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Cryopreservation techniques in genipap and mangaba: challengers and perspectives

Técnicas de criopreservação em jenipapeiro e mangabeira: desafios e perspectivas

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ABSTRACT
The genipap (*Genipa americana* L.) is a native species of Brazil, found in various regions of the country, from the North to the South, including Sergipe. On the other hand, the mangabeira (*Hancornia speciosa* Gomes), another Brazilian tropical plant, is recognized for its fruits rich in iron and vitamin C, widely exploited by the pulp, juice, and ice cream industry. However, it faces threats due to urban development in the coastal region. To preserve these species, techniques such as cryopreservation are fundamental. This approach, which maintains plant tissues at ultra-low temperatures, allows the conservation of genetic material indefinitely, contributing to the maintenance of biological diversity. Encapsulation technology, which involves enclosing plant material in a specific matrix, is also promising in this context, offering a long-term preservation method for plant explants and embryos. These methods represent significant advances in the field of plant species conservation, providing effective strategies to ensure the survival of these plants in the face of environmental challenges and human development. The objective of this review is to address some studies involving cryopreservation techniques for genipap and mangaba species. Through cryopreservation techniques, we can achieve *ex situ* conservation for the germplasm bank as well as for plant regeneration and reforestation of areas. It is evident that there is a need to increase studies on the conservation of both species.

Keywords: *Genipa americana* L, *Hancornia speciosa* Gomes, cryopreservation, encapsulation, *ex situ* conservation.

RESUMO
O jenipapeiro (G*enipa americana* L.), é uma espécie nativa do Brasil, encontrada em diversas regiões do país, desde o Norte até o Sul, incluindo Sergipe. Já a mangabeira (*Hancornia speciosa* Gomes), outra planta tropical brasileira, é reconhecida por seus frutos ricos em ferro e vitamina C, amplamente explorados pela indústria de polpas, sucos e sorvetes. No entanto, enfrenta ameaças devido ao desenvolvimento urbano na região costeira. Para preservar essas espécies, técnicas como a criopreservação são fundamentais. Essa abordagem, que mantém tecidos vegetais em temperaturas ultrabaixas, possibilita conservar o material genético por tempo indefinido, contribuindo para a manutenção da diversidade biológica. A tecnologia de encapsulamento, que envolve material vegetal em uma matriz específica, também é promissora nesse contexto, oferecendo uma forma de preservação de longo prazo para explantes e embriões vegetais. Esses métodos representam avanços importantes no campo da conservação de espécies vegetais, fornecendo estratégias eficazes para garantir a sobrevivência dessas plantas em face dos desafios ambientais e do desenvolvimento humano. Como objetivo esta revisão

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aborda alguns trabalhos que envolvem estudos sobre as técnicas criopreservação para as espécies jenipapeiro e mangabeira. Através das técnicas de criopreservação conseguimos a conservação *ex situ* para o banco de germoplasma e também para regeneração de plantas desta, e o reflorestamento de áreas. Percebe-se que precisa aumentar os estudos sobre a conservação das duas espécies.

Palavras-chave: *Genipa americana* L., *Hancornia speciosa* Gomes, criopreservação, encapsulamento, conservação *ex situ*.

RESUMEN
La genipapa (Genipa americana L.) es una especie nativa de Brasil, encontrada en diversas regiones del país, de Norte a Sur, incluyendo Sergipe. Por otro lado, la mangabeira (Hancornia speciosa Gomes), otra planta tropical brasileña, es reconocida por sus frutos ricos en hierro y vitamina C, ampliamente explotados por la industria de pulpas, jugos y helados. Sin embargo, enfrenta amenazas debido al desarrollo urbano en la región costera. Para preservar estas especies, técnicas como la criopreservación son fundamentales. Este enfoque, que mantiene los tejidos vegetales a temperaturas ultrabajas, permite la conservación del material genético de forma indefinida, contribuyendo al mantenimiento de la diversidad biológica. La tecnología de encapsulación, que consiste en encerrar material vegetal en una matriz específica, también es prometedora en este contexto y ofrece un método de conservación a largo plazo para explantes y embriones de plantas. Estos métodos representan avances significativos en el campo de la conservación de especies vegetales, proporcionando estrategias efectivas para asegurar la supervivencia de estas plantas frente a los desafíos ambientales y del desarrollo humano. El objetivo de esta revisión es abordar algunos estudios que involucran técnicas de criopreservación para especies de genipapa y mangaba. A través de técnicas de criopreservación podemos lograr la conservación ex situ para el banco de germoplasma así como para la regeneración vegetal y reforestación de áreas. Es evidente que existe la necesidad de incrementar los estudios sobre la conservación de ambas especies.

