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Comparative Study of Three Vitrification Solutions for an Effective Droplet-vitrification Cryopreservation Procedure for Pfaffia Glomerata (Spreng.) Pedersen

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IRIS designed the study, wrote the first draft and prepared the final version of the manuscript, Author DVS managed the literature searches, performed the experiments and recorded the data obtained, author ISM performed the statistical analysis of the data, author ANS reviewed the statistical analyses of the data, reviewed and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of this research was to establish a cryopreservation protocol for shoot tips (ST) of *in vitro P. glomerata* using the droplet-vitrification technique.
Study Design: The experimental design was a factorial, with four factors, arranged in a completely randomized design. Three vitrification solutions (PVS2, PVS3, PVS4), three times (20, 40, 60 min) and two temperatures (25 ± 2 °C and 0 °C) of treatment with the solutions, followed by freezing (LN+) or not (LN-) with liquid nitrogen (LN) were tested. All tests were performed using six replicates and the results analysed using Two-way ANOVA and Tukey's tests and expressed as the mean ± the standard error of the means (SEM) deviation.

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Place and Duration of Study: Laboratory of Plant Cryobiology, Embrapa Genetic Resources and Biotechnology, over a two-year period.
Methodology: ST excised from in vitro plantlets were pre-cultured overnight (19h), treated with a loading solution (LS) and three different vitrification solutions (PVS2, PVS3, PVS4) prior to freezing in LN. Treatment with the vitrification solutions was carried out at 0 or 25°C, for 20, 40 or 60 min. For freezing, drops of the vitrification solutions containing a single ST were dispensed on aluminum foil strips and the strips were submerged in LN (-196°C). For thawing, foil strips were submerged into unloading solution (US) at 40 ± 2°C, for three min. Thawed ST were transferred to regeneration medium and cultured *in vitro*.
Results: Highest regeneration percentages after cryopreservation were 82% for ST treated with PVS3, at 0°C, for 60 min; 32% for ST treated with PVS4 at 25°C for 60 min or 0°C for 40 min and 22% for those treated with PVS2 at 0°C for 60 min.
Conclusion: Droplet-vitrification is a suitable technique to ensure survival of *P. glomerata* ST after cryopreservation. This procedure can be applied to establish germplasm collections of this medicinal species in gene banks.

Keywords: Brazilian ginseng; cryopreservation; droplet-vitrification; germplasm conservation; Pfaffia glomerata; shoot tips.

1. INTRODUCTION

Species of *Pfaffia*, Amaranthaceae family, stand out for their medicinal properties [1]. The roots of *Pfaffia plants* are used in Brazilian folk medicine as tonic and general stimulant, aphrodisiac and for treatment of diabetes. Roots and leaves of these plants have also been used as tranquilizer, antirheumatic, antidiarrheal, anti-inflammatory, antidiabetic, febrile, internal, and external healing, antihemorrhic, improvement of vision and memory and for treatment of gastric disorders, arthritis, arthrosis, anemia, asthenia, and pain [2,3]. In Brazil, there are 27 different genera and 158 species of this family, but *P. glomerata* is of major medicinal and commercial importance [4].

Cryopreservation is considered the ideal method for the long-term conservation of germplasm of many plant species, because it allows the storage of biological materials for indefinite periods of time, maintaining high genetic stability, phenotypic characteristics, using little space, and requiring little maintenance [5]. Different cryopreservation techniques have been developed and they are applicable to different types of plant tissues, and cryopreservation has become an important strategy for the conservation of plant germplasm in gene banks around the world [5,6].

