**In Vitro Conservation and Cryopreservation of Medicinal and Aromatic Plants: A Review**

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**ABSTRACT**

Medicinal and aromatic plants are those containing special chemical components that enable them to relief pain, release pleasant aromas, and improve food flavors. Meanwhile, medicinal and aromatic plants are under real jeopardy due to the uncontrolled collection of these plants as a result of being extensively used in herbal medicine and food industry. So conservation action for these natural resources is of high priority. Plants conserved under *in situ* conservation conditions are exposed to natural disasters, pests and pathogens in addition to the fluctuating government policies. Also *ex situ* conservation is very difficult to be applied as adequate samples have to be collected for the conservation of genetic diversity. *In vitro* conservation is offering a strong and a multi package of techniques that do so good when other conservation methods are not feasible. Slow growth conservation is a very simple *in vitro* technique based on reducing the growth rates of the tissue cultured plant for short or mid-term storage and yet increasing the intervals between subcultures. In this conservation type, several techniques are used separately or in combinations to slow down the growth rate of the stored explants, such as, addition of elevated levels of osmotic agents or ABA in addition to storage under minimal growth conditions such as low temperature and dark incubation. Cryopreservation is another conservation technique that is usually described as the most reliable tool for long-term storage of plant germplasm and reported to be advantageous over most other conservation methods in terms of simplicity, applicability to a wide range of genotypes and ability to maintain the genetic stability of plant material. Thanks to the use of cryopreservation techniques, such as, encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet-vitrification, many medicinal plant species are conserved indefinitely for the next generations.

**Keywords:** Cryopreservation, Encapsulation-dehydration, Encapsulation-vitrification, Droplet-vitrification, Medicinal plants, Osmotic agents, Slow growth conservation, Vitrification.

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Meanwhile, medicinal plants are under real jeopardy due to the uncontrolled collection of this medicinal plant as a result of the growing reliance on herbal medicine along with extensive use in food and beverages industry (Pitman and Jorgensen, 2002; Akine et al., 2010).

Moreover, knowing that 70% of the world’s plants are currently on the Red List of endangered plants is extremely shocking (Paunescu, 2009) especially that many of these plants are medicinal herbs (Paunescu, 2009). Over-collecting, unsuitable agriculture and forestry practices, urbanization, pollution, habitat destruction and degradation, and climate change are the leading causal agents behind biodiversity loss and fragmentation (Pitman and Jorgensen, 2002). Jordan flora, is also exposed to fragmentation and loss due to urbanization, deforestation, and deterioration of rangelands by over-grazing in addition to depletion of the major water resources (Al-Esawi, 1998). Moreover, a red list is expected to be released in 2014 by World Conservation Union with big fears that a huge number of Jordan flora might be on the list (Royal Botanic Garden (RBG), 2012).

So in view of the importance of these plants in the health care, food and phytoindustry, it is imperative to have a global concern about the loss of such valuable herbs, and to establish new programs for the conservation of these plants. This should be in combination with strict full legal measures for efficient protection of these valuable genetic resources (Shamra et al., 2010).

**History of Medicinal and Aromatic Plants**

Medicinal and aromatic plants are those containing special chemical profiles that enable them to relief pain, release pleasant aromas, and improve food flavors (Foster and Anderson, 1978; Friedman and Adler 2001; Craker, 2007). How medicinal plant and aromatic production had initiated is lost to history, but it’s assumed that it started once man had recognized that smelling, chewing, and/or eating these plants was able to calm pain down or even cure from some diseases (Craker, 2007). So presently, these plants are known as medicinal and aromatic plants, and they became the main constituent for medicines, colorings, preservatives, and other similar items.

Records have reported that people had been using medicinal and aromatic plants since the Paleolithic age (50,000 B.C.) (Chadwick and Mann, 1950). Moreover; Sumerians, Assyrians and Acadians had classified many of these plants long time ago (3000 B.C.) according to their use such as healing plants, pain plants and illness plants as it was found many years later in their survived clay tablets (Mert et al., 2008; Ozturk, 2012). Later on (400B.C) the use of medicinal plants became a backbone in Unani medicine which is considered the root of all traditional medicine in the middle east and other Asian countries such India and China (Chadwick and Mann, 1950; Foster and Anderson, 1978).

Meanwhile, there were no chemical basis for the use of these plants in medicine until 19th century. But a rapid decline in the use of plants in medicine at the beginning of the 20th century was caused due to technological innovations and political and social forces. This happened precisely by the development of sulfa drugs in the 1930s and the manufacturing of organic chemicals in the 1940s which became the adapted method for treatment in some countries, especially in the United States (Craker et al., 2003).

However, a “U-turn” to the use of herbs in medicine has started in the 1960s driven by demographic, financial and health reasons, like the presence of populations of Asian and Hispanic origin in the USA (Craker, 2007). Also the fact that average consumers became more knowledgeable and interested in organic
and natural foods in addition to the benefits of using medicinal and aromatic plants instead of synthetic medicine due to both education and media (Powers, 2006).