Palabras clave: *Genipa americana* L, *Hancornia speciosa* Gomes, criopreservación, encapsulación, conservación *ex situ*.

1 INTRODUCTION

Genipa americana L., commonly known in Brazil as jenipapeiro or jenipapo (RUZZA et al., 2020), stands out as a native but not endemic species of Brazil and occurs in the North, Northeast, Midwest, Southeast, and South regions of Brazil (GOMES, 2015), in various Brazilian states, including Sergipe, which sparks the need for studies, as it has been used in restoration programs for degraded areas by the Restoration Group

ISSN: 1696-8352

of the Federal University of Sergipe (SANTOS et al., 2011). It has a tree habit, which can reach up to 25 m in height, and is distributed from Amapá to São Paulo and Mato Grosso, being cultivated in orchards throughout the country, including the southern states (CORRÊA, 1984).

Mangabeira *(Hancornia speciosa* Gomes) is a tropical fruit plant native to Brazil and found in various regions of the country, from the Coastal Tablelands and Low Coastal Plain of the Northeast to the cerrados of the Midwest, North, and Southeast regions. FONSECA et al. (2003) classified mangabeira as belonging to the Apocynaceae family. It is a latex-producing plant, its fruits are rich in iron and also a good source of vitamin C, presenting an incomparable flavor and aroma, being the main product exploited by pulp, juice, and ice cream industries. The fact that mangabeira is on the list of endangered species due to the increasing development of the construction industry in the coastal region makes cryopreservation techniques an ally for the preservation of this species (SARTOR et al., 2012).

Its basic definition is the aseptic cultivation of any living part of the plant (explants) consisting of tissue fractions, organs, or even cells in suspension in a synthetic culture medium (nutrients, growth regulators, etc.) under controlled conditions of temperature, humidity, and luminosity, to generate a new plant (LAMEIRA et al., 2000).

Cryopreservation of plant genetic materials enables the conservation of meristems, embryos, seeds, and other plant tissues for an indefinite period. This laboratory method is pioneering in the southern region for the conservation of germplasms at ultra-low temperatures (-196°C) within liquid nitrogen tanks. This technique halts the plant's growth and subsequent multiplication, enabling the formation of a germplasm bank in a reduced space (SARTOR et al., 2012). The exposure of plants to nitrogen, in addition to conservation, can eliminate viral particles from plant tissues, a technique called cryotherapy (LIMA, 2014).

It can be used for all types of explants, such as: meristems, cell suspensions, embryos (somatic, zygotic, or nucellar), and protoplasts. Generally, smaller structures are more suitable for freezing, as dehydration and freezing occur more quickly and uniformly in smaller structures. Various techniques are being studied in search of better storage

ISSN: 1696-8352

conditions, with the main one being based on reducing metabolism, either by removing water and/or lowering temperature (KOHOMA et al., 2006). According to TOWILL (2002), the efficiency of the cryopreservation process will depend on the results obtained in each of the following stages: preparation of the material, cryopreservation, and thawing of the material. The major challenge for cryopreservation is to freeze without the formation of ice crystals inside the cells.

There are different cryopreservation techniques, and the choice among them depends on the species and explant to be cryopreserved (PANIS & LAMBARDI, 2005). One of the recently developed cryopreservation techniques is droplet vitrification, which involves pretreating the explants with vitrification solution before they are placed on strips of aluminum foil with a drop of Plant Vitrification Solution 2 (PVS2) and then immersed in liquid nitrogen (ENGELMANN, 2011).

