REVIEW

ORCHID CRYOPRESERVATION

Criopreservação de orquideas

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ABSTRACT

Orchids are lush and highly valuable plants due to their diversity and the beauty of their flowers, which increases their commercialization. The family Orchidaceae comprises approximately 35,000 species, distributed among more than 1,000 distinct genera and 100,000 hybrids, totaling approximately 8% to 10% of all flowering plants. With the advance of agriculture and the constant destruction of their natural habitat, orchid species are collected in an indiscriminate manner by collectors and vendors, and this extractive activity threatens many species with extinction, drastically reducing their genetic variability in nature. Therefore, it is essential to seek alternatives that make the preservation of such species feasible using techniques with low maintenance costs that provide greater storage time and that enable good phytosanitary conditions for the plant material for commercial use. Cryopreservation involves the conservation of biological materials at ultra-low temperatures, generally in liquid nitrogen at -196 ºC or in its vapor phase at -150 ºC. This is the only technique currently available for the long-term preservation of the germplasm of plant species that are vegetatively propagated or that have unviable, recalcitrant or intermediate seeds. The objective of this bibliographic review is to report on the importance, methods and application of cryopreservation for orchids. According to the studies reviewed, this is an incipient, developing and relevant field that generates a lot of discussion and requires further research relative to the type of treatment to use for cryopreservation and the methodology to be applied according to the species. The types of methods that are used for cryopreservation and the large variation in the responses of orchids to the cryopreservation methods observed in this study emphasize the need for the development of more appropriate protocols for the preservation of orchids.

Index terms: Preservation, Orchidaceae, *ex situ* conservation, freezing tolerance, extinction.

RESUMO

As orquídeas são plantas exuberantes e apresentam alto valor agregado devido à diversidade e à beleza de suas flores, o que incrementa a sua comercialização. A família Orchidaceae compreende cerca de 35.000 espécies, distribuídas em mais de 1000 gêneros distintos e 100.000 híbridos, totalizando cerca de 8% a 10% de todas as plantas com flores. Com o avanço da agricultura e a constante degradação do seu habitat natural, as espécies de orquídeas são coletadas de maneira indiscriminada por colecionadores e comerciantes. Este extrativismo ameaça muitas espécies de extinção reduzindo drasticamente a sua variabilidade genética na natureza. Portanto, torna-se fundamental a busca de alternativas que viabilizem a preservação destas espécies, com a utilização de técnicas que apresentem baixo custo de manutenção, que proporcionem maior tempo de armazenamento, e que possibilite ao material vegetal boas condições fitossanitárias para utilização comercial. A criopreservação compreende a conservação de material biológico a temperaturas ultrabaixas, geralmente em nitrogênio líquido a -196 ºC, ou em sua fase de vapor a -150 ºC. Esta é a única técnica disponível atualmente para a preservação a longo prazo do germoplasma de espécies de plantas que são propagadas vegetativamente ou que possuem sementes inviáveis, recalcitrantes ou intermediárias. Esta revisão bibliográfica objetivou reportar a importância, métodos e aplicação do método de criopreservação para orquídeas. De acordo com os trabalhos revisados, pode-se verificar que essa é uma área relativamente nova e importante e encontra-se em pleno desenvolvimento, o que gera bastante discussão e requer ainda muita pesquisa em relação ao tipo de tratamento a utilizar para criopreservar e à metodologia a ser aplicada de acordo com a espécie utilizada. Devido à observação, neste trabalho, dos tipos de métodos que são utilizados para criopreservação e das grandes variações nas respostas das orquídeas aos métodos de criopreservação, o desenvolvimento de protocolos mais adequados para preservação das orquídeas torna-se necessário.

Termos para indexação: Preservação, Orchidaceae, conservação *ex situ*, tolerância ao congelamento, extinção.

INTRODUCTION

Orchidaceae represents one of the largest families of the plant kingdom, and its very specialized traits confer high adaptive ability to different environments, with flowers that exhibit marked particularities and perform an important role in attracting pollinators, which consequently favors cross-pollination. Although the diversity of orchids is high in Brazil and in other parts of the world, many species are victims of extractive activities, which have made them vulnerable, endangered or even extinct. Thus, the application of plant tissue culture techniques for the

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conservation of germplasm has made plant material from specific species available for future use.

In vitro germplasm banks of whole plants, derived from cells isolated from plant tissues and organs through the use of tissue culture techniques, constitute a very efficient manner of conservation for orchids, and germplasm collections have assumed a high-priority role in the conservation and preservation of species threatened with extinction. *In vitro* conservation is important because it makes possible the maintenance of cultures in active growth through the periodic subculture of buds and nodal segments. However, this technique allows for the conservation of plant germplasm for only a short period of time: nine to twelve months, depending on the procedure and the plant species. Cryopreservation is an innovative technique that bolsters existing techniques and is necessary for orchid conservation programs, offering adequate conservation and preservation potential as well as safe and good-quality genetic material for both research and industrial uses. This singular technique has the potential to guarantee the long-term conservation of the germplasm of species threatened with extinction and represents a valuable method for the conservation of the genetic resources of many orchid specimens.

The survival ability of plant tissues subjected to cryopreservation is variable, and protocols based on the biochemical and biophysical mechanisms associated with dehydration and freezing with the objective of preventing or minimizing damage are needed. This enables the recovery of the most plant tissue possible based on knowledge of the mechanisms associated with tissue responses to dehydration and freezing.