**Importance of Medicinal and Aromatic Plants**

There are nearly 50,000-80,000 flowering plant species classified as medicinal plants (Ozturk 2012; Sharma, *et al.*, 2010) with 500-1000 taxa that are used in folk medicine (Ozturk 2012). In Jordan about 485 species belonging to 99 different families were reported as medicinal plants (Al-Eisawi, 1996; Al-Qura’n, 2011; Royal Botanic Garden (RBG), 2012). Medicinal plants are the predominant constituents of medicines in most medical traditions (Sharma, *et al.*, 2010). Not that but also, these plants are currently in use as pharmaceuticals, herbal remedies, dietary supplements, homeopathies, medicinal and herbal teas, sweets, perfumes, cosmetics, coloring agents, varnishes, fireworks, detergents and other plant-based products (Lange, 2004).

Additionally, a great development in both systematic and methodological approaches in research was witnessed in last two decades to highlight many new plants with novel properties to be used commercially (Baser, 2005). However, due to the growing demand for such plants a quantum jump in global sales for medicinal and aromatic plants is estimated to reach more than 65 billion US$ (Ozturk, 2012).

**Threats Facing Medicinal and Aromatic plants**

The World Conservation Union has declared in its latest release that 70% of the world’s assessed plants were under real jeopardy (Paunescu, 2009). Also according to the United Nations Environment Program (UNEP) estimates, by the year 2032 more than 70% of the land’s surface will be destroyed or disturbed which leads to the fragmentation of the many habitat resulting in further loss of plant populations (Paunescu, 2009). Combinations of many factors such as, factors: over-collecting, unsuitable agriculture and forestry practices, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species and climate change are comprising the major sources of threats for these plants (Craker, 2007).

In Jordan, a red list is expected to be released by 2014 by World Conservation Union with big fears that a huge number of Jordan flora might be on the list (Royal Botanic Garden (RBG), 2012).

Medicinal plants are not far from such threats due to over harvesting and destruction of habitat, population growth, urbanization and the unrestricted collection of medicinal plants from the wild that ends with over-exploitation of these natural resources (Al-Qura’n, 2011). More than 70% of the plant collections involve destructive harvesting due to the use of specific parts like roots, bark, wood, stem and the whole plant in case of herbs which comprises a definite threat to these valuable genetic stocks (Shamra *et al.*, 2010). Moreover, the increase in demand for medicinal and aromatic plants is expected to continue threatening many species taking in account 90% of medicinal plants that are used by industries are collected from the wild while 20 species of plants are under commercial cultivation (Craker, 2007; Shamra *et al.*, 2010).

The differences in prices between wild and cultivated plants due to the high demand for the wild material or the lack of offered plant material has also resulted in destructive collection practices for medicinal plants (Craker, 2007) keeping in mind the financial gains that represent a none negotiable share of total income for many medicinal plant collectors in many localities (Schippmann, *et al.* 2005). Therefore taking strict full legal measures and establishing new programs for protecting medicinal plant resources have become a matter of urgency (Shamra *et al.*, 2010).
Conservation of Medicinal and Aromatic plants

Due to the novel value of the medicinal plants, conservation action for these natural resources is of high priority. Therefore, ensuring wild-collection of these plants sustainably can be achieved through the development and implementation of management programs that monitor trade and guideline a sustainable collection, must get adequate global support (Lange, 2004; Walter, 2002). However the growing global concern of loosing these valuable genetic resources has stimulated many methods for the conservation of medicinal plant genetic resources.

In Situ Conservation

It’s well known that conservation of wild life is almost based on in situ conservation (Reed et al., 2004). This type of conservation can be practiced in farmers’ fields in case of domesticated materials or in natural environments for both wild relatives and wild species (Gepts, 2006). This method was supported mainly by environmentalists and conservationists, who are dedicated mainly for the conservation of ecosystems and species diversity (Gepts, 2006). Also in situ conservation is encouraged because of many reasons. It’s directed to the conservation of landraces which comprise an essential component of indigenous cultures, it allows plant evolution to proceed, its cost is low, and it is the primary approach of conservation for wild relatives (Gepts, 2006). In situ protection is also practiced as a measure for threatened medicinal and aromatic plants conservation in their natural habitats; for example, in India there are 36 nature reserves for medicinal plants (Lange 2004).

Despite of in situ conservation benefits it is almost impossible to adopt only this approach for conservation due to the disappearance of large wild areas (Englemann, 1991). Moreover, plants conserved under such natural conditions are exposed to natural disasters, pests and pathogens in addition to the fluctuating government policies and urban development (Younes, 2012).

