Evaluation of cryopreservation of Petiveria alliacea somatic embryos based on stress caused for the method used

Avaliação da criopreservação de embriões somáticos de Petiveria alliacea com base no estresse causado pelo método usado

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ABSTRACT
Petiveria alliacea is a medicinal species with great potential for pharmacological use against several pathologies, including neoplasms. Many studies have been developed to optimize efficient production methodologies and long-term conservation of this species' genetic resources, with a view to phytochemical and pharmacological research. This study demonstrates the efficiency of the D-cryoplate technique, applied to somatic embryos from plants maintained in vitro. Leaf explants were inoculated in culture medium containing 20 μM PIC and incubated under standard conditions in plant tissue culture. After 60 days, the somatic embryos induced directly on the leaf tissue surface were transferred to the multiplication medium (MS0). For cryopreservation, samples of these embryos were precultured for 24 hours in medium supplemented with sucrose (0.5M), then groups of 3-5 somatic embryos were encapsulated in calcium chloride directly in aluminum cryoplates. The cryoplates with somatic embryos adsorbed were immersed in a conditioning solution (loading) for 20 min. After being removed from loading, the somatic embryos adhered to the cryoplates were exposed to laminar flow air for different periods of time (0 to 140 min) to assess the level of dehydration. Then, samples submitted at each time were immersed in liquid nitrogen for 2 min. After this time, the cryoplates were removed and kept at room temperature for 20 min, and the somatic embryos were cultivated in MS0 medium. Evaluation after each treatment showed a high survival rate (93%) in cryopreserved somatic embryos. After 90 days of culture it was observed that somatic embryos dehydrated for 120 min showed the highest multiplication rate (32 embryos/inoculated embryo) obtained so far with these explants. The D-cryoplate technique brought innovation to established protocols representing the best option for in vitro conservation of these structures biotechnologically produced that are so promising for phytochemical and pharmacological research.

Keywords: Guinea, Dehydration, D-Cryoplate, in Vitro Conservation.

RESUMO
Petiveria alliacea é uma espécie medicinal com grande potencial de uso farmacológico contra diversas patologias, incluindo neoplasias. Muitos estudos têm sidodesenvolvidos para otimizar metodologias eficientes de produção e conservação a longo prazo dos recursos genéticos desta espécie, com vistas à pesquisa fitoquímica e farmacológica. Este estudo demonstra a eficiência da técnica de D-crioplaca, aplicada a embriões somáticos, oriundos de plantas mantidas in vitro. Explantes foliares foram inoculados em meio de cultura contendo PIC 20 μM e incubados em condições padrão em cultura de tecidos vegetais. Após 60 dias, os embriões somáticos induzidos diretamente na superfície do tecido foliar foram transferidos para meio de multiplicação (MS0). Para a criopreservação, amostras destes embriões foram pré-cultivadas por 24 horas em meio suplementado com sacarose (0,5M), em seguida, grupos de 3-5 embriõessomáticos foram encapsulados em cloreto de cálcio diretamente em crioplacas de alumínio. As crioplacas com embriões somáticos assim adsorvidos foram imersas em solução de condicionamento (loading), por 20 min. Após serem retirados do loading, os embriões somáticos aderidos às crioplacas foram expostos ao ar do fluxo laminar por diferentes períodos de tempo (0 a 140 min) para avaliar o nível de desidratação. A seguir, amostras submetidas a cada tempo foram imersas em nitrogênio líquido por 2 min. Após esse tempo, as crioplacas foram retiradas e mantidas em temperatura ambiente em solução de unloading por20 min, sendo os embriões somáticos cultivados em meio MS0. A avaliação após cada tratamento mostrou alta taxa da sobrevivência (93%) nos embriões somáticos criopreservados. Após 90 dias de cultura observou-se que embriões somáticos desidratados por 120 min

apresentaram a mais alta taxa de multiplicação (32 embriões/embrião inoculado) obtida até o momento com estes explantes. A técnica de D-crioplaca trouxe inovação aos protocolos já estabelecidos representando a melhor opção para conservação in vitro destas estruturas produzidas biotecnologicamente e tão promissoras para pesquisa fitoquímica e farmacológica.