The use of cryopreservation facilitates the storage and rapid multiplication of plant germplasm in a pathogen-free aseptic environment as well as optimization of physical space and labor, which can also facilitate the exchange of germplasm. This simple method with low initial and maintenance costs, which allows for the storage of biological material for long periods of time, constitutes a technological investment that will provide for considerable growth in the research on and trade in orchids. Orchids are rapidly transforming into one of the primary cultivated ornamental plants and have aroused medicinal and dietary interest. In this context, the objective of this review is to report on the importance, methods and applications of the cryopreservation of orchids.

Family Orchidaceae

Orchids represent the most evolved group of the order Liliales, with approximately 35,000 species that exhibit specialized traits, conferring high adaptive ability to different environments. Orchid flowers are known for their natural beauty and for attracting the interest of various producers, making information about their cultivation and storage increasingly relevant. In addition to their ornamental potential, some genera provide food products such as vanilla, medicines and other products used in industry (Dressler, 1993; Lucksom, 2007; Zanenga-Godoy, 2003).

Orchidaceae are slow-growing plants with a long juvenile period, requiring four to five years on average for the evaluation of flower quality of the progeny and for the acquisition of new seeds. Some species display complex reproductive processes (cross-pollination and specific physiology for seed germination), and these factors hinder their propagation and preservation (Nikishina, 2001). Additionally, because a single capsule can produce more than one million seeds, many times it is impossible to sow all of the seeds and train all of the plants at the same time. This requires the development of preservation methods for maintaining the viability of the seeds for long periods of time. Similarly, the collection and storage of pollen must permit its use in crosses between plants that flower at specific times or places (Carvalho, 2006).

The constant destruction of orchid habitats contributes to the reduction of their genetic variability in nature (Nikishina, 2001). Due to the large number of species that are threatened with extinction (Ibama, 2008), a preservation method is necessary to guarantee their conservation and future use without losing their genetic variation. Plant specimens can be preserved *in situ,* i.e., in their natural habitat; however, this has a high maintenance cost, as it requires large areas of land, and the plants remain vulnerable to abiotic factors such as bad weather, pests and diseases (Santos, 2000).

Cryopreservation Technique

Cryopreservation is a methodology that enables the preservation of biological material (viable cells, tissues and organs) in a non-dividing state with almost zero metabolism. This state is attained through the exposure of the material to ultra-low temperatures as low as -196°C in liquid nitrogen (LN) or in nitrogen's vapor phase at -150°C (Kartha, 1985). At this temperature, molecule movements are drastically reduced, there is no liquid phase in the cells (Vasco, 2003), and all of the metabolic processes such as respiration and enzymatic activity are deactivated, which allows for the assurance of conservation of biological material over long periods because biological deterioration is arrested (Benson et al., 1998). The cells, however, must maintain their structure intact, which will enable them to return to their normal activities after thawing (Bajaj, 1995). This

technique has been shown to be efficient for the storage of various parts of the plants such as seeds, pollen, somatic and zygotic embryos, and plant parts such as roots, bulbs, tubers, buds, and apical meristems, among others (Bajaj, 1995). It also enables the acquisition of whole plants from cells isolated from plant organs and tissues through the use of tissue culture techniques, leading to the establishment of *in vitro* germplasm banks (Engelmann, 1991).

The Importance of Cryopreservation

Cryopreservation is an ideal method for the conservation of germplasm because it enables the storage of biological materials for an indefinite period of time while maintaining their genetic stability and phenotypic characteristics, as well as using little space and requiring little maintenance (Engelmann, 1997). Cryopreservation also facilitates the transport and exchange of germplasm (Vieira, 2000). Among the plant conservation technologies, two related to genomic banks merit emphasis: a) the cryopreservation of cells, and b) cryopreservation of DNA or its fragments (Mattick et al., 1992). In the first case, it is imperative that the obtaining of cells obeys the fundamentals established for sampling the genetic characteristics of populations because the cells preserve the different forms of control of heredity. In the second case, although the entire genetic structure of the population is not preserved, the conserved genetic material is invaluable for genetic improvement activities, primarily those that use biotechnological procedures, as in the case of genetic engineering (Mattick et al., 1992). This methodology is important for the maintenance of pollen viability and fertility for a long period of time and for the improvement and conservation of the genetic resources of plants (Towill, 1985; Carvalho, 2006, Panella, 2009; Chaudhury et al., 2010; Li et al., 2011; Custódio et al., 2011).

Distinct and adapted techniques have been used in different cases, including the cryopreservation of protoplasts, cell suspensions, calluses, apical and lateral buds, meristems, seeds, somatic and zygotic embryos (Table 1) and biotechnological products (cultures for the production of secondary metabolites of economic interest and genetically modified cell lines) constituting valuable methods for the conservation of the genetic resources of plants (Sakai, 1995; Harding et al., 1997).

Cryopreservation optimizes research such that the plant breeder does not need to wait for the growth and flowering of the plant to obtain the male parent. This brings obvious benefits to species that have a long vegetative period, that flower few times during the year or even for some plants that are propagated vegetatively; at the same time, the technique makes genetic material available, thus avoiding the loss of genetic material through conservation (Towill, 2000).

According to the International Board for Plant Genetic Resources - IBPGR (1982), cryopreservation is a recommended technique for species with vegetative propagation, recalcitrant seeds, rare germplasm or even species threatened with extinction and wild plant species. Additionally, conservation in liquid nitrogen can also be used for species of seeds with agricultural importance (Stanwood, 1987). Particularly for orchids, cryopreservation offers an adequate method for long-term storage of genetic material for genetic improvement programs (Vendrame et al., 2007).