Ex Situ Conservation

Ex situ conservation (off-site) stands for the conservation and maintenance of plants outside their natural habitat as samples which takes a form of whole plants, seed, pollen, vegetative propagules, tissue or cell cultures (Paunescu, 2009). Ex situ conservation methods of these plant samples is usually practiced in botanic gardens, seed storage, and in vitro cultivation (Ramsay et al., 2000; Paunescu, 2009). There are 2204 botanic gardens in the world that hold in secure more than one third of the world’s flowering plants (BGCI Report, 2001)

Comparing to in situ conservation, ex situ conservation is able to store larger number of accessions in a collection which are ready to be accessed for characterization, evaluation and distribution in addition to higher security level that guarantees a safe conservation of large numbers of accessions (Shibli et al., 2006). Meanwhile, ex situ conservation is very difficult to be applied as adequate samples have to be collected for the conservation of genetic diversity which can reach several hundreds for gene pool conservation and to 5,000-20,000 plants to maintain heterozygosity (Englemann, 1991) which makes land space requirement a none negotiable element especially in the case of forest trees (Paunescu, 2009). Additionally, labour, costs and trained personnel requirements are comprising additional major limitations for ex situ conservation (Shibli et al., 2006).

In Vitro Conservation

In vitro culture represents a wide range of techniques including growing plant parts such as shoot tips, meristems, somatic embryos or embryogenic callus under aseptic conditions (Vasil and Vasil, 1972; Pierik, 1997). Not only in vitro culture techniques have facilitated crop improvement and mass propagation, but also it has yielded great achievements in the scale of
Slow Growth Conservation

Slow growth conservation is a very simple in vitro technique that permits conservation plants material for periods ranging from 6 months to 5 years, depending on species (El-Dawayati et al., 2012). This technique is based on reducing the growth rates of the tissue cultured plant and yet increasing the intervals between subcultures (Englemann, 1991). Three basic methods are used in this technique including physical (reduced temperature and light conditions), chemical (using growth retardants such as osmotic agents and abscisic acid, limiting the availability of carbohydrate to sub-optimal levels), (Engelmann, 1997; Paunescu 2009) and may be used in combinations (Sahijram and Rajasekharan, 2004).

Slow growth conservation of plant germplasm has been widely applied and practiced for the conservation of many medicinal plants such as Ocimum species (Mandal et al., 2000), Adhatoda vasica (Anan and Bansal 2002), Phyllanthus amarus (Singh et al., 2006a), Artemisia herba-alba (Sharaf, et al., 2012) Achillea fragrantissima (Younis, 2012), Teucrium polium L. (Rababa’a, 2010), and Cannabis sativa L. Hemant et al., 2012). However investigating the influence of slow growth conservation on the chemical profile of the in vitro conserved plant material is an important prerequisite for any medicinal or aromatic plant containing natural products of pharmaceutical interest (Hemant et al., 2012).

Slow Growth Conservation using Osmotic Agents:

Plants growth and physiology are highly influenced by carbon source type and content in all tissue culture phases including conservation (Yun-peng et al., 2012). When carbohydrates are added to a culture medium they serve both as carbon sources and as osmotic stressors as they tend to reduce water potential and restrict water availability to the explants ( Shibli et al., 2006). This will hinder cell elongation and division, restrict growth and yet prolong the storage time (Yun-peng et al., 2012.). Sucrose and mannitol, are common osmotic stressors that serve as growth retardants by causing osmotic stress to plant material under conservation (Shibli, et al., 2006). Moreover, the responses to these osmotic agents when added to the storage media were reported to be species dependent (Silva and Pereira 2011).

Sucrose

Sucrose is considered the most suitable energy source for plant in vitro propagation (Yun-peng et al., 2012.). It comprises the major proportion of most tissue culture media comparing to the other constituents (Shibli, et al., 2006). Also, sucrose could act as an osmotic agent when used at high concentrations in the growth media. Using sucrose as osmotic agent was reported to reduce growth rate of the in vitro grown explants. As sucrose level in the media
increases this would lead water content of the plant cell to decreases, and yet the cells volume decreased (Taiz and Zeiger, 2002). Consequently, this decrease in cell volume would result in lower turgor pressure in the cells, and as cell expansion is a turgor-driven process, loss of turgor would result in growth reduction (Taiz and Zeiger, 2002).

Elevated levels of sucrose were successfully used to reduce growth in vitro in many plant species (Bertrand-Desbrunais, et al., 1992). For example in Phoenix dactylifera callus culture, 0.1 M sucrose was able to extend subculture intervals and to yeild 86.7% survival after 12 weeks of conservation (Shibli, et al., 2005). Also Moges, et al., (2003) had used elevated levels for slow growth conservation of Saintpaulia ionantha microshoots. Moreover, Gopal, et al., (2010) had used sucrose for the conservation of Saintpaulia ionantha. Microshoot elongation was found to decrease in Solanum tuberosum (Sarkar and Nail, 1998) and Pyrus syriaca (Tahtamouni, et al., 2001) as concentration of sucrose increased in the medium. More over using (0.2 M) sucrose was highly recommended for in vitro conservation of many plant species due to its ability to reduce growth and to maintain recovery after storage at full capacity (Rabbaa, et al., 2012; Sharaf et al., , 2012, Tahtamouni, et al., 2001; Younis, 2012).

