus although growth during the first 2 months of culture was inhibited. Studies on the cryopreservation of date palm for germplasm collections were initiated by Towill et al. (1989). Shoot-tips were excised from 2-month-old seedlings of Medjool cv., precultured for 2 days and then cooled to LN temperatures. Viability of treated shoot-tips was assessed by growth *in vitro*. Dimethyl sulfoxide (DMSO) in concentrations up to 10% was not toxic, although growth was slower than untreated shoot-tips. Several combinations of DMSO and sucrose were effective in obtaining survival after LN exposure. In most cases, the LN-treated shoot tips developed directly into a shoot without callus formation. In this respect, Bagniol et al. (1992) suggested that gradients may be exhibiting both the outflow of water and the penetration of the cryoprotectants during the process of date palm cryopreservation. MyCock et al. (1995, 1997) developed date palm plantlets using somatic tissue previously frozen for several months in LN. They reported that late globular/early torpedo stage date palm embryos were successfully cryopreserved. Drying on its own resulted in a better percentage survival than when the samples were pre-treated with cryoprotectants only; as before, when the pre-treatments were combined the survival rate was substantially improved.

A number of steps are followed for cryopreservation, which include: preculture in media with osmotically active compounds, treatment with cryoprotective agents, cooling and storage at −196°C, thawing, post-thaw treatments and recovery of growth. Capacity to survive storage in LN is dependent upon many factors including the physiological status of the explant. Type and nature of cells determine the ability of cells to withstand freezing stress. In general it is recommended that explants be taken from rapidly growing cultures since actively dividing cells have dense cytoplasm and little developed vacuolar systems which make them more likely to withstand freezing and remain viable. Three types of meristematic and regenerative *in vitro* tissues can be obtained from date palm: (1) individual meristems isolated from shoot-tip cultures; (2) highly proliferating meristem cultures containing nodular-like meristem clusters; (3) somatic embryos. Cryopreservation methods have been developed for nodular tissue by Bekheet et al. (2007). Undifferentiated tissue cultures (nodular cultures) were successfully cryopreserved by freezing methods; subsequently the plants were regenerated.

The potential for dehydration caused by air drying in the cryopreservation of date palm tissue cultures through direct immersion in LN was studied. Among different types of sugars (e.g. fructose, glucose, sorbitol and sucrose) used as osmotic agents in preculture medium, sucrose proved to be the best for the survival of cryopreserved date palm tissue cultures. To determine the potential of vitrification on freezing tolerance, cultures were exposed to a vitrification solution [22% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) propylene glycol and 7% (w/v) dimethyl sulfoxide] for 20–100 min. The highest survival rate was obtained

with cultures exposed for 80 min at 0°C followed by 40 min at 25°C. Cultures were kept in LN (−196°C) for 48 h. The storage duration in LN is not a critical factor affecting the recovery of cryopreserved plant material. Once the plant material is plunged into LN, the internal solutes will be vitrified and biochemical or biophysical events will no longer occur. Thawing is done at room temperature using a laminar airflow cabinet. For recovery of cryostored tissue cultures, various techniques have been used to detect viable plant cells, including fluorescein diacetate (FDA) staining (Ashmore 1997) and a triphenyl tetrazolium chloride (TTC) reduction assay (Wang et al. 2002). The TTC test distinguishes between survived and non-survived plant cells, tissues and organs on the basis of their respiration rate. Regrowth of the cryopreserved plant material is another indicator of plant viability, as evidenced by cellular growth and greening of the apical regions, or in the form of callus.