One of the benefits of cryopreservation of seeds is an increase in the probability of germination. These results are more pronounced after long periods of storage, which can most likely be explained by the damage that occurs in the membrane; accessibility to nutrients in the medium is greater for an embryo with a damaged membrane than for an embryo with an intact membrane (Nikishina, 2001). Breeders have also considered pollen storage in liquid nitrogen as a means of advancing genetic improvement programs (Carvalho, 2006) because pollen is important for the conservation of genetic material from various species and has been stocked at a number of institutes (Engelmann, 2004). The cryopreservation of pollen enables crosses between plants that flower at different times or plants that grow in different and distant locations. It also lowers transmission rates of diseases for which pollinators can act as vectors and can be used for germplasm conservation for long periods of time (Bajaj, 1995).

CRYOPRESERVATION METHODS

Cryopreservation methods can be divided into stages, such as pre-growth, cryoprotection, cooling, storage, heating, and recovery growth (Withers; Williams, 1998). In the cryoprotection phase, compounds with osmotic activity or other supplements are added to improve freezing tolerance. Cryoprotection is intended to minimize the physical and chemical changes that occur during freezing and thawing (Withers; Williams, 1998) and involves the application of compounds based on dimethylsulfoxide [DMSO], glycerol, sucrose and ethylene glycol (Vieira, 2000). The cooling of biological materials can be performed according to different methodologies, such as the classical methodology (slow freezing) or the contemporary methodology (vitrification, encapsulation-dehydration) (Kartha, 1985; Sakai, 1995, Toribio; Celestino, 2000; Santos, 2000; Sakai et al., 2000; Lopes, 2005; Sakai; Engelmann, 2007).

Table 1 – List of orchid species cryopreserved using different explants and techniques.

Explant	Group/Species	Technique	Survival (%)	Reference
Cell suspension cultures	*Doritaenopsis*	Vitrification	Obtaining of protocorms without morphological variation	Tsukazaki et al., 2000.
Leaf segments	*Aerides odorata* Lour.	Encapsulation -dehydration	-	Hongthongkham;_ Bunnag, 2014.
Immature seeds	*Bletilla striata*	Vitrification	-	Hirano et al., 2004.
Pollen	*Dendrobium* hybrids (D. `Sena Red´, D. `Mini WRL´)	Vitrification	Equal to or greater than 60%	Vendrame et al., 2008.
Pollen and seeds	*Dendrobium* sp.	Vitrification/ PVS2	-	Carvalho, 2006.
Pollen	*Dendrobium*, *Vanda*, *Cymbidium* and *Arachnis*	Vitrification (4–6 º C)	-	Shijun, 1984.
Protocorms	*Doritis pulcherrima*	Vitrification (PVS2)	(0 to 90 min) 0%	Thammasiri, 2000.
Protocorms	*Dendrobium candidum*	Air-drying method dehydration	-	Bian et al., 2002.
Protocorms	*Dendrobium cariniferum*	Encapsulation-Vitrification 20 min PVS2 (25 ± 2°C)	15%	Pornnchuti; Thammasiri, 2008.
Protocorms	*Dendrobium cruentum*	Vitrification 240 min PVS2 (0 ± 2°C)	33%	Thammasiri, 2008.
Protocorms	*Dendrobium cruentum*	Encapsulation - dehydration 50 min PVS2 (25 ± 2°C)	27%	Thammasiri, 2008.
Protocorms	*Dendrobium cariniferum*	Encapsulation/Vitrification 60 min PVS2 (25 ± 2°C)	15%	Thammasiri, 2008.
Protocorms	*Rhynchostylis gigantea*	Vitrification 60 min PVS2 (25 ± 2°C)	19%	Thammasiri, 2008.
Protocorms	*Seidenfadenia mitrata*	Vitrification 80 min PVS2 (25 ± 2°C)	67%	Thammasiri, 2008.
Protocorms	*Dendrobium candidum* Wall.	Encapsulation Vitrification	85%	Yin; Hong, 2009.
Protocorms	*Dendrobium* Bobby Messina	Vitrification 20 min PVS2 (0 ± 2°C)	-	Antony et al., 2010.
Protocorms	*Dendrobium* Bobby Messina	Encapsulation-dehydration	-	Antony et al., 2011.
Protocormslike bodies (PLBs)	*Phalaenopsis bellina*	Encapsulation-dehydration	43,5%	Khoddamzadeh et al., 2011.
Protocorms	*Dendrobium nobile*	Vitrification	68% survival	Vendrame; Faria, 2011.

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Table 1 – continued.