Droplet-vitrification is a cryopreservation technique that has been successfully applied to many plant species [7,8]. This technique consists of dripping vitrification solution containing one single explant on aluminum foil strips, which are immersed quickly in liquid nitrogen (LN) -196°C. It was developed from the droplet-freezing method first reported by Kartha and colleagues [9] for the cryopreservation of cassava (*Manihot esculenta* Crantz) meristems. On account of aluminum being an efficient thermal conductor, and that freezing explants displayed on aluminum foil strips allows direct contact of the droplets containing the explants with liquid nitrogen, higher and more homogeneous rates of temperature change are achieved during cooling or warming, promoting freezing at a uniform rate of the constituent cells of the explants [10]. Additionally, high cooling rates, about 130 °C / min^{-1} [11], facilitate intracellular vitrification, whereas rapid reheating reduces the possibility of recrystallization of intracellular ice, leading to greater recovery and survival of the material frozen in liquid nitrogen [12, 13]. The technique of droplet-vitrification has been used for the cryopreservation of shoot tips of different species of herbaceous and woody plants, such as *Rosa* x *hibrida* L. [14], *Colocasia esculenta* var. *esculenta* (L.) Schott [15], *Malus domestica* Borkh [14], cultivars of *Lilium* x *lancifolium*, *Lilium* x *longiflorum*, *Lilium* x *siberia* [16]. Even though satisfactory cryopreservation of *P. glomerata* shoot tips by vitrification has been reported previously [17], this research was carried out to investigate the possible beneficial effect of the droplet - vitrification technique on *P. glomerata* shoot tips survival to further improve the cryopreservation of this species.

2. MATERIALS AND METHODS

2.1 Explants and Growth Medium

The explants used for this study were 2-4 mm-long shoot tips (ST), consisting of the meristematic dome plus 1 to 2 leaf primordia and a portion of the shoot, excised from 15-day old shoots sprouting on nodal segments that were obtained from *P. glomerata* plantlets growing *in vitro* on MS medium [18] A sample of 120 ST were used at each stage of the procedure. 60 of these ST received the treatment, but were not frozen in LN (LN-, control) and 60 ST received tre treatment and then were frozen in LN (LN+). The basic medium was supplemented with 0.02 mg/l BAP (6-benzylaminopurine), 0.01 mg/l of NAA and 0.1 mg/l of GA3 (gibberellic acid), 8 g/l agar, 30 g/L anhydrous sucrose, pH adjusted to 5.7 (before the addition of the agar) and sterilized by autoclaving at 121 °C, for 15 min, and dispensed after autoclaving in glass test tubes (150 x 25 mm). This growth medium was used in all the steps of this cryopreservation procedure, except for the preculture treatment. All the steps of the cryopreservation procedure described here were performed in a laminar flow hood, under aseptic conditions, at ambient temperature (25 ± 2 °C).

2.1.1 Culture conditions

After each step of the procedure, ST were individually transferred to test tubes containing 10 ml of MS medium (**2.1**), transferred to a growth room and cultivated at 25 ± 2 ºC, with a 12-hour photoperiod and light intensity of 40 $\mu m.m^{-2}.s^{-1}$, supplied by cool LED bulbs, to access the effect of each step of the procedure on ST viability and their growth and regeneration capacity. Cryopreserved ST (LN+) were initially cultivated in the dark for 48 h and subsequently for 72 h in diffuse light prior to exposure to the regular light conditions of the growth room, specified above.

2.2 Droplet - vitrification Cryopreservation Procedure

The droplet - vitrification technique tested in this study consisted of several successive steps, and prior to cryopreservation in LN (-196°C), excised ST were pre-cultured, treated with a loading solution (LS, basic MS supplemented with 2.0 M glycerol and 0.4 M sucrose) and three vitrification solutions (Plant Vitrification Solution) containing different combinations of cryoprotectants: PVS2 (30% glycerol, 15% ethylene glycol, 15% dimethylsulfoxide and 0.4 M sucrose, diluted in MS medium), PVS3 (50% of glycerol, 50% of sucrose, diluted in MS medium) and PVS4 (35% glycerol, 20% ethylene glycol and 0.6 M sucrose, diluted in MS medium). The vitrification solutions were sterilized by filtration using a 0.22 µm, 150 ml analytical filter unit. Three exposure times (20, 40 and 60 min) and two temperatures (25 ± 2 °C and 0 °C) were tested to determine the best exposure duration and temperature for treatment with the vitrification solutions tested.