17.4 Synthetic Seeds

Advances in biotechnology, especially with *in vitro* culture techniques and molecular biology, provide important tools for improving conservation and management of plant genetic resources. Somatic embryos are excellent material for cryopreservation of plant germplasm. Somatic embryogenesis is a technique that lends itself to the production of synthetic seeds. Cryopreservation of encapsulated somatic embryos/synthetic seed is yet another viable proposition for long-term conservation and exchange of germplasm. Synthetic or artificial seeds have been defined as somatic embryos engineered for use in the commercial propagation of plants. Through the combination of vegetative propagation, long-term storage, and clonal propagation, synthetic seeds can have many applications in agriculture. Somatic embryogenesis is the production of embryo-like structures from somatic cells. A somatic embryo (SE) is an independent bipolar structure, not physically attached to the original tissue that develops in the same way as a zygotic embryo. Two pathways of development are possible for SEs; they can either develop directly from the tissue being cultured or indirectly from callus. The latter method, indirect embryogenesis from liquid cell suspensions, is desirable for micropropagation, providing that clonal integrity can be guaranteed. Using this system, large numbers of SEs can potentially be produced in small volumes of culture media in a synchronous manner, thereby allowing partial mechanization and reduced labor costs.

Somatic embryos can be easily managed using bioreactors. Previously, synthetic seeds were simply hydrated somatic embryos produced from vegetative cells in plant tissue culture. These had the advantage of rapid multiplication, but the system was labor intensive, costly and the propagules were very delicate. To improve the process a new technique was developed in which a single embryo is enclosed in an alginate capsule. Prolonged storage of these synthetic seeds is now possible as the somatic embryos can be dried to a moisture content of less than 20%. Capsules of synthetic seeds are produced by mixing embryos in sodium alginate followed by dropping them into a calcium salt solution (i.e. calcium chloride) or by inserting them into a drop of

sodium alginate just as they are falling into the calcium chloride. Gel capsules can be amended with nutrient, growth regulators and carbohydrates to facilitate the rapid growth and survival of embryos. In this regard, it was reported that the maturation phase and quality of somatic embryos are critical for achieving high conversion frequencies of any encapsulation or coating systems (Redenbaugh et al. 1986).

By combining a vegetative propagation system with the capability of long-term storage and clonal multiplication, synthetic seeds have many diverse applications. Establishment of synthetic seeds has multiple advantages, including ease of handling, potential long-term storage and low cost of production and subsequent propagation. In this respect, natural unipolar propagules like microbulbs, rhizomes, protocorms, nodal cuttings and shoot buds, besides bipolar somatic embryos, have been subjected to encapsulation for storage, easy handling, transport, delivery and their establishment under *in vitro* and/or *extra vitrum* conditions. Although a variety of natural and synthetic polymers are available for encapsulation, sodium alginate is the most commonly used gel-matrix because of its favorable gelling properties, non-toxicity and low cost. Different concentrations (1.5–6%) of sodium alginate have been used for different systems. Higher or lower levels of sodium alginate reduced the conversion frequency.

Synthetic seed research through somatic embryos has been achieved on numerous crop plants including conifers (Fowke et al. 1994), high-value vegetables like celery and lettuce (Sanada et al. 1993) and papaya (Castillo et al. 1998). At present, the alfalfa synthetic seed system is the most advanced (Redenbaugh 1993) and has progressed to direct field planting of synthetic seeds (Fujii et al. 1992). The use of encapsulated shoot-tips as artificial seeds is reported on banana (Ganapathi et al. 1992). A synthetic seed protocol for garlic conservation as an asexually propagated plant was established using *in vitro* regenerated bulblets (Bekheet 2006).

In date palm, a reliable system for preservation of germplasm via artificial seeds was recognized by Bekheet et al. (2005). Somatic embryos proliferated *in vitro* from shoot-tip cultures were encased in sodium alginate capsules and stored for 12 months. Somatic embryos in four maturation stages i.e. globular, torpedo, cotyledon and late cotyledon were taken and dried in a laminar flow bench (Fig. 17.2a) and then mixed with gel of 3% sodium alginate prepared in distilled water (Fig. 17.2b). An antibiotic mixture containing rifampicin (60 mg), cefatoxine (250 mg) and tetracycline HCL was used to avoid contamination. The embryos were placed into calcium chloride solution (2.5%) for 30 min and then stored at 5 and 25°C. After 12 months, the encapsulated embryos were sown in distilled water and then cultured on MS-hormone-free medium (Fig. 17.2c). Using 3% sodium alginate as a gel matrix was the best for viability and conversion to plantlets (Fig. 17.2d). For plantlets recovery and development from encapsulated somatic embryos, 20 g/L of sucrose was added to the culture medium. Observations indicated that the highest percentages of viability were recorded with the cotyledon stage (80% at 5°C and 40% at 25°C). However the highest percentages of conversion to plantlets were registered with the late cotyledon stage (65% at 5°C and 35% at 25°C). Similarly, somatic embryogenesis and synthetic seeds of Egyptian date palm cvs. Gondeila, Sewi and Zagloul were studied by Ibrahim et al. (2006).