Explant	Group/Species	Technique	Survival (%)	Reference
Protocorms	*Dendrobium* Swartz. hybrid 'Dong Yai'	Vitrification	79% germination (seed)	Galdino et al., 2012.
Protocorms	Dendrobium nobile	Encapsulation–vitrification (EV), Encapsulation-dehydration (ED)	(EV) survival 78.1% and growth 75.9% ED survival 53.3% and growth 50.2%	Mohanty et al., 2012.
Protocorms	*Dendrobium* sonia-28	Encapsulation-dehydration	-	Pouzi et al., 2011.
Protocorms	*Dendrobium* sonia-28	Encapsulation-vitrification (PVS2, 0º C)	-	Ching et al.; 2012.
Seeds	*Anoectochilus formosanus* Hayata	Cross-pollination	90% seed-derived plants	Shiau et al., 2002.
Seeds	*Cattleya aurantiaca*	Room Temperature	40%	Knudson, 1924; 1934.
Seeds	*Dendrobium* and *Brassolaeliocattleya*	Room Temperature	0 (after 3 months)	Kano, 1965.
Seeds	*Dendrobium* and *Brassolaeliocattleya*	Room Temperature (after 3 months, with reduction of water content)	70 and 7 (after 17 months)	Kano, 1965.
Seeds Pollen	*Dendrobium* and *Calanthe*	Temp. -79ºC (Dehydrated)	-	Ito, 1965.
Seeds	Hybrids of *Cattleya* and *Dendrobium nobile*	Temp. -79ºC (Dehydrated or immersed in glycerine)	Germinated well (209 days and 465 days)	Ito, 1965.
Seeds	*Dendrobium phalaenopsis, Phalaenopsis amabilis*	Temp. 25–28ºC	30% (after 20 days)	Limartha, 1975.
Seeds	*Calanthe discolor* x *Calanthe sieboldii* and *Calanthe discolor*	Temp. -15 ºC and -17 ºC	52%, 30% (after 1 year)	Hasegawa et al., 1978.
Seeds	Epiphytic orchids	Temp. -196°C	-	Pritchard HW, 1984.
Seeds	*Encyclia vitellinum*	Temp. - 40ºC	100% (5 weeks)	Koopowitz; Ward, 1984.
Seeds	*Cattleya aurantiaca*	Temp. 20ºC (with water contents of 2.2 and 5.6%)	5% (after 1 year)	Seaton, 1985.
Seeds	*Dactylorhiza fuchsii*	Temp. 62ºC	0% (a few days)	Pritchard, 1985.
Seeds	*Cattleya aurantiaca*	5ºC (water contents of 6.5 and 10.4%)	33-37% (after 6 years)	Seaton; Hailes, 1989.
Seeds	*Dendrobium candidum*	Vitrification (PVS2)	95%	Wang et al., 1998.

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Table 1 – continued.

Explant	Group/Species	Technique	Survival (%)	Reference
Seeds	*Dactylorhiza fuchsii, Dendrobium anosmum, Eulophia gonychila, Paphiopedilum rothschildianum*	Temperatures below zero including (0 to -20º C)	On average, respectively 60% 50% 30% 65% 30%	Pritchard et al., 1999.
Seeds	*Doritis pulcherrima*	Vitrification 50 min PVS2 (2º C)	62%	Thammasiri, 2000.
Seeds	Tropicals orchids	Liquid nitrogen (-196° C)	Equal or greater than (69±3) growth of protocorms	Nikishina et al., 2001.
Seeds	*Bratonia* orchids	Cryopreserved in nitrogen	-	Popov et al., 2004.
Seeds and protocorms	*Dendrobium candidium*	Air drying	Unclear data After freezing from 0 to 3% (with and without ABA)	Bian et al., 2002.
Seeds and protocorms	*Bratonia* sp	Cryopreserved in nitrogen	-	Popova et al., 2003.
Seeds	*Bletilla striata*	Vitrification	82%	Hirano et al., 2005.
Seeds	*Cattleya intermedia, Encyclia pygmaea, E. odoratissima, Grobya* sp.*, Oncidium flexuosum, Oncidium pumilum* and natural hybrid *Laeliocattleya*	Temp. 5 ºC	90% (24 months)	Pardo-Alvarez; Ferreira, 2006.
Seeds	*Oncidium bifolium* Sims	Encapsulation-Dehydration	80% of the protocorms survived after 7 hours of dehydration 11.3% protocorms resulted in plants	Flachsland et al., 2006.
Seeds	*Dendrobium* sp.	Dehydration/PVS2 vitrification	50% higher than the control	Vendrame; Carvalho; Days, 2007.
Seeds and protocorm	Rare orchids	Vitrification	9% protocorms	Nikishina et al., 2007.
Seeds	*Vanda coerulea* Griff. ex Lindl	Vitrification	0%	Thammasiri & Soamkul, 2007.
Seeds	*Dendrobium chrysotoxum*	Vitrification 50 min PVS2 (25 ± 2°C)	99%	Thammasiri, 2008.
Seeds	*Dendrobium cruentum*	Vitrification 30 min PVS2 (25 ± 2°C)	32%	Thammasiri, 2008.

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Table 1 – continued.

Explant	Group/Species	Technique	Survival (%)	Reference
Seeds	*Dendrobium draconis*	Vitrification 30 min PVS2 (25 ± 2°C)	95%	Thammasiri, 2008.
Seeds	*Dendrobium hercoglossum*	Encapsulation-Vitrification 80 min PVS2 (25 ± 2°C)	80%w	Thammasiri, 2008.
Seeds	*Doritis pulcherrima*	Vitrification 50 min PVS2 (25 ± 2ºC)	62%	Thammasiri, 2008.
Seeds	*Rhynchostylis coelestis*	Vitrification 50 min PVS2 (25 ± 2ºC)	85%	Thammasiri, 2008.
Seeds	*Vanda coerulea*	Vitrification 70 min PVS2 (25 ± 2°C)	67%	Thammasiri, 2008.
Seeds and protocorms	*Bletilla striata* Mature	Vitrification (Droplet-Vitrification)	-	Jitsopakul et al., 2008.
Seeds	*Cyrtopodium hatschbachii* Pabst	Encapsulation -dehydration	64% survival	Surenciski et al., 2012.
Seeds	*Oncidium flexuosum* Sims	Vitrification	-	Galdiano Jr et al., 2013.
Seeds	*Doritis pulcherrima*	Vitrification 50 min PVS2 (0 ± 2°C)	62%	Thammasiri, 2000.
Shoot primordia	*Vanda pumila*	Vitrification	-	Hai-yan; Kondo 1996.
Shoot tips	*Dendrobium* Walter Oumae	Encapsulation/dehydration	-	Lurswijidjarus; Thammasirib, 2004.
Shoot tips	*Dendrobium* Walter Oumae	Vitrification	-	Lurswijidjarus; Thammasiri, 2004.
Shoots	*Mokara* Golden Nugget Orchid	Vitrification	-	Safrinah et al., 2009.
Zygotic embryos	*Bletilla striata* Rchb. f.	Vitrification	60%	Ishikawa et al., 1997.
Zygotic embryos	JP. terr. orchid *Bletilla striata*	Vitrification	-	Ishikawa et al, 1997.