**Mannitol:**

Mannitol is a sugar-alcohol which was also used as an osmotic stressor for slow growth conservation in many plant species (Shibli, et al., 2006; Tahtamouni, et al., 2001). It has been used to mimic osmotic stress assuming that its only occasionally metabolized by tissue cultured plants (Yun-peng, et al., 2012.). Mannitol was also able to suppress shoot growth of Chrysanthemum morifolium (Shibli, et al., 1992). In Pyrus syriaca physiological disorders were reported to increase and conservation period were extended as mannitol concentration increased in the medium (Tahtamouni, et al., 2001). Additionally Rabbaa et al. (2012) reported that using (0.1 M) mannitol was able to reduce growth and extended subculture interval in (Teucrium polium L.) but it had adversely affected regrowth as only (36.3%) of the explants had recover after conservation. Similar responses were also reported by Sharaf et al. (2012) and Younis (2012) when they stored A. herba-alba and A. fragransima respectively at same mannitol level. On the other hand mannitol was found not to support tissue growth in Nicotiana tabacum (Lipavska and Verugdenhil, 1996).

**Sorbitol**

Sorbitol is a sugar alcohol, which is systemically used in slow growth conservation protocols (George, et al., 2008). When added at elevated levels sorbitol changes water potential of the culture media, and consequently as sorbitol is taken up by the plants growth rate is reduced in response to cellular osmotic regulation (Pierik, 1997). Sorbitol was reported to inhibit shoot growth of Chrysanthemum morifolium (Shibli, et al., 1992). Also when used as an osmotic agent, sorbitol decreased Saintpaulia ionantha shoot height irrespective of its concentration (Moges, et al., 2003). Sorbitol has also reduced growth in Amygdalus communis and extended the subculture intervals to up to 4 months.( Shibli, et al., 1999). Also sorbitol at (0.1 M) concentration was found effect in decreasing growth in (Teucrium polium L.) and A. fragransima respectively as reported by Rabbaa et al. (2012) and Younis (2012).

Additionally, a complete fail in survival and regrowth was reported in calli of Phoenix dactylifera (Shibli, et al., 2005) when 0.2 M sorbitol was used for conservation. Simillar results were obtained when sorbitol concentration in the media exceeded (0.1 M) in (Teucrium polium L.) and A. fragransima respectively (Rabbaa et al., 2012; Younis, 2012). Moreover, Tahtamouni, et al. (2001) reported physiological
disorders in wild pear that were positively correlated to the level of sorbitol as the conservation period of wild pear was prolonged which agrees with Moges, et al. (2004) results on Saintpaulia ionantha when microshoots were conserved on 0.49 M or 0.66 M sorbitol for 12 weeks. Meanwhile, high death percentages of explants conserved under elevated levels of sorbitol were reported in many plants which was due to the continuous accumulation of carbohydrates in the cells that might have reached toxic levels (Moges, et al., 2004; Shibli, et al., 2005; Tahtamouni, et al., 2001).

**Slow Growth Conservation Using ABA:**

Abscisic acid is an endogenous growth retardant and it was often used for growth reduction of in vitro conserved cultures (Gopal et al., 2004). It serves as both a signal and a response to many stresses and yet increases plant tolerant to these stresses by dehydration resistance (Finkelstein, et al., 2002). For example, ABA was found to regulate expression of many genes that are responsible for the syntheses of proteins needed for osmotic adjustment in the cell, such as, membrane stabilization proteins, and the LEA proteins that would modify water state in the cell to cope with osmotic stress (Taiz and Zeiger, 2002). Also, during storage, ABA tends to modify carbohydrate metabolism of cells to increase the ability of conserved tissues to sustain higher residual water content and yet to decreases the adverse effects of osmotic stress and dehydration (Shibli, et al., 2004; Shibli, et al., 2006). ABA was successfully used to reduce the growth of shoots of potato, grape, persimmon and date palm (Westcott, 1981; Roca et al., 1982; Ai and Luo, 2004 Yun-peng et al., 2012). Also using (3.8 μM) ABA was reported to reduce growth in microshoots of (Teucrium polium L.) and A. frarantissima respectively (Rabbah et al., 2012; Younis, 2012). Moreover, Lilium oriental hybrids microshoots were reported to be conserved for more than nine months at normal growth room conditions in the media supplemented with 1.0 to 3.0 mgL-1 ABA (Chen et al., 2006 Yun-peng et al., 2012). However, these authors reported that ABA was lethal to some plant species such as pepper (Silva and Pereira, 2011), sweet potato (Gopal et al., 2004) and apple (Kovalchuk et al., 2009).