2.2.1 Pre-culture of shoot tips

Excised ST were transferred to Petri dishes containing 20 ml of solid MS medium plus 0.3 M sucrose and cultivated in the dark, at 25 ± 2 ºC, in the growth room, in the conditions described above (**2.1.1**) for a period of 18 ± 2 h (overnight treatment). After pre-culture, 60 ST (pre-culture control, PCC) were inoculated in test tubes containing 10 ml of the growth medium described above (**2.1**) and transferred to the growth room and cultured as described above (**2.1.1**).

2.2.2 Loading, vitrification, and freezing procedures

Pre-cultured ST were transferred to cryovials containing 2.0 ml of LS and held for a total of 60 min; after 30 min the LS was removed with a pipette, 2.0 ml of fresh LS was added, and the ST remained immersed in the LS for a further 30 min. The loading treatment was carried out at 25 ± 2 °C (room temperature). After loading, a control sample (LSC) consisting of 60 ST was transferred to test tubes, placed in the growth room, and cultivated under the same conditions described previously (**2.1**) to access the effect of the loading treatment on ST viability. For the vitrification with the cryoprotectant solutions, the LS was drained, three subsamples of the ST were transferred to three different cryovials and 1.0 ml of each of the three vitrification solutions tested (PVS2 or PVS3 or PVS4) was added to each cryovial, and the ST were held in the solutions for 20, 40 or 60 min. Halfway through the vitrification time, the vitrification solution was removed with a pipette and another 1.0 ml of the vitrification solution was immediately added to the vial. ST remained immersed in the solutions until the total vitrification period (20, 40 and 60 min) was completed. The vitrification solutions were at 25 ± 2 °C (room temperature) or chilled to 0 °C in an ice bath, and they were maintained

at these same temperatures for the duration of the treatment. After the vitrification step, samples of the treated ST (60 ST for each of the treatments performed) were transferred to test tubes containing growth medium and cultured in the growth room, and under the same conditions described previously (**2.1**) to access ST viability after the vitrification treatments (LN-).

Finally, for the freezing test, ST treated with each vitrification solution (PVS2, PVS3 or PVS4) were removed from the cryovials, individually transferred to drops of fresh vitrification solution displayed on sterile aluminum foil strips (50 x 7 mm). The strips were then immersed into LN and held for at least 60 min.

2.2.3 Thawing and dilution and regeneration

ST were thawed rapidly by removing strips from LN and immersing them in a glass jar containing 10 ml of dilution solution (liquid standard MS supplemented with 1.2 M sucrose) previously warmed to 40 ± 2 °C. The jar was stirred constantly for about 2 min and after this time, the dilution solution and the aluminum foil strips were removed from the jar and 10 ml of fresh dilution solution was added and allowed to act for 5 min. After this time, the dilution solution was removed, and ST were transferred to a Petri dish lined with one disc of autoclaved filter paper. This step of the procedure was conducted at 25 ± 2 °C (room temperature). After thawing ST were individually transferred to test tubes containing 10 ml of solid MS medium (**2.1**) and cultured under the same conditions described previously (**2.1.1**), to access ST viability after cryopreservation (LN+). ST were monitored daily up to 7 days after innoculation (DAI) and then weekly for up to three months. ST that remained green, enlarged, resumed growth, and formed new leaves and roots were recorded as viable.

2.3 Statistical Analysis

The experimental design was a factorial, with four factors (vitrification solution type, time of exposure, temperature of exposure, and presence or absence of liquid nitrogen). Three vitrification solutions (PVS2, PVS3 and PVS4), three exposure times to these vitrification solutions (20, 40 and 60 min), two temperatures of treatment with the vitrification solutions (25 ± 2 °C and 0 °C) followed by freezing with liquid nitrogen (LN+) or not (LN-). These 36 treatments (combinations of factors) were replicated six times and the 216 experimental units were arranged in a completely randomized design. For the statistical analysis, the data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey's test, at 1% probability level ($p < 0.01$). The analyzes were performed using the softwares GraphPad Prism 5.01 (Inc., 2007, San Diego California USA) and Genes [19]. Data are presented as mean percentage of total number of ST that showed regrowth after cryopreservation ± the standard error of the means SEM).