Palavras-Chave: Guiné, Desidratação, D-Crioplaca, Conservação in Vitro.

1 INTRODUCTION

Petiveria alliacea is a medicinal species, popularly known as guinea, is native to tropical regions, and can be found in Central and South America and in some regions of the Caribbean and Africa. (Rzedowski and Rzedowski, 2000). Due to the large production of secondary metabolites in different parts of the plant, its extracts have several biological activities, such as: analgesic (Sertie et al., 1995), anti-inflammatory (Gutierrez and Hoyo-Vadillo, 2017), antimicrobial (Guedes et al., 2009), antifungal (Illnait-Zaragozi et al., 2011), neuropharmacological (Luz et al., 2016), antileukemic (Blainski et al., 2010), and antitumor (Rosner et al., 2001), and many others. Among the substances produced by the secondary metabolism of the species, dibenzyl trisulfide (DTS), isolated for the first time of this species (De Souza et al., 1990), stands out as the main antitumor agent whose purification process forms more potent substances with a broad spectrum of activity against tumors in various organs, encouraging the development of new chemotherapeutics (An et al., 2006, Gu et al., 2008; Bao et al., 2008, Wauchope et al., 2021).

In-depth phytochemical and pharmacological studies require a large amount of plant material, with high phytosanitary quality and genetically uniform, and plant tissue culture is the most suitable alternative to supply material for these studies. The in vitro cultivation of organs and structures producing substances of interest has received increasing attention, since, due to the molecular complexity of some metabolites, chemical synthesis by the pharmaceutical industry has low yield (Rao and Ravishankar, 2002). In addition to enabling the production of plants in a faster way, when compared to field development, the culture of isolated tissues, cells and organs, also allows the obtaining of substances produced in each plant structure, in addition to enabling modulation, with to the increase in the concentration of substances of interest from the cultivated organs and structures.

Efficient suspension cell culture and micropropagation protocols via axillary meristems (Castelar et al., 2011; Castelar et al., 2014; Soares et al., 2014) and via somatic embryogenesis from leaves and roots have already been reported for P. alliacea (Cantelmo et al., 2013; Soares, 2016). However, the in vitro conservation of germplasm under cultivation, aiming at the further development of phytochemical studies, as well as the supply of these polysulphides is inappropriate, since remaining in culture for long periods of time can lead to explants losing vigor and altering the original genetic constitution. (Larkin and Scowcroft, 1981). Therefore, cryopreservation has been recognized as the most suitable methodology for the long-term storage of plant genetic resources, due to the blocking of cell metabolism, reducing the risk of genetic and epigenetic alterations (Watt et al., 2000; Panis et al., 2005; Keller, 2008; Engelmann, 2011).

Cryopreservation is considered an economical technique, safe from the point of view of long-term conservation (Salaj et al., 2010; Engelmann, 2012). Exposure to liquid nitrogen (LN) at -196°C or its vapor phase at -150°C inactivates cell metabolism, preventing any change (Mazur, 1970; Engelmann, 2004). However, the large amount of water present in plant cells can lead to the formation of ice crystals, causing damage to the cell structure, preventing its recovery after LN removal (Engelmann, 2004; Reed, 2008; Kaczmarczyk et al., 2019). Therefore, protective dehydration is the most critical step in cryopreservation protocols, since excessive dehydration can trigger stress mechanisms, such as membrane changes and lipid peroxidation, compromising cell function and material recovery (Fang et al., 2009).

Some factors are crucial for choosing the most appropriate cryopreservation method, especially the size of the explant and its tolerance to desiccation (Pence, 2020). In this context, embryonic tissues, composed of young, homogeneous, small-sized cells with few vacuoles, consequently little intracellular water, are quite tolerant to the ultra-low temperatures of LN (Gonzalez-Arnaoet al., 2014).