The first cryopreservation protocols for plant tissues, known as the classical method, called for freezing in two phases: phase I, in which freezing is slow and the temperature is reduced at a set rate (1 to 10 ºC min^{-1}) to values close to -40 ºC using a programmable freezer (cryostat), followed by phase II, in which freezing is induced rapidly through direct immersion of the material in LN (Engelmann, 1997). The technique using slow freezing has been used since the development of the first cryopreservation protocols, and it is a complex procedure in which the freezing rate and pre-freezing temperature play a critical role in preserving the viability of the material. More efficient results were obtained with small units with uniform morphology, such as cultures of protoplasts, cell suspensions and calluses, and less efficient results were observed with larger units such as zygotic and somatic embryos and shoots. Another limitation of this method is the need for a programmable freezer to obtain precise and reproducible results (Abdelnour, 1999).

Vitrification, or the formation of a vitreous state, a process through which the water undergoes a transition from a liquid phase to an amorphous, metastable solid phase, constitutes the contemporary cryopreservation technique (Santos, 2000). Vitrification of the cytoplasm is performed by dehydrating tissues with a high-viscosity supersaturated solution to the point where there is no free water remaining in the cells to crystallize when the tissue is immersed in liquid nitrogen. The materials are pre-treated with concentrated solutions of cryoprotectants and then frozen in liquid nitrogen to achieve a state of vitrification of the internal solutes. However, these cryoprotectants can cause cytotoxicity and osmotic stress to the cell. Recently, sugars (sucrose, trehalose and glucose) have been used as cryoprotectants because, in addition to not being toxic, they appear to aid in the vitrification process (Santos, 2001). Dehydration can be performed through water evaporation or by treatment with a very concentrated solution of cryoprotectants (DMSO, ethylene glycol, glycerol, propylene glycol) (Gamarano de Melo, 2008).

Vitrification and encapsulation-dehydration techniques make use of sophisticated equipment and have been shown to be adequate for the cryopreservation of apices and meristems. Depending on the species, some chemical cryoprotectants display cytotoxicity and cause undesirable alterations during cultivation. For this reason, a protocol is sought that can guarantee cryobiological viability and eliminate the use of chemical agents by reducing the water content present in the cells of plant structures, the primary limiting factor in cryopreservation, through desiccation in a sterile air flow (Lopes, 2005).

Another variation of the vitrification technique is encapsulation-dehydration, which consists of encapsulating meristems, apices and somatic embryos in capsules of sodium alginate gel. The tissues in this case are pre-cultivated in media containing high levels of sucrose, which is considered to be an important technique for long-term conservation without genetic alteration (Santos, 2000; Abdelnour, 1999). The encapsulation-dehydration process has been described by Fabre and Dereuddre (1990) and consists of the encapsulation of plant material in a matrix of calcium alginate followed by a pre-treatment in high-efficiency liquid sucrose media and rapid freezing of explants in liquid nitrogen. As a result, intracellular solutes vitrify and ice formation is impeded. This technique has been used successfully in different plant species (Takagi, 2000).

CLASSICAL METHOD OF CRYOPRESERVATION

In classical cryopreservation techniques, the first cryopreservation protocols for plant tissues were based on slow freezing. In this technique, the critical points would be the removal of intracellular water and the behavior of the water remaining in the interior of cells during the freezing and thawing processes (Santos, 2000; 2001). These classical freezing methods were based on physical-chemical events that occur during the freezing process under natural conditions, which were described by Mazur (1969).

As temperature decreases, nearing 0 ºC, the cell and its external medium achieve a supercooled state and, later, the formation of ice occurs in the extracellular medium. The content of the supercooled cell remains unfrozen, possibly because the cell wall and the plasma membrane impede the ice crystals in the intercellular spaces from penetrating the cell and triggering freezing of the cytoplasm. If freezing occurs slowly, water migrates from the interior of the cell to the external medium due to the difference in water vapor pressure, which is greater within the cell than in the frozen intercellular spaces and is converted into ice on the cell surface or between the protoplast and the cell wall. This phenomenon is called freeze-induced desiccation. In this manner, the cell is dehydrated and the free water present is reduced to a minimum or completely removed, thus avoiding the formation of ice in the cell's interior. As a result, the concentration of the cell solution increases and the cell loses turgor. When the water potential of the partially dehydrated cells equals that of the extracellular ice, equilibrium is established and dehydration stops as long as the temperature remains constant. If the cooling of the cell is very rapid, however, freeze-induced desiccation does not occur and the cells become more supercooled and eventually the intracellular solution, which still contains a high concentration of free water, freezes, forming ice crystals that cause mechanical injury to the cells (Steponkus; Webb, 1992).