3. RESULTS AND DISCUSSION

Regrowth percentages of *Pfaffia* ST after freezing in LN using the droplet-vitrification technique were determined by the type of cryoprotectant solution, the temperature of the solution and the duration of the treatment with each solution (Figs. 2 and 3). The analysis of variance and Tukey's test showed that the regrowth percentages of control and cryopreserved ST was significantly different ($p < 0.001$), that the type of vitrification solution had a significant positive effect ($p < 0.001$) on ST regrowth after freezing in LN, and that PVS3 and PVS4 were superior to PVS2 (Fig. 2). Fig 2 shows that when the dehydration of the ST was done with the cryoprotectant solutions chilled to 0 °C, in an ice bath, the regrowth percentages of ST treated with PVS3 were significantly higher ($p < 0.001$), regardless of the duration of the treatment, and the highest percentage of regrowth (81%) was obtained for ST treated with PVS3 for 40 min (Fig 2B). The lowest regrowth percentages were obtained for ST treated with PVS2, and no ST treated with PVS2 for 20 min survived after freezing in LN (Fig 2A). The regrowth percentages of ST treated with PVS4 were lower than those obtained for ST treated with PVS3 and higher than the regrowth percentages of ST treated with PVS2. Regrowth percentages of ST treated with the vitrification solutions at room temperature (25 ± 2 °C) showed a similar pattern of response to the type of vitrification solution and the duration of the dehydration treatment with each solution. As can be seen in Fig. 3, the regrowth percentages of ST treated with PVS3 at 25 °C (room temperature) were higher, regardless of the duration of the treatment, and the highest percentage of regrowth (50%) was obtained for ST treated with PVS3 for 40 min (Fig 3B). The lowest percentages of regrowth were obtained for ST dehydrated with cryoprotectant solutions for 60 min, and no ST treated with PVS2 for 40 min (Fig 3B) survived after freezing in LN.

Typically, viable cryopreserved ST turned green within a week of culture in regrowth medium, and within 30 days of culture new plantlets developed from ST and had similar morphological traits to the stock plants, with expanded leaves showing a characteristic pink hue, well developed roots and the stem segmented in an average of 3 - 5 nodes (Fig 1D). Regrowth percentages of control ST, that is, those that were treated with the same cryoprotectant solutions, at the same temperatures and for the same periods of time, but were not frozen in LN, ranged from 83% to 100% (Figs. 2 and 3). The lower regrowth percentages were observed for control ST treated with PVS2 (Figs. 2 and 3). Only 83% and 86% of control ST treated with PVS2 chilled to 0 °C in an ice bath for 20 and 40 min, respectively, remained viable and could regrow and originate new plantlets (Fig 2A and B). The lowest regrowth percentages for control ST treated with the cryoprotectant solutions at 25 ± 2 °C (room temperature) were also for those treated with PVS2 (Fig 3A, B and C).

The results obtained in the present work demonstrated that of the three vitrification solutions tested (PVS2, PVS3 and PVS4), PVS3 was the most favorable to preserve the viability and regrowth capacity *P. glomerata* ST after freezing using the droplet – vitrification technique. These results suggest that the combination and / or concentration of cryoprotectants present in this solution might have promoted proper dehydration of the ST and caused less pronounced chemical toxicity and osmotic stress, resulting in better survival rates after freezing. PVS2 was also the least favorable of the three vitrification solutions tested, probably because the dehydration promoted by this solution could have been too intense, resulting in significant osmotic stress and damage to *P. glomerata* cells, and a low regrowth response of the ST. Treatment of *P. glomerata* ST with PVS4 (35% glycerol, 20% ethylene glycol and 0.6 M sucrose) also resulted in lower regrowth rates, suggesting that this solution might have failed to promote suitable dehydration of the ST, resulting in intracellular damage during freezing, and in the low percentages of regrowth observed in explants immersed in LN.