The cryopreservation of somatic embryos (SEs) of P. alliacea was established through vitrification techniques with the use of high concentrations of cryoprotective solutions (Pettinelli et al., 2017; Vaz et al., 2020). However, the use of aluminum cryoplates to support explants (Yamamoto et al., 2011), in conjunction with osmotic dehydration that induces vitrification, in the V-cryoplate technique, or with an evaporative dehydration step, in the D-cryoplate technique, significantly improved the recovery of different explants, including somatic embryos of different species, enabling the development of simpler and more reproducible protocols (Yamamoto et al., 2011;

Engelmann-Sylvestre and Engelmann, 2015, Tanaka et al., 2020). A previous study, using V-cryoplate, evaluated the ideal dehydration level for the recovery of SEs obtained from roots of P. alliacea, after cryopreservation (Pettinelli et al., 2020). However, the large amount of polysulfides synthesized in P. alliacea SEs, compared to other plant structures (Webster et al., 2008), justify the demand for more comprehensive, optimized, and increasingly efficient protocols. Thus, the objective of this work was to evaluate the use of the D-cryoplate technique in SEs from leaf explants.

2 MATERIALS AND METHODS

Plant material

Petiveria alliacea plants kept in the in vitro collection of the Plant Biotechnology Center of the State University of Rio de Janeiro (NBV/UERJ), were used as leaf explant donors for the production of somatic embryos.

Induction of somatic embryogenesis

Leaf segments of about 1 cm^2 were excised from plants in vitro and inoculated in MS medium (Murashige and Skoog, 1962), supplemented with 20µM Picloram (PIC), according to a previously established protocol (Cantelmo et al., 2013). After 60 days of culture, somatic embryos (SEs) were isolated from the explant surface and transferred to MS medium, free of growth regulators (MS0), and subcultured monthly. The cultures were maintained in a growth chamber, under an average luminous intensity of 46 $\mu mol.m^2.s^{-1}$ at 38° ± 2°C, with a photoperiod of 16h.

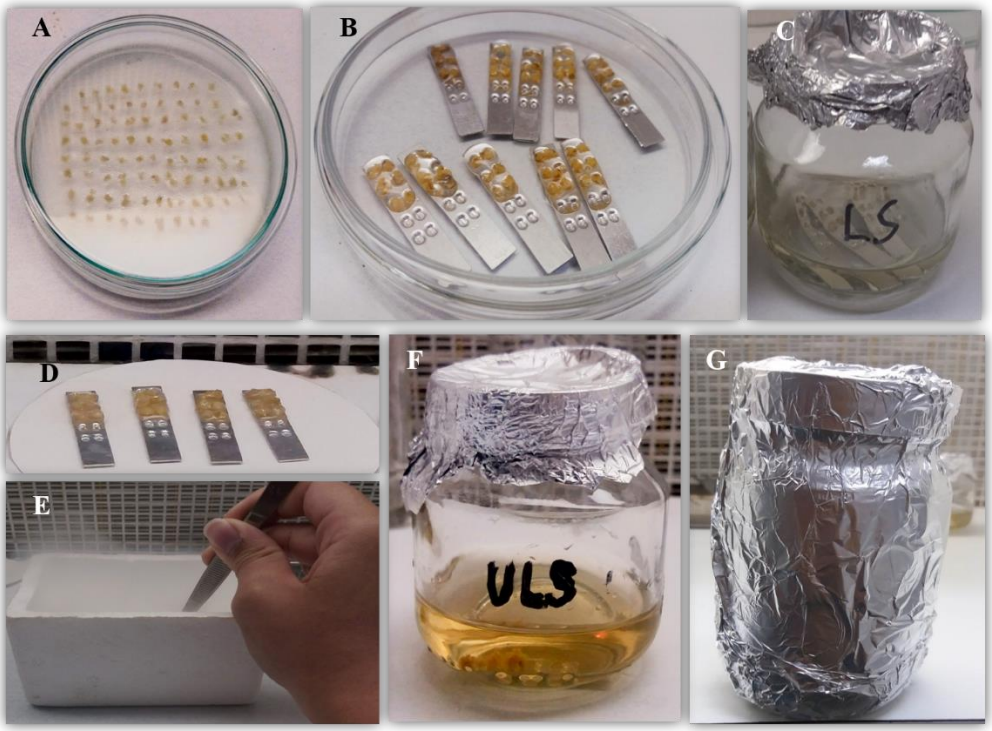
Cryopreservation by D-Cryoplate

Embryos at early stages of development (globular and cordiform) were precultured for 24 hours in MS medium with half the salt concentration, supplemented with 0.5M sucrose and solidified with FITAGEL® (Pettinelli et al., 2017) (Figure 1A). The D-cryoplate method described by Niino et al., 2013, with modifications, was used. Thus, part of the ESs sample was cultivated in MS0, as a pre-culture control.