The encapsulation technology or synthetic seeds, developed by Kitto & Janick (1982), was based on the use of somatic embryos as functional seeds, which consists of encapsulating plant material such as shoot apices or lateral buds in a matrix of sodium alginate and calcium chloride. FEBRE & DEREUDDRE (1990) conducted studies with the aim of combining cryopreservation with synthetic seed technology. Initially, synthetic seeds were proposed for somatic embryos, which can be stored short-term in cold chambers and/or sown directly in soil (FIGUEIREDO et al., 2018; SILVEIRA & SIBOV, 2019). Over time, other protocols have emerged, using synthetic seeds for the conservation and/or preservation of explants, such as microshoots, axillary buds, and shoot apices (BENELLI et al., 2013; AHMED et al., 2015). When sown under in vitro and ex vitro conditions, synthetic seeds have the ability to regenerate and form a new plant, maintaining this potential even after storage (ARA et al., 2000).

2 CRYOPRESERVATION TECHNIQUES

There are several cryopreservation techniques available, and the choice among them depends on the species and type of explant to be preserved (PANIS & LAMBARDI, 2005). One of these techniques is droplet vitrification, which involves a series of important steps including pretreatment, preconditioning, preculture, osmoprotection,

ISSN: 1696-8352

dehydration, cooling, warming, and protection (SAKAI et al., 2008). The application of a vitrification solution (PVS - Plant Vitrification Solution) to explants is one of the most significant advancements in this field and involves the use of a mixture of cryoprotectants (ENGELMANN, 2011). The most common solutions for plant species are PVS2, composed of 30% glycerol, 15% ethylene glycol, 15% DMSO, and 0.4 M sucrose, and PVS3, composed of 50% glycerol and 50% sucrose, optionally in liquid MS medium (NISHIZAWA et al., 1993).

Vitrification is a physical process that occurs at extremely low temperatures, where cellular content freezes due to the presence of a highly concentrated solution of cryoprotectants. During this process, cellular content transitions from a liquid to a vitreous state, without the formation of ice crystals. This phenomenon occurs because, during freezing, solutes inside cells concentrate, maintaining osmotic balance with the external environment and protecting tissue from excessive dehydration, thereby preserving cellular volume. Additionally, freezing leads to a metabolic block, preventing molecular diffusion inside and outside cells and avoiding the formation of ice crystals (ARMITAGE & RICH, 1990).

This transition to the vitrified state allows the material to tolerate rapid temperature variations during cooling and warming, maintaining cellular stability during storage in liquid nitrogen. This stability is crucial to prevent physical and chemical alterations during the dehydration process (ARNÃO et al., 2008). Vitrification techniques are derived from this natural process and are achieved by dehydrating tissues through artificial exposure to concentrated solutions of chemical cryoprotectants during pretreatment (ENGELMANN, 2004; SOUZA et al. 2023). Currently, different vitrification protocols are being developed, varying in the composition of cryoprotectant solutions and exposure time, adapting to the characteristics of each species.

This technology encapsulation is becoming an important tool in studies on multiplication and preservation of genetic material through in vitro cultivation. The encapsulation process has proven to be an effective strategy for maintaining genotypes under *in vitro* conditions, especially through cryopreservation (PERREIRA et al., 2008). Several approaches have been developed to improve the preservation of synthetic seeds,

ISSN: 1696-8352

with notable mention of encapsulation-vitrification and encapsulation-dehydration, as highlighted by renowned researchers (WANG et al., 2005; PINTOS et al., 2008; RAI et al., 2009). Generally, research in this area focuses on the use of somatic embryos as a source of explants, while studies exploring other types of explants as encapsulable units are still limited.

Sodium alginate, derived from seaweed, has been widely used as an encapsulating agent due to its solubility at room temperature, ability to form a permeable gel, and characteristics of low cost and easy handling (NOGUEIRA, 2010). The alginate matrix surrounding the propagules provides protection to somatic embryos, facilitates storage, transportation, and conversion into plants. This technique has been continuously improved to allow the incorporation of various elements into the encapsulation matrix, including phytohormones, nutrients, and vitamins (GUERRA et al., 2001). However, the resistance of the alginate hydrogel can hinder the rupture of capsules by encapsulated explants, requiring additional steps to facilitate this process, such as decomplexation with potassium nitrate (MONDO & CICERO, 2008). These aspects are crucial for the development and effective application of synthetic seed technology.