Results like the ones observed in the present research were reported by MARKOVIĆ and colleagues [20] for shoot tips of grapevine cryopreserved using the droplet-vitrification technique. The authors reported that there was an interaction between dehydration time in PVS2 and exposure to liquid nitrogen, and that this interaction interfered negatively on the regrowth rate of the explants. They reported that longer dehydration periods in PVS2, resulted in lower regeneration capacity of grapevine explants [20]. Similarly, a better effect of dehydration with PVS3 was also observed for cherry (*Prunus cerasus* x *Prunus canescens*) cryopreserved shoot tips, which retained high survival rates (95%) and regeneration (40%) after a 60 min dehydration treatment with PVS3 [21]. WANG and colleagues [22] in a study comparing three vitrification-based cryopreservation procedures for shoot tips of potato, cv. “Zihuabai”, reported that dehydration with PVS2 was the step that caused the most damage to cells of shoot tips, regardless of the cryogenic procedure tested. PVS3 also produced significantly higher survival and regeneration percentages of cryopreserved shoot tips of *Chrysanthemum* [12]. In the case of *Galanthus elwsii*, however, dehydration with PVS2 for 20 and 30 min did not compromise the survival of the explants after freezing in liquid nitrogen [23]. There is a great number of reports of positive effect of PVS2 on plant cells and tissues survival after cryopreservation. However, even though PVS2 has been effective to promote adequate desiccation of explants of many plant species, there has been many cases in which this solution was not ideal, and other vitrification solutions proved more effective. In this regard, Panis and colleagues [13] state that the effect of the treatment with PVS2 varies considerably among plant species, but that, generally, the longer the time of exposure, more lethal effects to the cells are observed. In other studies, it was reported that the best exposure times to PVS2 were 60 min for *Stevia*. [8], 20 min. for *Rosa hybrida* L. [14], 60 min for *Malus domestica* Borkh. [7], 30 min for *Byrsonima intermedia* A. Juss [24], 20 min for *Rosa agrestis*, *R. canina* and *R. dumalis*, and 30 min for *Rosa rubiginosa* [25]. Barraco and collaborators [26] observed that the sprouting of two sugarcane cultivars from Hawaii (H70-144 and E CP68-1026) showed relatively high values of tolerance to PVS2 for exposure periods of 20 and 40 min, with an average of 70% survival. Regarding the duration of the treatment with the vitrification solutions, the highest ST regeneration rates occurred after 40 min, and longer periods of treatment with these solutions resulted in decreased regrowth rates. These results suggest that the cryoprotectant solutions might have become toxic and caused injury due to cumulative chemical toxicity or osmotic shock to ST cells after prolonged periods of treatment.

Successful cryopreservation for *P. glomerata* shoot tips was established previously using the vitrification technique [17]. The present work focused on the droplet-vitrification technique and was undertaken to optimize shoot tip regrowth percentages and to provide an alternative cryopreservation procedure for this species. Establishment of different cryopreservation procedures is important to provide safer cryopreservation alternatives and to meet genotype-specific requirements of plant species, which can be a limiting factor for the wide application of cryopreservation technology for plant germplasm conservation in gene banks. It has been shown that injury to plant cells during cryopreservation are correlated to the cryogenic procedure. In the case of potato cv. Zihuabai, there was clear evidence that vitrification with PVS2 caused the most severe injury to shoot tip cells, whereas the droplet-vitrification procedure produced moderate injury [22]. PANIS and collaborators [13] verified a 42% regeneration increase for banana shoot tips, of four genomic groups, which were cryopreserved adopting the droplet-vitrification technique, in comparison with the traditional vitrification method used previously. In the case of the present study, shoot tip regrowth rates were higher and plantlet regeneration and growth was faster than when the vitrification procedure was used [17]. The droplet-vitrification technique has been developed for many other plant species [7,8,12,13,14,15,16,21,22,23,24,25,26,27]. It is a practical, user-friendly technique and is now routinely applied for the cryopreservation of shoot tips of a wide range of plant species and genotypes and could be an alternative for implementing long-term storage of *P. glomerata* germplasm.