The other embryos were arranged in groups of three to five embryos per well in the cryoplates (7mm X 37mm X 0.5mm). Then, a sodium alginate solution (3%) was dropped into the cryoplate wells and then a calcium chloride solution (0.1M) promoting polymerization and fixation of the ESs after 15 min of contact (Figure 1B). Cryoplates were then immersed in a conditioning solution (loading) containing 2M glycerol with 0.4M sucrose, for 20 minutes (Figure 1C) and placed on filter paper with ES facing upwards (Figure 1D), being exposed to laminar flow air for 0, 30, 60, 90, 120 and 140

minutes for physical dehydration. Then, the cryoplates were immersed in NL for about two minutes (Figure 1E), except for the non-cryopreserved control sample. After removing the LN, the cryoplates were immersed in a unloading solution (MS + 1.2M sucrose) (Figure 1F), and kept at room temperature for 20 minutes, with the groups of ESs still in alginate capsules, inoculated in MS medium supplemented with 0.6 μM AIA (Cantelmo et al., 2013) and incubated for 7 days in the dark (Figura 1G), then transferred to MS0 medium and kept under culture conditions already described.

Figure 1. Steps of the D-cryoplate technique: A) Preculture in sucrose; B) Polymerization in calcium chloride and fixation in cryoplates; C) Osmoprotection in loading solution; D) Exposure to airflow; E) Immersion in NL; F) Rewarming unloading solution; G) In vitro culture in the dark.



Survival and recovery assessment

Survival was evaluated by the TTC (2.3.5-triphenyl – tetrazolium chloride) viability test (Towill and Mazur, 1975), immediately after each treatment: preculture (PC), cryopreserved (+NL) and controls (-NL). The ESs were immersed in 1 ml of TTC solution (0.6%) and incubated at 30 ± 2°C for 24 hours in the absence of light. The chloride triphenyltetrazolium is a vital dye and on entering living cells is reduced to an insoluble red compound called formazan. Thus, the survival rate was estimated by changing the ESs color to red. Recovery was evaluated by multiplication of ESs, by secondary embryogenesis, after 90 days of culture, under the conditions described above.

In addition to the quantitative evaluation, qualitative observations of the regenerative response were also carried out, in relation to the formation of friable calluses, roots or somatic embryos in early conversion.

Data analysis

Three experiments were carried out, with 10 ES per treatment. The viability rate and the average number of embryos/treated embryo were evaluated by analysis of variance (ANOVA) and Tukey-Kramer mean comparison test, with the aid of the Graphpad InStat program, considering significant the values with $p \leq 0.05$.