Recently developed adaptive techniques, such as encapsulation/vitrification or encapsulation/dehydration, involve enclosing the explant in a sodium alginate capsule before dehydration. These techniques allow the use of higher concentrations of cryoprotectants during preculture, resulting in greater tolerance to dehydration. The sodium alginate capsule provides protection and supplies nutrients during all pretreatment stages (PEREIRA et al., 2008; NASCIMENTO et al., 2020).

3 MAIN RESULTS PUBLISHED

There are few existing studies published on the long-term conservation of genipap and mangaba species. SARTOR et al. (2012) aimed to cryopreserve apical and lateral buds of mangaba using vitrification and encapsulation-dehydration techniques. In the vitrification method, concentrations of sucrose (0, 0.25, 0.5, and 0.75 M) and dimethyl sulfoxide (DMSO) (0, 5, 10, and 15%) were used to aid in the dehydration of the material, followed by freezing the buds in liquid nitrogen for five days and then cultivating them

ISSN: 1696-8352

in a regeneration medium. For the encapsulation-dehydration technique, capsules were formed using solutions of alginate gel (5%) and calcium chloride (0.2 M), with dehydration periods of 0, 1, 2, and 3 hours in a laminar flow chamber. It was observed that encapsulated buds frozen in liquid nitrogen did not regenerate when sub-cultured in nutrient medium; the buds that survived vitrification were those subjected to DMSO and sucrose and subsequently cultivated in Wood Plant Medium (WPM), showing better regeneration for concentrations of 5% DMSO and 0.5 M sucrose, respectively.

SANTANA et al. (2022) evaluated the viability of cryopreserving apical buds of mangaba using the droplet vitrification technique. In Experiment I, when comparing the regeneration percentage with the control treatment (without cryopreservation NL-), it was observed that the AB accession presented 100% surviving explants on the 30th day of cultivation when subjected to 30 minutes of exposure to PVS2 solution (30% (v/v) glycerol, 15% (v/v) ethylene glycol, and 15% (v/v) DMSO). This result suggests that the PVS2 solution was not toxic or deleterious to the AB accession, at least during this exposure period. In Experiment II, it was observed that the Japaratinga (JA) accession had a lower rate of dead explants and a higher rate of surviving explants compared to the Terra Caída (TC) accession on days 30 and 60 of cultivation. However, regarding the number of oxidized explants, the JA accession proved to be more susceptible to the effects of oxidative stress during the droplet vitrification process, registering rates of 67% and 80% on days 30 and 60 of cultivation, respectively. The authors highlight the need for adjustments in the concentrations of post-culture medium components to achieve higher regeneration rates.

SANTOS et al. (2015) performed cryopreservation techniques in mangaba (*Hancornia speciosa* Gomes) to establish a protocol for long-term storage. Their main objective was to evaluate the efficiency of droplet vitrification and vitrification in cryopreserving shoot apices of mangaba. The regrowth of mangaba apices cryopreserved by the droplet vitrification technique was evaluated using different treatment times with the vitrification solution (15, 30, 45, and 60 min.) and subjected to different pre-culture periods (absence, 24, or 48h) in a medium with 0.3 M sucrose before cryopreservation. In addition to this technique, the effect of the classic vitrification technique using four

ISSN: 1696-8352

exposure times to the vitrification solution (15, 30, 45, and 60 min.) was evaluated. Significant differences were observed in the exposure time for both vitrification and droplet vitrification techniques. For both techniques, the 60-minute immersion time promoted regrowth of over 70% in the cryopreserved apices. Pre-culture promoted increased survival of cryopreserved explants. The cryopreservation techniques of shoot apices by droplet vitrification and vitrification were shown to be viable for prolonged mangaba conservation.