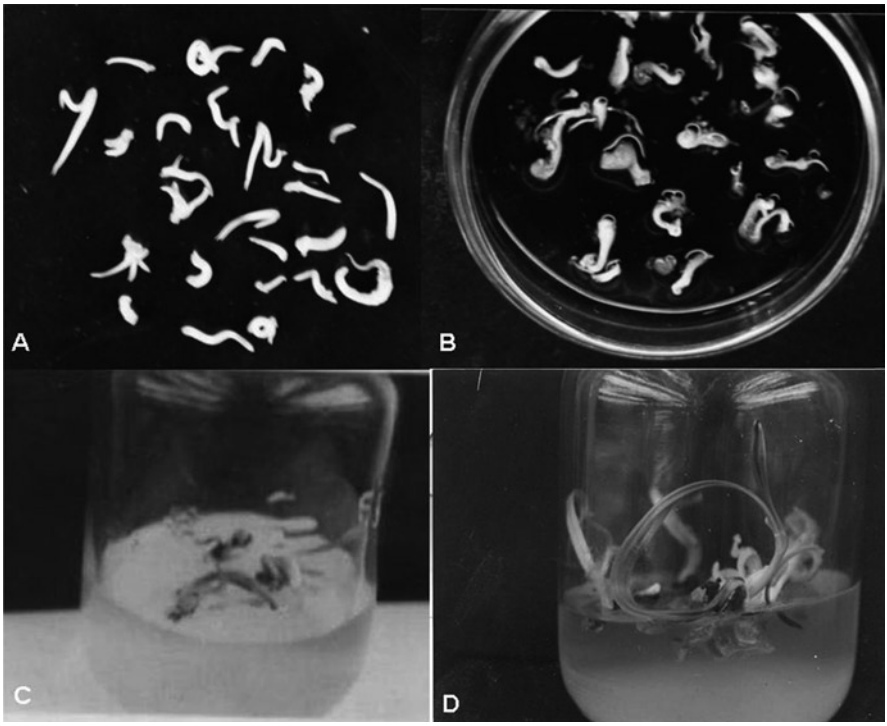

Fig. 17.2 (**a**) Air drying of proliferated somatic embryos of date palm, (**b**) somatic embryos coated with 3% sodium alginate, (**c**) coated somatic embryos recovered on MS-hormone-free medium, and (**d**) plantlets development from encapsulated somatic embryos

17.5 Characterization of Diversity and Genetic Stability

17.5.1 Biochemical and Molecular Characterization

Germplasm characterization is important for varietal identification, genetic conservation and utilization for genetic improvement of date palm (Al-Moshileh et al. 2004). Correct identification of cultivars usually is not possible until fruits are produced. In addition the characterization of cultivars and the evaluation of genetic diversity require a large set of phenotypic data that are often difficult to assess and sometimes variable due to environmental influences. The ability to identify genetic variation is indispensable to effective management and use of genetic resources. Traditionally, diversity is assessed by measuring variation in phenotypic traits such as flower color, growth habit or quantitative agronomic traits like yield potential, stress tolerance, etc., which are of direct interest to users (Rao 2004). The genetic information provided by morphological characters is often limited and the expression of quantitative traits is subjected to strong environmental influence. Earlier

biochemical methods based on seed protein and enzyme electrophoresis were introduced. They proved particularly useful in analysis of genetic diversity by revealing differences between seed storage proteins or enzymes encoded by different alleles at one (allozymes) or more gene loci (isozymes). Isozymes represent biochemical markers which are successfully used as a possible alternative or complementary method for characterization of crop plant cultivars (Cardy and Kannenberg 1982; Torres and Tisserat 1980)