**Slow Growth Conservation using low temperatures:**

Storage under low temperature conditions is another slow growth conservation technique that is used for the conservation a wide range of plants (Engelmann, 1991). Under low temperature conditions, growth rate is reduced as many biochemical activities are slowed down, such as, carbohydrate translocation, respiration rates and protein synthesis (Taiz and Zeiger, 2002). Meanwhile, the origin of stored species usually determines how cold the storage temperature should be (Engelmann, 1991; Paunescu, 2009). For example, temperate species could be stored at 4°C, while the tropical plants are usually stored at 15-20°C (Paunescu, 2009). Many plants were successfully conserved under low temperature conditions, such as, potato (Westcott, 1981), Kiwi (Monette, 1986), cassava (Roca et al., 1984) and oil palm (Corbineau et al., 1990).

**Long- Term Conservation (Cryopreservation)**

Cryopreservation is usually described as the most reliable tool for long-term storage of plant germplasm (Sakai and Englemann, 2007). This conservation method offers a unique opportunity for the long-term preservation of endangered species as well as cultured cells and somatic embryos with unique attributes (Sakai and Englemann, 2007). The principle of cryopreservation, is the storage of plant material at ultra low temperature (-196°C) that takes a place in a cryogenic condition which is liquid nitrogen (Wen and Wang, 2010). At this temperature, all forms of cellular divisions and metabolic activities of plant cell are ceased and consequently plant material can be stored
unprotected for an indefinite time scale (Gonzalez-Arnao et al., 2008; Shatnawi, et al., 2011). As a technique, cryopreservation is based on eradication of all freezeable water from tissues using physical or osmotic dehydration, followed by ultra rapid freezing (Rees et al., 2004). As a result, water removal comprises a backbone in preventing freezing injury and in retaining post-thaw viability (Gonzalez-Arnao and Engelmann, 2006). Crystallization during freezing is considered the main factor affecting survival of cells subjected to cryopreservation. As ice crystals are formed during warming procedure it becomes detrimental to cellular integrity. Due to slow warming the tendency for large ice crystals to grow becomes greater and yet higher rates of recovery will be achieved (Gonzalez-Arnao et al., 2008). There is a series of successive, potentially stressing events that should be practiced to obtain a successful cryopreservation includes submitting selected explants during preculture, cryoprotection, dehydration, rapid immersion in LN, thawing, recovery, and plant regeneration (Reed, 2001, 2008).

Cryopreservation was always reported to be advantageous over most other conservation methods in terms of simplicity and the applicability to a wide range of genotypes (Paunescu, 2009). Moreover, the cryogenic storage is practiced in a small volume, which excludes contamination risks and minimizes maintenance requirements (Amir, et al., 2011 Younis 2012). In most base storage strategies cryopreservation is usually adopted as its safe and cost-effective for long-term preservation.

**Cryopreservation Techniques:**

Recently, most of cryopreservation researches had focused on the development of techniques that are simple and reliable and would be applicable to various plant material types such as cultured cells, shoot tips and somatic embryos (Sakai and Englemann, 2007). This had outcome different procedures for cryopreservation, like encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet-vitrification (Engelmann, 2004).

**Encapsulation-Dehydration:**

This method, developed by Fabre and Dereuddre (1990) in which shoot tips, somatic embryos or callus cells are encapsulated within alginate beads (Kaczmarczyk et al., 2008) and subsequent culture in a medium containing elevated concentrations (0.7–1.5 M) of sucrose(Gonzalez-Arnao and Engelmann, 2006; Shatnawi, et al., 1999, Younes, 2012) for 1 to 3 days (Figure 1). The beads are then allowed to dehydrate using silica gel or by air under the laminar air flow until the moisture content drops to 20-30% (Fabre and Dereuddre, 1990), before being immersed in LN (Figure 1). Encapsulation-dehydration is practiced in a large scale because it is simple, inexpensive and provides a high level of genetic stability (Shibli, et al., 2006; Shatnawi, et al., 2011). Also the encapsulating material keeps the tissue in a vitrified state that will reduce the hazard of ice crystal formation (Scottez et al., 1996).

Additionally in this technique mechanical damage is minimized due to the protection provided by the beads to the tissues and during handling. Another advantage of this method is eliminating the use of high concentrations of cryoprotectants in addition to the nutritive nature of the bead, which will promote survival and regrowth of the cryopreserved explants (Panis and Swennen, 2005). However, high survival rates had been recorded in several species after freezing when water contents of the beads was reduced to 20% (Cho, et al., 2002; Engelmann, 1997; González-Benito, et al., 1998; Shatnawi, et al., 1999, 2004; Reed, 2008). In Jordan...
many plant species were successfully cryopreserved using encapsulation dehydration such as; *Amygdalus communis* shoot tips (Al-Ababneh, *et al.*, 2002), *Artemisia herba-alba* shoot tips (Sharaf, *et al.*., 2012.), *Capparis spinosa* shoot tips (Shatnawi, 2011), embryonic callus of *Crocus hyemalis* and *Crocus moabiticus* (Shibli, *et al.*., 2009), *Phoenix dactylfera* embryogenic callus (Subaih, *et al.*, 2007), and *Teucrium polium* shoot tips (Rabba’a, *et al.*, 2012). Meanwhile, compared to the other cryopreservation methods, encapsulation-dehydration technique requires much long time to be applied which comprises a major disadvantage for this method.