SÁ et al. (2015) aimed to evaluate the effect of different dehydration times in a laminar flow chamber and immersion times in a cryoprotective solution (MS+0.5 M sucrose) on the regenerative capacity of shoot apices of *Genipa americana* L. for the establishment of future cryopreservation protocols. Shoot apices were obtained from seedlings, Oiteiros accession, germinated and cultivated in vitro. The explants were subjected to encapsulation and different immersion times in a cryoprotective solution and dehydration times in a laminar flow chamber. Significant effects of immersion times in the cryoprotective solution (MS+0.5 M sucrose) and dehydration times in a laminar flow chamber on the moisture content of shoot apices were observed. Immersion for 24 and 48 hours in the cryoprotective solution reduced the moisture content by 23.31% and 28.47%, respectively, compared to the initial moisture content of the capsules (66.68%). Encapsulated shoot apices, regardless of immersion time in the cryoprotective solution, showed higher moisture content (47.96%). High regeneration rates of encapsulated shoot apices, immersed in cryoprotective solutions (0.5 M sucrose) and dehydrated in a laminar flow chamber for 0, 2, and 4 hours, reaching 91%, 67%, 100%, and 91.67% regeneration, respectively, were observed. Immersion for 24 hours in the cryoprotective solution (MS+0.5 M sucrose) and dehydration for two hours in a laminar flow chamber showed potential for use in future encapsulation-dehydration cryopreservation studies.

Studies conducted by SANTOS & SALOMÃO (2016) aimed to evaluate the effect of dehydration and storage in liquid nitrogen (-196°C) on the viability of zygotic embryos of *G. americana* L., as a step towards developing a long-term conservation protocol for this species. Excised embryonic axes from seeds of *Genipa americana* L. dehydrated to different water contents were successfully cryopreserved by quickly immersing seed

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samples directly in liquid nitrogen. Control and cryopreserved embryonic axes were excised and cultured in WPM culture medium to assess viability. All control embryonic axes (LN2-) excised from fully hydrated seeds (43.89% moisture) germinated after 21 days of in vitro cultivation. These high germination percentages persisted even after irrigation. When the moisture content of the seeds was as low as 6.79%, germination percentages of 93%, 96%, and 93% were observed after freezing in liquid nitrogen for embryonic axes excised from seeds dehydrated to 13.26%, 9.57%, and 6.79% moisture, respectively.

FIGUEIREDO et al. (2018) aimed to attempt genipap conservation using encapsulable unit storage and slow growth techniques. They used nodal segments of the *Genipa americana* plant and subjected them to different storage methods, including encapsulation and slow growth, as part of a conservation study. In the case of encapsulation, the effects of different concentrations of sodium alginate (3% and 4%), pre-treatment with sucrose solutions (0, 0.25, and 0.50 M), and storage temperatures (8ºC and 15ºC) over 30 days, with subsequent evaluation of capsule integrity after 50 days, were investigated. For slow growth, different concentrations of sucrose (30, 45, and 60 g L-1) and storage temperatures (8°C and 15°C) after 90 and 180 days, with analysis of survival, regeneration, aerial part length, number of shoots, and leaves were tested. Only encapsulated units stored at 15°C were able to survive, with better capsule integrity observed at this temperature with pre-treatment of 0 M sucrose. In the case of slow growth, the 8ºC temperature did not allow explant survival. Consequently, the best results after 180 days were obtained at 15°C with the addition of 60 g L-1 sucrose to the culture medium. Therefore, the experiments indicate the feasibility of species storage using these techniques.

Nascimento et al. (2020) aimed to investigate the impact of dehydration duration on the regeneration capacity of embryonic axes of two jenipapo accessions (Umbaúba and Núcleo Bandeirante). Seeds were subjected to different dehydration periods in a polycarbonate box with silica gel, ranging from 0, 12, 16, and 20 hours, at room temperature. After each period, the seed moisture content was determined. Subsequently, the seeds were inoculated on germination medium, and samples were prepared in

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cryotubes and immersed in liquid nitrogen at -196°C. Dehydration using silica gel effectively reduced the moisture content in both accessions. Umbaúba accession exhibited 100% germination in all dehydration treatments prior to cryopreservation, while the Núcleo Bandeirante accession proved to be more sensitive, with only 10% germination after 20 hours of dehydration. A reduction in growth variables of the Umbaúba accession was observed with increased dehydration time, while the treatments did not affect the growth variables of the Núcleo Bandeirante accession. Despite the changes observed in the ultrastructure of the embryonic axes, dehydration did not have a significant impact on the percentage of regeneration of cryopreserved accessions.