The use of biochemical methods eliminates the environmental influence; however, their usefulness is limited due to their inability to detect low levels of variation. Earlier isoenzyme marker analysis proved useful in identification of date palm cultivars (Baaziz and Saaidi 1988; Bendiab et al. 1993). However, they provide limited number of informative markers and give no direct assessment of the genomic variation. By using polymorphic isoenzyme markers, Bendiab et al. (1998) studied genetic diversity of the Moroccan palm groves and estimated variability more than 90% of total diversity of the date palm within-populations, whereas variability between the populations is limited to about 10%. In this regard, a study to assess levels of alloenzym diversity in collection of 29 date palm cultivars belonging to three main date-growing regions in Tunisia was conducted by Salem et al. (2001). A total of 19 different profiles were detected in the 29 cultivars using four enzyme systems i.e., glutamate oxaloacetate transaminase (GOT), phosphoglucomutase (PGM), shikimate dehydrogenase (SDH) and phosphoglucoisomerase (PGI). Results of electrophoretic analysis revealed five polymorphic loci at the four enzymes and 27 (93.3%) of the 29 cultivars were identified uniquely.

Recently introduced DNA-based techniques have the potential to identify polymorphisms represented by differences in DNA sequences. These methods are being used as complementary strategies to traditional approaches for the assessment of genetic diversity. The major advantage of DNA-based techniques is to analyze variation at the DNA level without environmental influences. The analysis can be performed at any plant growth stage using any plant part and requires only small amount of material (Rao 2004). Molecular characterization has a number of applications in the management of germplasm collections. These include elucidating systematic relationships between accessions; assessing gaps and redundancies in the collection; development of core subsets; characterizing newly acquired germplasm; maintaining trueness-to-type; monitoring shifts in population genetic structure in heterogeneous germplasm; monitoring genetic shifts caused by differential viability in storage or *in vitro* culture; exploiting associations among traits of interest and genetic markers; and genetic enhancement (Bretting and Widrlechner 1995). The DNA polymorphisms are exploited by an ever-increasing number of molecular marker techniques for differentiation between individuals, accessions and plant species. Their higher resolution compared with all other markers makes them a valuable tool for varietal and parental identification. Although there has been a small amount of molecular characterization of the date palm holdings, various molecular marker systems, such as isozymes, restriction of fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPD) and

amplified fragment length polymorphism (AFLPs) have been tested for their application in date palms (Ben-Abdalla et al. 2000; Bennaceur et al. 1991; Corniquel and Mercier 1994; Sedra et al. 1998).

17.5.2 Genetic Stability of Conserved Collections

Cryopreservation and cold storage are the most appropriate techniques to conserve large collections of plants (Ashmore 1997). Both techniques involves *in vitro* culture manipulation of the stored tissues that could be considered a potential risk for the regeneration of genetic instability (Brar and Jain 1998). Number of studies indicate that plants recovered from cold storage or slow growth have no genetic alteration (Hao et al. 2004; Renau-Morata et al. 2006). In this respect, Scocchi et al. (2004) tested the genetic fidelity of Chinaberry tree (*Melia azedarach* L.) plants obtained from cryopreserved apical meristem-tips using isozyme and RAPD markers. The isozyme systems presented identical electrophoretic bands and Rf values. In regard to RAPD analysis, none of the primers generated a unique profile for the control and the plants obtained from cryopreserved apical meristem-tips.

Concerning the stability of date palm tissue cultures, genetic variations of *in vitro* grown date palm Deglet Noor cv., tissue cultures were tested by random amplified polymorphic DNA (RAPD) profiles (Othmani et al. 2009). Nine arbitrary 10-mer primers were used to amplify DNA from 180 plantlets. RAPD patterns of the plantlets were identical with the original mother plant, indicating that no somaclonal variation was detected by the RAPD technique. Genetic stability is the key to reliability in the *frozen storage* of date palm germplasm. In this regard date palm callus previously stored at −196°C for 3 months had no observed changes in the patterns of survival or morphology of resulting plantlets (Tisserat et al. 1981). Ulrich et al. (1982) observed the stability of the date palm plantlets, regenerated from cryopreserved callus. Isozyme analysis of leaves revealed that within the Medjool cv. no enzyme differences were expressed among the leaflets of plantlets from untreated, PGD-treated, and frozen cultures.