![Diagram](image.png)

**Figure 1:** Steps of encapsulation-dehydration procedure:

A: Shoot tips isolation and preculturing.
B: Encapsulation of precultured shoot tips then incubation on sucrose supplemented MS liquid media.
C: Air dehydration under laminar.
D: Transfer of dehydrated shoot tips to cryovials and plunge into LN.
E: Rapid thawing.
F: Subculture to recovery media.
G: Incubation for several days under dark before transfer to normal light conditions (to avoid exposure to photooxidation)

**Vitrification:**

Vitrification is defined as supercooling a highly concentrated cryoprotectant at ultra low temperature and yet the cryoprotectant will solidify into a metastable glass state without undergoing crystallization (Sakai and Englemann, 2007). Thus, vitrification is an effective freeze-avoidance mechanism. Also as a glass state is attained it will stop all chemical reactions that require molecular diffusion and yet will lead to metabolic inactivity and stability over time (Sakai and Englemann, 2007). Vitrification comprises one of the main and most widely applied plant cryopreservation techniques. The successful application of vitrification-based protocols was firstly reported on somatic embryos and shoot-tips of asparagus.
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(Uragami, et al., 1989), and pear (Dereuddre, et al., 1990).

Vitrification is based on three major phases (Figure 2): the loading phase, dehydration with the highly concentrated vitrification solutions, and unloading phase (Ashmore, 1997). In loading phase samples are exposed to cryoprotectants or diluted vitrification solutions (Withers, 1991) the samples are dehydrated by a highly concentrated vitrification solution before being plugged in LN, (Sakai, et al., 1991).

In unloading phase vitrification solution is drained out of the cryovials after rapid thawing, and then replaced routinely with 1.2 M sucrose for 10-20 min (Shibli, et al., 2006; Shatnawi, et al., 2011). Plant vitrification solution (PVS2) is described as an aqueous cryoprotectant solution in which living material can be cooled without appreciable ice formation either intra or extra-cellularly (Fahy, et al., 1987; Kim, et al., 2006). The protective property of this solution is due to its ability to increases the osmotic potential of the external medium (Reed, 1990). This will allow water to flow out of the cells and dehydration of tissues (Ashmore, 1997). Cryoprotectants must be optimized at a proper concentration to be not toxic, also it should be readily miscible with water, and with high ability to penetrate cells rapidly (Reinhoud, et al., 1995; Shibli, et al., 2006).

Plant vitrification solution 2 (PVS2) is commonly used in most vitrification protocols (Reed, 2008). It is composed of a mixture of cryoprotectants includes 30% w/w glycerol, 15% w/w ethylene glycol, 15% w/w dimethylsulfoxide DMSO in MS medium supplemented with 0.4 M sucrose (Sakai and Engelmann, 2007; Shatnawi, et al., 2004; Rabba’a, et al., 2012). In a vitrification protocol, there are several requirements for successful vitrification such as; tolerance to PVS2 which is obtained by optimizing the preconditioning and loading treatments, as well as the duration and temperature of exposure to the vitrification solution in addition to unloading and post-LN handling of the samples (Sakai and Englmann, 2007). In Jordan several species were reported as cryopreserved successfully using vitrification technique such as, Artemisia herba-alba (Sharaf, et al., 2012), Teucrium polium (Rabba’a, 2010), C. aurantium (Al-Ababneh, et al., 2002), Capparis spinosa (Shatnawi, 2011), Phoenix dactylifera (Subaih, et al., 2007) and Achillea fragrantissima L. (Younis, 2012).

**Figure 2: Steps of vitrification procedure:**

A: Shoot tips isolation and preculturing.
B: Treatment with loading solution and PVS2 solutions.
C: Transfer of treated shoot tips to cryovials and plunged into LN.
D: Rapid thawing, unloading and subculture to recovery media, then incubation for several days under dark before transfer to normal light conditions (to avoid exposure to photooxidation).
Encapsulation-Vitrification:

Encapsulation-vitrification (Figure 3) is the dehydration of explants encapsulated in alginate beads using vitrification solutions before freezing instead of dehydration by air or silica gel (Younes, 2012). In vitrification technique the explants are allowed to freeze within a short time period which is difficult to be applied in case of large number of samples. This is due to the duration of the successive steps of a vitrification protocol that is very short and requires a very precise duration and small sized explants are difficult to manipulate (Sharaf, et al., 2012). On the other hand, the encapsulation-dehydration technique requires much longer time to be applied and manipulating the encapsulated explants is very easy, due to the relatively large size of the alginate beads (Sakai and Englemann, 2007). So it’s a new technique which combines the goods of vitrification and encapsulation-dehydration techniques (Sakai and Englemann, 2007) which makes it relatively suitable for cryopreservation on a large scale (Hirai and Sakai, 2000; Rabbaa, 2010; and Sharaf, et al., 2012). An increasing number of species were cryopreserved successfully using encapsulation-vitrification including yam (Hirai and Sakai, 2001), Ananas comosus (Gamez- Pastrana, et al., 2004), Ipomea batatas (Hirai and Sakai, 2003), Manihot esculenta (Charoensub, et al., 2004b) Artemisia herba-alba (Sharaf, et al., 2012 ), Teucrium polium (Rabba’a, 2010), C. aurantium (Al-Ababneh, et al., 2002), Capparis spinosa (Shatnawi, 2011), Saintpaulia ionantha (Moges, et al., 2004), Phoenix dactylifera (Subaih, et al., 2007), Achillea fragrantissima L. (Younis, 2012) and many other species (Sakai and Engelman, 2007). Not only that but also better results were reported when this technique was applied in comparison to encapsulation-dehydration method in terms of rate of shoot formation and speed of growth (Hirai and Sakai, 2000; 2001).

Figure 3: Steps of encapsulation- vitrification

A: Shoot tips isolation and preculturing.
B: Encapsulation of precultured shoot tips then treatment with loading solution and PVS2 solutions.
C: Transfer of treated shoot tips to cryovials and plunge into LN.
D: Rapid thawing, unloading and subculture to recovery media then incubation for several days under dark before transfer to normal light conditions (to avoid exposure to photooxidation).

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**Droplet Vitrification:**

This technique was developed by Kartha et al. (1982) in which the shoot tips of cassava shoot tips were placed in droplets of cryoprotective medium before ultracooling (Sakai and Englemann, 2007). In this technique shoot tips are plated on aluminium foils and dehydrated with cryoprotectants, such as PVS2. Using aluminium foils facilitates the transfer of shoot tips into and out of LN which can be beneficial during application of PVS2 as slightly longer incubation times can be toxic for shoot tips (Kaczmarszyk et al., 2011). Shoot tips introduced to droplet-vitrification protocol, are loaded, treated individually with 5-10 μl droplets of PVS2 on a piece of aluminium foil, which is then immersed in LN (Figure 4). After warming, the aluminium foils are plunged in liquid medium containing 1.2 M sucrose for 20 min unloading and then placed on recovery medium (Figure 4). The major achievement of this technique is the possibility of obtaining very high cooling/warming rates due to the very small volume of cryoprotective medium in which the explants are placed (Sakai and Englemann, 2007).

Although its a very recent technique which has been applied until now to a limited number of plant species only (Sakai and Englemann, 2007) this protocol was reported as successful when applied to some plant species such as several thymus species (*T. moroderi, T. longicaulis* and *thymus vulgaris*), potato, sweet potato, chicory, strawberry, pelargonium, date palm, thyme, olive and shih (Panta et al., 2006, Gallard et al. 2008, Sanchez-Romero et al., 2009, Marco-Medina et al., 2010, Ozudogru and Kaya, 2012, and Sharaf et al., 2012).

**Figure 4: Steps of droplet vitrification procedure:**

A: Shoot tips isolation and preculturing.
B: Treatment with loading solution and PVS2 solutions.
C: Transfer of treated shoot tips on the aluminum strips to cryovials and plunge into LN.
D: Rapid thawing, unloading and subculture to recovery media, then incubation for several days under dark before transfer to normal light conditions (to avoid exposure to photooxidation).
Genetic Stability after Cryopreservation

Besides conservation, cryopreservation aims to maintain the genetic stability of plant material. Metabolic activities are theoretically ceased once the explant is stored cryogenically and consequently plant material is expected to be true to type after rewarming from cryopreservation (Panis et al. 2002 Kaczmarczyk et al., 2012). So, validity of cryopreservation is only achieved if genetic stability is unchanged after exposure to liquid nitrogen (Zarghami et al., 2008). As a result, only differentiated tissues such as shoot tips are targeted as plant material for cryogenic storage instead of undifferentiated plant material like to avoid any genetic alteration (Benson et al., 1996; Harding, 2004). Evaluation of plant genetic uniformity to validate newly established cryopreservation protocols was carried out as some evidence of epigenetic alterations after cryopreservation of in vitro-derived plant material was noticed (Harding 2004; Micula et al., 2011). Unlike genetic changes; the original DNA sequence is not altered by epigenetic modifications as these modifications are usually featured with alterations in DNA methylation (Boyko and Kovalchuk 2008; Smulders and Klerk, 2011). Although these alterations may affect gene transcription; epigenetic modifications are usually temporary and plants might get back to their normal phenotypes relatively easily (Smulders and Klerk, 2011). Still, few epigenetic alterations might be long lasting and may even be inherited to offsprings during sexual propagation (Brettell and Dennis 1991). DNA methylation influences plant vigor and morphogenesis and is highly affected by environmental conditions and stresses during tissue culture and cryopreservation (Chen et al., 2011).