In experimental conditions, when the cells reach the pre-freezing temperature, the majority of the freezable water has already escaped, forming ice in the external medium. Thus, exposure to the LN temperature has very little adverse effect (Kartha, 1985; Sakai, 1995).

The material undergoes a pre-treatment with cryoprotective substances followed by slow freezing to a preset temperature (approximately -40 ºC) at a controlled freezing velocity (1 to 10 ºC/hour), using a programmable freezer followed by direct immersion in liquid nitrogen. In this process, freeze-induced desiccation occurs (Santos,

2000; Withers; Williams, 1998). However, if freezing is very fast, the intracellular water can freeze, forming crystals that cause mechanical injury to the cells (Santos, 2000). Other problems that can occur are damages caused by the high concentration of intracellular solutes and the loss of the plasma membrane in highly plasmolyzed cells (Withers; Williams, 1998), which are both results of excessive dehydration.

CONTEMPORARY METHOD OF CRYOPRESERVATION

The more recently developed contemporary methods of cryopreservation are simpler and are based on vitrification (Fahy et al., 1984; Sakai et al., 1990, 1991a, 1991b). In contrast with the classical method, which is based on freezing, the procedures based on vitrification and dehydration of the cells occur prior to freezing. Dehydration occurs by placing the material in contact with concentrated chemical substances followed by rapid freezing. As a consequence, the factors that affect the formation of intracellular ice are avoided. Thus, the critical point of this process is the dehydration of the material and not, as in the classical method, the freezing itself. Thus, the material must have appropriate and compatible water content to lead to high survival rates. Another advantage of vitrification over the classical method is that cryostats are not necessary, which facilitates the cryopreservation process and reduces costs (Engelman, 1997). The contemporary method offers various advantages over conventional cryopreservation techniques, for example, the ease of handling the cryoprotectant, the elimination of programmable freezers, the independence of survival from the freezing velocity, and an increase in the size of the explants that are able to survive exposure to liquid nitrogen (Bachiri et al., 1995).

The vitrification method was used successfully in the cryopreservation of plant cells at the beginning of the 1990s, with emphasis on the studies by Sakai et al. (1990, 1991a, 1991b), Yamada et al. (1991) and Nishizawa et al. (1993) (Table 1). These studies enabled great advances in plant cryobiology, making possible the use of simpler and less expensive methods for the preservation of plant germplasm (Sakai et al., 1990, 1991a, 1991b, Yamada et al., 1991, Nishizawa et al., 1993). Among the cryopreservation protocols for orchids, vitrification is an effective, simple, safe, inexpensive technique that is applicable to a wide range of orchid explants (Galdiano et al., 2012).

The vitreous state brings various benefits to the dehydrated cell: it limits water loss, crystallizes salts and proteins in the cytoplasm, protects against pH changes due to the removal of water, and prevents cell collapse when dehydration is excessive (Carvalho, 2006).

Vitrification restricts the diffusion of substances within the cell, leading to a quiescent metabolic state and resulting in the prevention of chemical reactions that are dependent on the diffusion process. Due to these characteristics of the vitreous state, biological deterioration is reduced to insignificant levels, ensuring stability during the quiescent period (Koster, 1991). This cryopreservation process involves two stages. In the first stage, called pre-vitrification, there is an increase in the osmotic concentration due to the application of cryoprotectants capable of passing through the cell membrane. In the second stage, the dehydration phase, the material is immersed in a vitrification solution. The pre-vitrification solution described by Nishizawa et al. (1993) is composed of 2 mol L^{-1} glycerol and 0.4 mol L^{-1} sucrose. The material is immersed in this solution for 20 to 60 minutes. This solution, in reality, does not lead to a significant increase in the osmotic concentration on the interior of the cell because glycerol and sucrose are not capable of penetrating the cells in so short a period of time. However, the application of this solution has been shown to be beneficial, as it increases the permeability of the cell membrane to the cryoprotectants and prevents damage to the cells during exposure to the vitrification solution (Towill, 2002).

Because the osmotic concentration of the vitrification solution is very high and the duration of contact with the biological material is relatively short, the main function of this solution is to dehydrate the material that will be cryopreserved, concentrating permeable cryoprotective agents and other cytoplasmic substances on the cell interior. The vitrification solution removes water from the cell interior, causing the intracellular solution to solidify and form an amorphous or vitreous state when placed in LN (Grout and Roberts, 1995; Wang et al., 1998).

Another method that has been used for cryopreservation of a large variety of plant species using the vitrification procedure is encapsulation-dehydration. In this technique, the explants are encapsulated in sodium alginate gel and the capsules containing the material are pre-cultivated in a medium containing high concentrations of sucrose (0.3 to 0.7 M), partially dehydrated by exposure to a flow of dry air (laminar flow hood or with silica gel) to approximately 20% humidity (based on fresh weight), directly immersed in liquid nitrogen, frozen rapidly (Vieira, 2000; Withers; Williams, 1998) and thawed slowly. This technique has been successful for cryopreservation of various species, including some species whose apical and lateral buds had previously been shown to be intolerant to freezing (Santos, 2000; 2001).