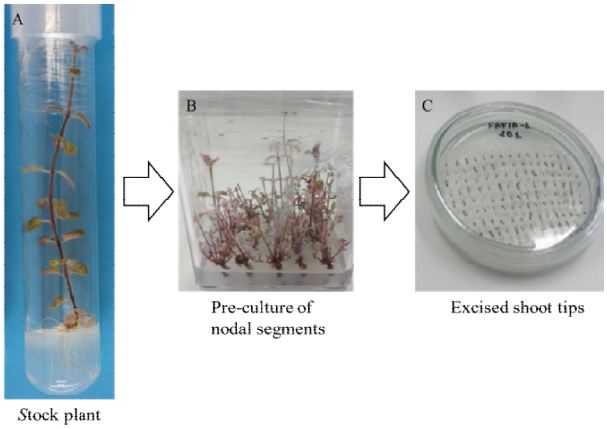

Fig. 1. *Pfaffia glomerata* at different stages of the cryopreservation procedure. A: stock plantlets growing *in vitro*; B: new shoots produced on nodal segments after 15 days of culture on growth medium (MS salts and vitamins supplemented with 0.02 mg/L BAP, 0.01 mg/L of NAA and 0.1 mg/L of GA3, 8 g/L agar, 30 g/L anhydrous sucrose, pH = 5.7); C: excised shoot tips (2 - 4mm) ready for use at different steps of the droplet – vitrification cryopreservation technique tested in this study

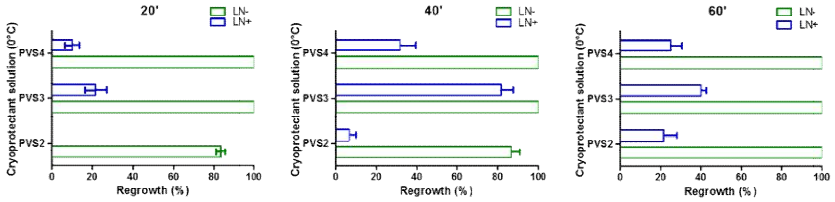

Fig. 2. Regrowth percentages of shoot tips of *Pfaffia glomerata* after treatment with PVS2, PVS3 and PVS4 at 0 °C for 20, 40 and 60 min. Blue bars show regrowth after cryopreservation in liquid nitrogen (LN+) and green bars regrowth of controls (LN-), which had the same treatments except freezing in liquid nitrogen. Data is presented as means of regrowth percentages with the standard error of the means (SEM), significant at P < 0.001

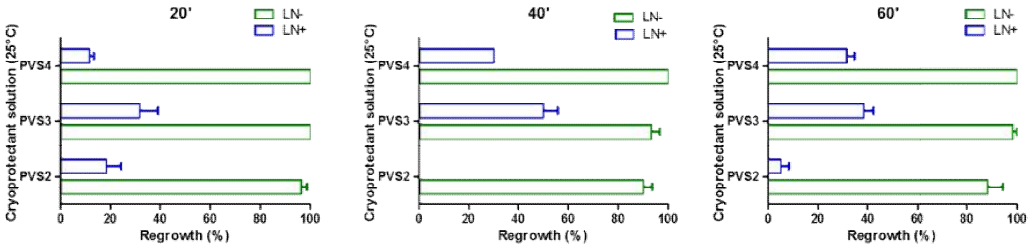

Fig. 3. Regrowth percentages of shoot tips of *Pfaffia glomerata* after treatment with PVS2, PVS3 and PVS4 at 25 °C for 20, 40 and 60 min. Blue bars show regrowth after cryopreservation in liquid nitrogen (LN+) and green bars regrowth of controls (LN-), which had the same treatments except freezing in liquid nitrogen. Data is presented as means of regrowth percentages with the standard error of the means (SEM), significant at P < 0.001