Examples on different genotypes that were reported with epigenetic variation after cryopreservation are Carica papaya (Kaity et al., 2008), Fragaria gracilis (Hao et al., 2002a), Citrus sinensis (Hao et al., 2002b), Malus pumila (Hao et al., 2001), Ribes (Johnston et al. 2009), Carica papaya (Kaity et al., 2008, 2009) and Prunus dulcis (Channuntapipat et al., 2003).

Several molecular markers have been used for the assessment of genetic stability of plants (Harding 2004). The most popular markers used in this domain are randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) (Micula et al., 2011). However, sometimes more than one molecular marker was used, e.g. RAF (randomly amplified DNA fingerprinting) and AMP (amplified DNA methylation polymorphism) for Carica papaya (Kaity et al. 2008, 2009), SSR (simple sequence repeats) for (Quercus suber L.) (Fernandes et al., 2008) in addition to AFLP and MSAP (methyl sensitive amplified polymorphism) for Malus pumila and Fragaria gracilis (Hao et al. 2001, 2002a).

Conclusion

Medicinal and aromatic plants became the main constituents for medicines, food and other phytindustries. Meanwhile these plants are endangered due to their extensive use in medicine, food and beverages industry. So, conservation of these natural resources is of high priority. In situ, ex situ and in vitro conservation approaches are comprising the three pillars of the conservation plan triangle for medicinal and aromatic plants. In vitro conservation is offering a package of techniques that are widely used for the conservation of plant germplasm. Slow growth conservation is a tissue culture technique based on reducing the growth rates of the stored explants by modifying the growth media composition (addition of elevated levels of osmotic agents or ABA) or storage under minimal growth conditions, such as, low temperature and dark incubation. Moreover, cryopreservation is consist of a collection of
conservation techniques (encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet-vitrification) that are simple, applicable to a wide range of genotypes and able to maintain the genetic stability of the stored plant material.

REFERENCES


In Vitro Conservation... Reham W. Tahtamouni et al

Jordan.


Roca W.M., R. Reyes and Beltran, J. 1984. Effect of various factors on minimal growth in tissue culture storage of cassava germplasm. Tropical Root Crops, 441-446.


In Vitro Cellular and Developmental Biology - Plant, 40: 403-407.
Westcott, R. J. 1981. Tissue culture storage of potato germplasm 2: Use of growth retardants. Potato -165-
Research. 24: 343-352.
حفظ النباتات الطبية والعطرية داخل الأنبوب والتمجيده بالنيتروجين السائل: مراجعة

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ملخص

يتضمن النباتات الطبية والعطرية على العديد من المواد الكيميائية التي تعمل على تخفيف الألم وإطلاق الروائح الطبية وتحسين النكهة. ت تعرض النباتات الطبية والعطرية لخطر حقيقي بسبب الجمع العشوائي لاستخدامها في طب العشاق والصناعات الغذائية بشكل كلف، لذا يعد حفظ هذه الأصول الطبيعية أولوية كبيرة. إن حفظ الأصول الوراثية الطبيعية داخل موائعها يعرضها لكارثة طبيعية كالإنهيارات والأمراض إضافة إلى التقلبات السياسية للحكومات، كذلك يعد حفظها خارج موطنها في غاية الصعوبة نظراً إلى الحاجة لأعداد كافية من هذه الأصول لحقوق على تنوعها الحيوي. تقدم طريقة الحفظ داخل الأنبوب زراعة متعددة من النباتات التي تعد ناجحة جداً عندما تفشل الطرق الأخرى. تعد طريقة الحفظ البسيطة للنمو طريقة سهلة تتم على إعطاء النمو للأقسام النباتية لسدة صغيرة أو متواضعة وبالتالي إطالة المدة الزمنية بين كل عملية إعادة للزراعة. في هذا النوع من الحفظ، تعتبر عدة تقنيات متفردة أو مجمعه في إعطاء النمو للأجزاء النباتية المحفزة مثل تراكيب عالى من المواد الأسموزية أو إضافه إلى التخزين تحت ظروف مفيدة للنمو مثل التخزين في الحالة المنخفضة أو في الظل، كذلك تعد ABA طريقة الحفظ بالتمجيده داخل النيتروجين السائل الطريقة الأفضل لحفظ النبات في المدى الطويل وذلك لتسهيلها وامكانيتها تطبيقها على مدى واسع من الأنواع الوراثية، إضافة إلى حفظها على النبات الحياني للنبات، وفضل استخدام تقنيات الحفظ بالتمجيده داخل النيتروجين السائل (الكوسة والتمجيده، الترجم، الكبسولة، الترجم بالنقاط) تم حفظ العديد من النباتات الطبية لوقت غير محدود للأجيال القادمة.

الكلمات الدالة: الحفظ بالتمجيده السائل، الحفظ بالنيتروجين والكبسولة، الحفظ بالتمجيده والكبسولة، حفظ-التمجيده عن القطرات، النباتات الطبية، المعالبات الأسموزية، الحفظ البسيط للنمو، الحفظ بالتمجيده.

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