3 RESULTS

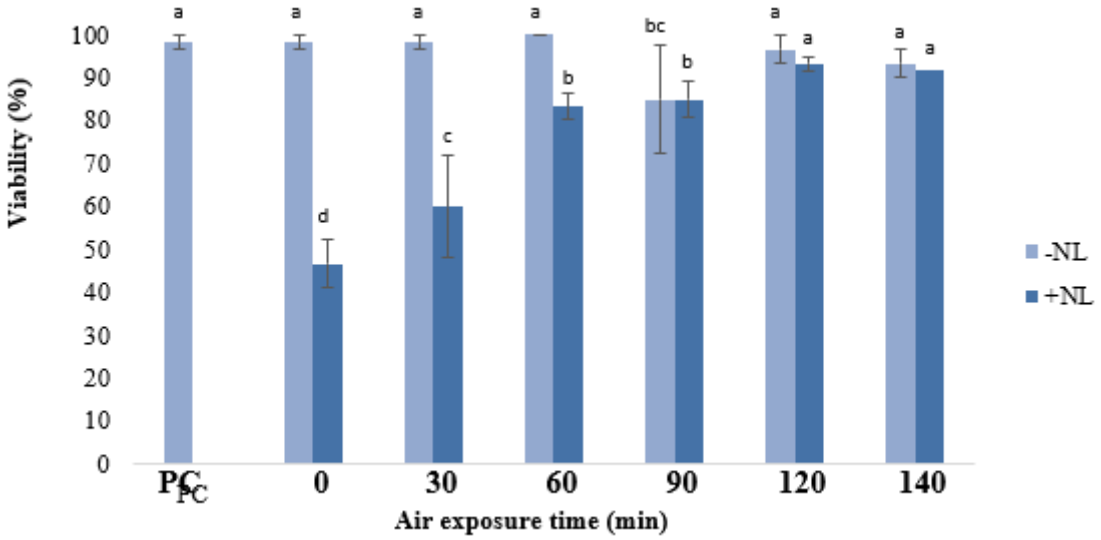
Induction of somatic embryogenesis

After 60 days of leaf segment culture in MS0 medium supplemented with PIC at 20μM, the emergence of ESs directly from the leaf tissue was observed, with a regeneration frequency of 90-100%, confirming the reproducibility of the culture protocol used. The same leaf segments subcultured in MS medium supplemented with PIC (20 μM) maintained their proliferative capacity, with about 150 ES per explant, after 30 days of culture. The primary embryos were isolated and inoculated in growth medium (MS0) where they presented high multiplicative rates via secondary embryogenesis, reaching about 80 secondary embryos for each inoculated embryo (data not shown). These secondary embryos were used in cryopreservation experiments.

Recovery after withdrawal from LN

The survival test indicated that control ESs (-NL) exposed to longer dehydration times showed a small drop in viability, compared to preculture (Figure 2). On the other hand, the increase in dehydration time allowed the best viability results in the cryopreserved group (+NL) (Figure 2). In time 120 minutes of exposure to the flow air, the highest viability rate of cryopreserved embryos was observed, with 93% survival, while the control, in this same dehydration time, reached 97% survival (Figure 2).

Figure 2. Viability rate of somatic embryos after different times of evaporative dehydration, immersed or not in LN.



The evaluation showed that ESs free of any treatment (general control) provided the highest multiplication rate, reaching about 85 secondary embryos for each treated embryo and a total of 846 SEs (data not shown). After preculture a total of 693 SEs were obtained indicating a reduction in recovery (data not shown). On the other hand, based on previous work, this treatment was considered essential for recovery after cryopreservation, regardless of the time of evaporative dehydration tested. The cryopreserved group reached a maximum rate of 32.2 embryos/treated embryo, in a dehydration time of 120 min (Table 1). In general, both groups (-NL and +NL) presented high multiplication rates, with the exception of time 0 min, that is, without going through evaporative dehydration, where no sign of regeneration was observed (Table 1).

Table 1 - Recovery after 90 days of culture of cryopreserved somatic embryos, depending on the time of exposure to air