CRYOPROTECTANTS USED WITH ORCHIDS

Cryoprotectants are chemical substances that reduce cell injury during freezing and thawing. The most commonly used, which are capable of passing through cell membranes, are propylene glycol, ethylene glycol, methanol, glycerol and DMSO. These cryoprotectants, however, can be toxic or can cause osmotic stress, leading to cell death or modification of their morphogenetic response in culture (Sakai, 1995). Glycerol is a cryoprotectant that has a distinct behavior because it penetrates the membranes if it is added at room temperature, but is incapable of penetration if it is used at 0ºC (Carvalho, 2006). Sugars (sucrose, trehalose and glucose) have been used as cryoprotective substances because they do not display cytotoxicity even when they are accumulated in large quantities in the cytoplasm. The latter also exhibit greater efficiency in the stabilization of cell membranes during freezing (Withers, 1991; Yamada, 1993).

Cryoprotectants that do not penetrate the cells (starch, polyvinylpyrrolidone (PVP) and polyethylene oxide) act by dehydrating the cells at temperatures close to freezing and, thus, are capable of reducing water activity in the cell interior (Efendi, 2003).

Phloroglucinol (1,3,5-benzenetriol) is a benzenetriol known to protect cells against oxidative stress, inflammation and damage from free radicals (Kang et al, 2006; Kim and Kim, 2010) and has been shown to be effective in the recovery and survival of cryopreserved protocorms of *Dendrobium nobile* (Vendrame and Faria, 2011).

CRYOPRESERVATION OF ORCHIDS

Different plant organs have been used for cryopreservation of *Orchidaceae*, including zygotic embryos (Ishikawa, 1997), seeds (Nikishina, 2001), immature seeds (Hirano, 2005), protocorms (Bian et al., 2002), pollen and seeds (Vendrame, 2007; 2008), and cell suspensions (Tsukasaki, 2000) (Table 1).

Embryos and protocorms easily regenerate, giving rise to new plants; thus, the development of a protocol for the conservation of the germplasm of orchids is important for future use in breeding and conservation programs (Ishikawa, 1997).

CRYOPRESERVATION OF ORCHID SEEDS

The storage of seeds plays an important role in the long-term conservation of seeds of orchid species because it requires little space for storage, enables preservation, facilitates the distribution of germplasm at reduced costs (Pritchard; Seaton, 1993), and represents an important tool for breeding programs of hybrid seeds (Vendrame, 2007). Although researchers have demonstrated that seeds from various orchid species maintain their viability when stored by conventional procedures, the longevity and the results obtained are highly variable. Thus, the storage of orchid seeds by the traditional method (-18 ºC and 5% humidity) does not appear to be highly recommended for seed conservation for long periods of time in germplasm banks (Pritchard; Seaton, 1993). Various studies have been conducted in recent years to determine the best storage conditions for orchid seeds. The results are highly variable depending on the method used, the species and even the lot of seeds tested (Table 1).

Bowling and Thompson (1972) stored seeds from 30 different orchid species at -10 ºC. Although these seeds remained viable for three years, an analysis after 10 years of storage showed that all of the seeds were dead (Pritchard, 1986). Pritchard (1984) cryopreserved orchid seeds in the 1980s and verified that ten different species satisfactorily resisted the cryopreservation process, maintaining their initial viability after the process. Seaton and Hailes (1989) found an almost total loss of viability in one lot of *Cattleya aurantiaca* seeds stored for 50 days at -18 ºC, whereas in another lot of this same species, the seeds stored for 400 days under the same conditions as the previous lot exhibited high viability (Thornhill and Koopowitz, 1992).

In the study of cryopreservation of seeds of hybrid *Dendrobium* exposed to the cryoprotectants phloroglucinol and Supercool X1000, Galdiano et al. (2012) observed that 1% phloroglucinol resulted in high *in vitro* germination rates (79%) for the seeds, and therefore this was effective as a cryoprotectant for the cryopreservation of hybrid *Dendrobium* seeds using the vitrification method. Conversely, the addition of 1% Supercool X1000 ® did not increase seed germination, demonstrating that this chemical is not effective as a cryoprotectant for the seeds. The authors reported that the seeds were germinated and that the seedlings exhibited normal growth and development without alterations. It was concluded that the use of cryopreservation for long-term storage of hybrid *Dendrobium* orchid seeds for germplasm conservation, reproduction or commercial use can perform better when PVS2 [plant vitrification solution 2] and phloroglucinol are used as cryoprotectants (Galdiano et al., 2012).

In a recent experiment, immature seeds of *Cyrtopodium hatschbachii* Pabst (Orchidaceae) were cryopreserved by an encapsulation-dehydration technique, with a significant 64% increase in seed germination and survival of the acclimatized plants after cryopreservation

(Surenciski et al., 2012). Based on the studies reviewed, one can confirm that this is a relatively new and underdeveloped area that, according to Seaton and Pritchard (1999), generates a great deal of discussion and demands further research.

CRYOPRESERVATION OF ORCHID POLLEN

Pollen storage is a manner of conserving a portion of the genetic diversity of a species in an accessible and easily usable manner for plant breeders. It can also be a rapid and practical manner for starting a germplasm bank of rare or threatened species (Connor; Towill, 1993; Grout and Roberts, 1995; Sacks; St. Clair, 1996; Reed, 2002).

The storage of pollen is similar to that of seeds, although the concentration of water content in pollen is easier to adjust. Pollen quality is very important for later use in plant reproduction. The pollen grains collected from very new or old anthers do not survive storage. Stressed plants produce pollen with low fertility. The storage of pollen, similarly to that of seeds, requires very little physical space and, more importantly, preserves large quantities of genetic material (Towill, 2002). According to Vendrame (2008) the reproduction and production of orchids is dependent on the adequate storage of pollen enabling crosses between plants that exhibit temporal and spatial separation in their periods of sexual reproduction. The storage of orchid pollen can also be useful for the development of haploid plants through the development of embryos from pollen grains (Pritchard; Prendergast, 1989).