Salman et al. (1988) analyzed isozyme polymorphism and chromosome number of tissue culture derived palm plants and found that all regenerated plants had the basic chromosome numbers 2n = 36 except one 2n = 70. A histo-cytological study was performed on apices of *in vitro* date palm plantlets cv. Bou Sthammi Noir subjected to cryopreservation (Bagniol et al. 1992). After freezing in LN, the apices showed cellular heterogeneity. Some cells conserved their meristematic characters. This was the case in the cellular layers corresponding to the meristem itself, whereas in the underlying zone, where the cells were more vacuolated, some were damaged, showing broken cell walls in some samples. During culture, prior to the cryoprotective treatment, obvious starch synthesis occurred in some cells of the samples. RAPD analysis was used to examine the genetic stability of cryo-stored date palm tissue cultures (Bekheet et al. 2007). Both treated and non-treated date palm tissue cultures in addition to field grown plants were identical with the primers used (Fig. 17.3).

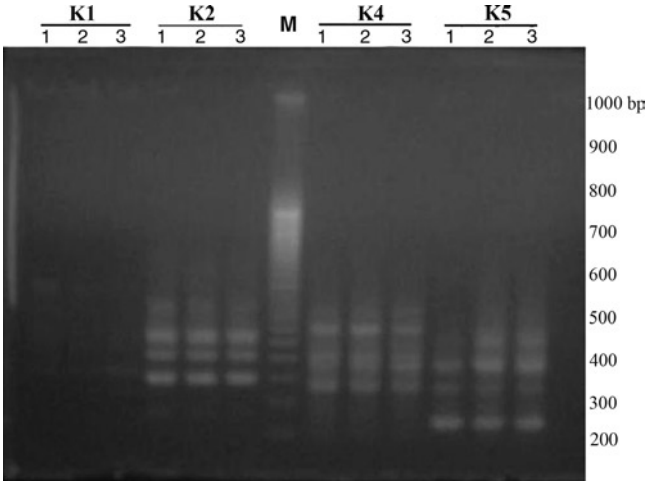

Fig. 17.3 RAPD profile of *in vivo* grown plant (lane 1), non-treated tissue cultures (lane 2), cryopreserved tissue cultures of date palm (lane 3) and the DNA marker (M) using random primers i.e., K1, K2, K4 and K5

17.6 Conclusions and Prospects

Conservation of plant genetic resources via tissue cultures had an immense effort by research work in the last three decades. *In vitro* techniques and storage methods are enabling the establishment of extensive collection using minimum space. Storage under low temperature is considered one of the major tissue culture techniques used for preservation of genetic resources of date palm. Maintaining date palm material *in vitro* under slow growth conditions could reduce cost associated with less frequent subculturing. However, cryopreservation offers long-term storage capability, maximal genetic stability of stored germplasm, minimal storage space and maintenance requirements. Cryopreservation is, so far, the only viable procedure for long-term germplasm conservation of vegetatively-propagated species such as date palm and, therefore, can be used for base collections. Slow-growth techniques are in a more advanced state of development than cryopreservation techniques, which still require improvement before they can be used on a routine basis in date palm. Adjustment of these protocols to the gene bank level would be necessary to actually exploit the advantages of *in vitro* preservation. Otherwise, establishment of synthetic seeds system is of great value in storage and propagation of valuable germplasm. Encapsulated embryos of date palm can be handled and *in vitro* planted providing large numbers of disease-free propagules. The capsules surrounding the somatic embryos would maintain the hydration of embryos, allowing temporary storage. In parallel, molecular and biochemical markers can be used to analyze date palm genetic diversity and detection of *in vitro* preserved derived plants.

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