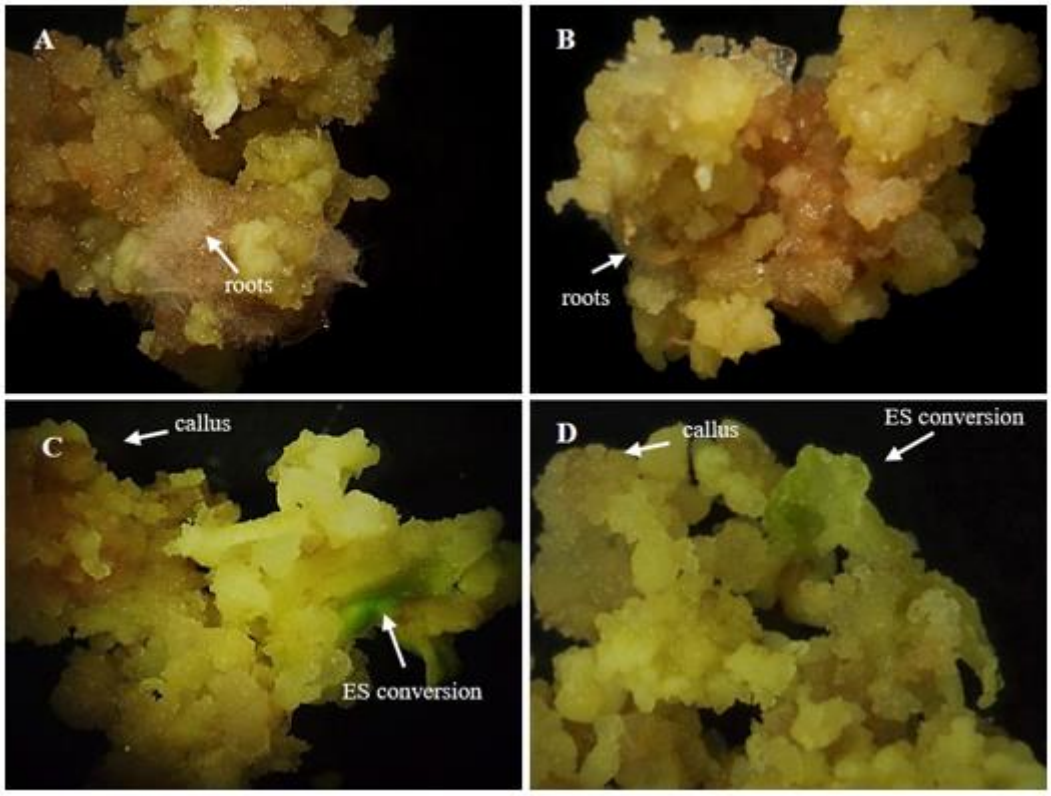
Air exposure time	-NL		+NL	
(min)	Total SEs*	SEs/SE*	Total SEs*	SEs/SE*
0	570 ± 2.0^{a}	57 ±0.2^{a}	10 ±0.0^{f}	1 ± 0.0^{e}
30	330 ± 1.5^{c}	33 ±0.1^{c}	86 ±1.0^{c}	8.6 ±0.1^{d}
60	405 ± 2.6^{b}	40.4 ±0.2^{b}	214 ±1.5^{c}	21.4 ±0.1^{c}
90	295 ±1.5^{c}	2.,5 ±0.1^{c}	192 ±1.5^{d}	19.2 ±0.1^{c}
120	192 ± 2.0^{d}	19.2 ±0.2^{d}	322 ±1.5^{a}	32.2 ± 0.1^{a}
140	170 ± 2.0^{d}	17 ±0.2^{d}	250 ±1.5^{b}	25 ± 0.1^{b}

OBS. N° SEs/SE = number of secondary somatic embryos for each treated somatic embryo; Control: Those not immersed in liquid nitrogen (-NL) and immersed in liquid nitrogen (+NL). * Means followed by different letters in the column differ significantly by the Tukey-Kramer test ($p<0.05$).

In addition to the quantitative evaluation through the multiplication of ESs, a qualitative evaluation of the recovery of materials exposed or not to liquid nitrogen (+NL

and -NL) was performed, which showed the presence of root formation (Figures 3A, 3B), ESs in conversion and late callus formation (Figures 3C, 3D), in the materials under cultivation.

Figure 3. Recovery of SEs submitted to different times of evaporative dehydration, after 90 days of culture in MS0: A) 90 min (-LN); B) 90 min (+LN); C)120 min (-LN); D)120 min (+LN). (16 x)



4 DISCUSSÂO

In this work, the somatic embryogenesis protocol of Petiveria alliacea (Cantelmo et al., 2013), used to produce somatic embryos from leaves, was reproductive, as demonstrated by the high rate of direct regeneration from leaf explants in response to Picloram. Furthermore, these primary embryos, when isolated and inoculated in growth medium (MS0) presented high multiplication rates, via secondary embryogenesis. The culture of somatic embryos is a very interesting system for phytochemical studies of this species, with recognized interest in pharmacology, due to the polysulfides produced in large quantities by these structures, notably dibenzyl trisulfide (DTS) (Webster et al., 2008). This polysulfide has proven activity against cancer, having already been used for the production of chemotherapy (An et al., 2006, Gu et al., 2008; Bao et al., 2008, Wauchope et al., 2021). Somatic embryogenesis has been a strategy widely used in metabolite-producing plants, targeting, among other purposes, the massive propagation

of elite genotypes (Vázquez-Flota et al., 2016) and the obtainment of secondary metabolites. The success of somatic embryogenesis applied to the production of substances of interest has already been reported in a range of medicinal species (Eleutherococcus koreanum, Kalopanax septemlobus, Allium victorialis var. platyphyllum Makino, Rosa rugosa Thunb.) (Park & Paek, 2014).