Pollen grains can be stored at temperatures of -20, -4 and +4 ºC; however, their longevity is reduced at these temperatures. At low intracellular water levels, the lower the storage temperature, the greater the longevity of seeds and also pollen (Towill, 2002). Seaton and Pritchard (1999) recommend that cryopreservation in liquid nitrogen be used in the formation of germplasm banks of orchid pollen and seeds, although they emphasize the importance of further proving the efficacy of this method.

The vitrification method has been used in the cryopreservation of pollen from two different cultivars of the genus *Dendrobium*: *D.* 'Sena Red Thailand' and *D.* 'Mini W/RL'. However, there was no significant difference, either between the treatments or between these and the controls, for the two tested orchid cultivars. All of the capsules in all of the treatments produced viable seeds for the two orchid cultivars. However, the vitrification method was not necessary to cryopreserve pollen from the cultivars of *Dendrobium* used in that experiment. For the tested cultivars, the simple storage of pollen in LN is sufficient for its cryopreservation (Carvalho, 2006). With a basis in the reviewed literature, the studies by Shijun (1984), Carvalho (2006) and Vendrame et al. (2008) addressed the conservation of orchid pollen at ultra-low temperatures (Table 1).

CRYOPRESERVATION OF ORCHID PROTOCORMS

Orchid protocorms are structures that easily regenerate, giving rise to new plants. The development of a protocol for orchid protocorm conservation is important to enable their future use in breeding and conservation programs (Ishikawa, 1997).

Popova et al. (2003) studied the effect of different culture media on the development of protocorms from hybrid seeds of cryopreserved *Bratonia*. After the cryopreservation of the seeds, the frequency of budding and new protocorms was greater in the MS and Morel media.

In the procedure for the cryopreservation of *Dendrobium nobile* protocorms, the exposure of these plant parts to cryoprotectants was necessary prior to storage in liquid nitrogen (Vendrame and Faria, 2011). These authors exposed *Dendrobium nobile* protocorms to seven procedures with the objective of confirming the influence of phloroglucinol on the recovery, survival and development of protocorms in this orchid species and confirmed that the *Dendrobium nobile* protocorms exposed to a 2 M glycerol solution for 20 min and vitrification (PVS2) with 1% phloroglucinol for 10 min produced a better result in the form of an increase in the number of recovered protocorms and a 68% survival index. According to the researchers, phloroglucinol combined with glycerol, and PVS2 showed, therefore, a positive effect. The supplementation with 1% phloroglucinol prevented an increase of greater than 100% in the recovery and survival of *D. nobile* protocorms in comparison with the same treatment without phloroglucinol. The addition of sucrose to the treatments of *D. nobile* protocorms showed that this sugar at a concentration of 0.04 M reduced by 90% the survival of the *D. nobile* protocorms, the effect of its use in the treatment being significantly negative (Vendrame and Faria, 2010).

In the evaluation of the cryopreservation of hybrid *Dendrobium* protocorms exposed to the cryoprotectants phloroglucinol and Supercool X1000, Galdiano and collaborators reported that cryopreserved protocorms of hybrid *Dendrobium* sp., pre-treated with 0.3 M sucrose for 24 hours, followed by exposure to PVS2 solution containing 1% phloroglucinol for 15 min at 0°C resulted in 14% survival of the protocorm after 75 days. This result indicated limited success compared with the survival of

0-6% observed for the controls and the other treatments with cryopreserved protocorms using droplet-vitrification, with phloroglucinol improving the survival and the number of seedlings formed after cryopreservation. However, there was no increase in the survival of protocorms with the addition of Supercool X1000® (Galdiano et al., 2012). The cryopreservation of protocorms of hybrid *Dendrobium* was shown to be more laborious and result in limited success when these cryoprotectants were used. Additionally, a genotypic effect may have contributed to the weak response of the hybrid *D. Swartz* "Dong Yai" protocorms cryopreserved by vitrification, although additional studies using genotypes of *Dendrobium* with Supercool X1000® and phloroglucinol at different concentrations are needed to improve the recovery and survival of protocorms after cryopreservation (Galdiano et al., 2012).

CRYOPRESERVATION OF MERISTEMS AND STEM APICES CAULINARES OF ORCHIDS

In the methodology for the cryopreservation of plant tissues based on slow freezing, the material undergoes pre-treatment with cryoprotective substances (DMSO, ethylene glycol, methanol, glycerol, propylene glycol). Subsequently, slow freezing is performed until a preset temperature (approximately -40 ºC) is reached at a freezing velocity (1 to 10 ºC/hour) controlled using a programmable freezer, followed by direct immersion in liquid nitrogen. More efficient results are found with small units with uniform morphology, such as suspended cell cultures and calluses, and it is less efficient with larger units such as zygotic and somatic embryos and shoots. Another limitation of this method is the need for programmable freezers to obtain precise and reproducible results (Santos, 2000; Abdelnour, 1999). Tsukasaki et al. (2000) cryopreserved calluses obtained from apical meristems of *Doritaenopsis*.

CONCLUSIONS

The reviewed studies indicate that orchid cryopreservation is a relatively new and important research area that is still developing, generates a great deal of discussion and requires further research in terms of the types of cryopreservation treatment to be used and the best methodology for particular species. Given the observations in this study, the types of methods that are used for cryopreservation and the large variations in the responses of orchids to different methods of cryopreservation, the development of more appropriate protocols for the preservation of orchids is necessary.

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