There are currently no results for droplet vitrification in genipap, due to its seeds having moderate tolerance to dehydration during storage. They can be dried to a level of about 10.0% moisture without a significant reduction in germination rate. However, when the moisture content of genipap seeds is reduced to levels equal to or less than 11.7%, this can increase the average germination time due to the difficulty of water absorption by the seed. Furthermore, freezing genipap seeds, regardless of their moisture content, compromises germination capacity and may induce or exacerbate seed dormancy. However, there is little information on genipap seed tolerance to freezing (SANTOS & SALOMÃO, 2016), and there is currently no well-established cryopreservation protocol for this species using seeds as material (SANTOS & SALOMÃO, 2023).

ISSN: 1696-8352

Table 1: Main cryopreservation technique articles published with genipap and mangaba.

TITLE	SPECIE	EXPLANT	TECHNIQUE (S)	RESULTS	REFERENCES
Techniques for cryopreservation of mangaba buds	*Hancornia speciosa* Gomes	Apical and lateral buds	Cryopreservation	The highest regeneration rates in NL were observed with concentrations of 5% DMSO and 0.5 M sucrose	SARTOR et al. (2012)
Encapsulation, cryoprotection, and dehydration on the regenerative capacity of shoot tips of *Genipa americana*	*Genipa americana* L.	Shoot tips	Encapsulation, Cryoprotection, and Dehydration."	Immersion for 24 and 48 hours in the cryoprotective solution (MS medium + 0.5M sucrose) reduced capsule moisture to 23.31% and 28.47%, respectively. Regeneration rates in NL were high for encapsulated shoot tips, ranging from 91% to 100%	SÁ et al. (2015)
Cryopreservation of the mangaba tree (*Hancornia speciosa* Gomes): a protocol for long-term storage.	*Hancornia speciosa* Gomes	Shoot tips	Cryopreservation	The immersion time of 60 minutes for vitrification techniques resulted in a growth resumption of over 70% in cryopreserved shoot tips	SANTOS et al. (2015)
Viability assessment of *Genipa americana* L. (Rubiaceae) embryonic axes after cryopreservation using in vitro culture.	*Genipa americana* L.	Embryonic axes	Cryopreservation	High germination rates (93%, 96%, and 93%) were observed for the embryonic axes after NL from dehydrated seeds, with moisture content of 13.26%, 9.57%, and 6.79%, respectively	SANTOS & SALOMÃO (2016)
Conservation of *Genipa americana*: encapsulation and slow growth techniques	*Genipa americana* L.	Nodal segments	Encapsulation and Dehydration.	After 180 days, the best results were observed at 15°C with the addition of 60 g L^{-1} of sucrose to the culture medium	FIGUEIREDO et al. (2018)

ISSN: 1696-8352

Long term conservation of embryonic axes of genipap accessions	*Genipa americana L.*	Embryonic axes	Dehydration duration on the regeneration capacity	The Umbaúba accession showed high germination (100%), while the Núcleo Bandeirante accession had low germination (10%) after 20 hours of dehydration. Umbaúba had its growth affected by dehydration, unlike Núcleo Bandeirante	NASCIMENTO et al. (2020)
Effects of droplet vitrification on the regeneration of apical tips of mangaba accessions	*Hancornia speciosa* Gomes	Shoot tips	Cryopreservation	The PVS_2 solution did not harm the AB accession. The JA accession had more surviving explants than the TC accession on days 30 and 60 of culture. However, the JA accession was more affected by oxidative stress, showing higher rates of oxidized explants on the same days	SANTANA et al. (2022)

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4 CONCLUSION

Jenipapo and mangaba, due to their significant socioeconomic importance, primarily in the utilization of fruits for making sweets and juices, have been increasingly studied to enable the storage of different accessions to ensure the species' survival in the event of any disaster in field Gene Banks. In light of the studies conducted, we realize how important the use of cryopreservation techniques is to protect and keep plant species like jenipapo and mangaba under security conservation.

These techniques not only help us conserve the genetic diversity of plants but can also be a lifeline for species at risk of disappearing, such as the mangaba. However, the results also remind us that there is still much to learn and refine in these techniques. Each plant has its own needs and peculiarities, and it is essential to adjust the methods according to these characteristics to ensure they survive and can regenerate properly after the cryopreservation process.

These studies show us that there is hope and potential in cryopreservation techniques, but they also highlight the ongoing importance of research and improvement in these techniques to ensure that we can effectively protect our precious plants and all the genetic wealth they carry.

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