Different methodologies have already been used for the cryopreservation of P. alliacea germplasm, from explants such as roots and somatic embryos (Pettinelli et al., 2017). Among these, the encapsulation-dehydration technique in tubes, combined with evaporative dehydration, provided insufficient results, both in terms of embryo viability and recovery (Pettinelli et al., 2017). The V-cryoplate technique, in SEs from root explants, enabled the recovery of 21 embryos/embryo treated with PVS2 solution (Pettinelli et al., 2017), representing an increase in the recovery rate compared to classical vitrification in cryotubes, where the maximum rate was 12 embryos/treated embryo (Pettinelli, 2017). However, the same technique (V-cryoplate) used in preliminary experiments with leaf somatic embryos did not allow its recovery after LN removal. The disruption of the plasma membrane is positively correlated with the degree of sensitivity to the dehydration step (Pettinelli et al., 2020; Vaz et al., 2020) and it was found that these leaf embryos still had 52% of plasmolysis in their meristematic cells after 60 days of culture, whereas SEs from roots had only 2% of plasmolysis after 30 days of culture (Pettinelli et al., 2017). These results indicated that the origin of somatic embryos affected the response to cryoprotective treatments and could be hypothetically explained by the greater tolerance to dehydration expressed by embryos of root origin.

In the search to optimize an efficient protocol for leaf embryos, the D-cryoplate technique, considered safer for not using cryoprotectants toxic to tissues (Niino et al., 2019), was used in this work. This technique has been successfully applied to different explants (stem apexes, axillary buds and polyembryogenic masses) (Ochaat et al., 2021), of many economically important species, such as: potato (Yamamoto et al., 2015; Valle Arizaga et al., 2017), sugarcane sugar (Rafique et al., 2016), persimmon (Matsumoto et al., 2015), cherry and plum (Vujovic et al., 2015), dates (Salma et al., 2014), blueberry (Dhungana et al., 2017) and garlic (Tanaka, 2018).

In this work, the recovery rates of leaf embryos after LN removal reached about 32 embryos/inoculated embryo and were much higher than the rates previously obtained with ESs from root explants, which resulted in only 19 embryos/inoculated embryo (Pettinelli

et al., 2017), confirming the influence of the ES origin on the tolerance to cryogenic treatments.

In cryopreservation protocols, the dehydration step is crucial for cell survival after ultracooling (Reed, 2008). In this work, recovery was not obtained after immersion in NL, of the non-dehydrated controls. On the other hand, when dehydration was excessive, after long times of exposure to air, in controls not immersed in NL, the drastic reduction severely affected recovery. Therefore, the ideal level of dehydration is one in which the water content has been reduced to the maximum, without the removal of structural water, which characterizes dehydration injuries (Engelmann, 1991).

The cryoplate dehydration methodology represents an innovation to cryopreservation protocols and the overall success can be explained by factors such as: thermal conductivity of aluminum, which promotes ultra-fast cooling and heating rates, reducing the damage associated with this step, the ease in handle the explants, avoiding tissue damage, in addition to the absence of cryoprotective solution toxic to cells, such as DMSO present in the composition of PVS2 (Volk et al., 2007, Matsumoto, 2017).

Regarding biological aspects during recovery, the formation of rhizogenic/embryogenic callus was observed in all treatment conditions, including controls, requiring further morphogenic studies to elucidate this phenomenon. Friable calluses have previously been observed in cultures of root-derived P. alliacea SEs (Pettinelli et al., 2017). Although the conversion of SEs has not been explored in this work, previous studies have shown that SEs of P. alliacea are able to convert to phenotypically normal plants (Cantelmo et al., 2013; Soares, 2016).

The cryopreservation of ESs from leaves using aluminum cryoplates, with evaluation of progressive evaporative dehydration, reached unprecedented rates in culture recovery, surpassing the rates obtained with ESs from root explants. Furthermore, the comparison with other techniques previously applied to the species clearly shows the better performance of D-cryoplate for the recovery of cryopreserved P. alliacea ESs.

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