The success of the cryopreservation of plant cells and tissues is determined by many factors, and among them the free water content of the explant at the moment of freezing has a preponderant role. Free water will undergo crystallization at liquid nitrogen temperature and cause irreversible damage to the cell membranes and cellular structure and cause enzymatic alterations [28,29,30]. Therefore, one of the key factors in the design of a cryopreservation protocol is to select and optimize a dehydration strategy for the explants prior to freezing them in liquid nitrogen, to avoid the formation of ice crystals inside the cells and tissues Treatment of explants with vitrification solutions containing combinations of different cryoprotectants, such as DMSO (dimethyl sulfoxide), ethylene glycol, glycerol, and sucrose, added in specific concentrations has become a common strategy in the design of cryopreservation protocols. However, these cryoprotectant solutions must be used with caution since they might have a toxic effect or cause osmotic stress to cells of some plant species, compromising viability of the cells in the explant, resulting in cell death, especially if immersion in them occurs at temperatures above 0 °C or for prolonged periods of time [8,31]. Toxic effects can be prevented by selecting adequate cryoprotectants, adjusting the concentration of cryoprotectants in the solution, and the temperature and duration of the treatment.

4. CONCLUSION

The aim of this study to develop a cryopreservation procedure based on the droplet–vitrification technique for shoot tips of *Pfaffia glomerata* (Spreng.) Pedersen was achieved. Shoot tips remained viable after cryopreservation in liquid nitrogen, and the highest regrowth rate (81%) was obtained for shoot tips dehydrated with PVS3 vitrification solution, for 40 min, at 0 ºC. Viable shoot tips resumed growth and developed into new plantlets with well-formed leaves and roots. This procedure can be applied to establish a germplasm collection to ensure the long-term conservation of *P. glomerata* germplasm and, additionally, it is a relevant contribution to the advancement of plant cryobiotechnology.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Montanari Jr I, Magalhães PM, Queiroga CL. Influence of plantation density and cultivation cycle on root productivity and tenors of β-ecdysone in *Pfaffia glomerata* (Spreng) Pedersen. Acta Hortic. 1999;3(502):125-129.
2. Freitas CS. Actions of crude hydroalcoholic extract of *Pfaffia* spp on gastrointestinal tract. Braz Archives of Biol and Technol. 2003;46(3):1167-1179. DOI:10.1590/S1516-89132003000300007
3. Rates SMK, Gosmann G. *Pfaffia*: available chemical and pharmacological data and

their implications for its therapeutic use. Braz J Pharmacognosy. 2002;2-85-92. DOI:10.1590/S0102-695X2002000200005

4. Zimmer AR, Bruxel F, Bassani VL, Gosmann G. HPLC method for the determination of ecdysterone in extractive solution from Pfaffia glomerata. J Pharm Biomed Anal. 2006;40:450-453. DOI:10.1016/j.jpba.2005.07.016
5. Jenderek MM, Reed BM. Cryopreserved storage of clonal germplasm in the USDA National Plant Germplasm System. In Vitro Cell.Dev.Biol.—Plant. 2017;53:299–308 DOI:10.1007/s11627-017-9828-3
6. Sakai A, Engelmann F. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. Cryo Letters. 2007;28:151–157.
7. Condello E, Caboni E, Andre E, Piette B, Druart R, Swennen R, PANIS B. Cryopreservation of apple in vitro axillary buds using droplet-vitrification. Cryo Letters. 2011;32:175-185.
8. Benelli C, Carvalho LSO, Elmerzougui S, Petruccelli R. Two advanced cryogenic procedures for improving *Stevia rebaudiana* (Bertoni) cryopreservation. Plants. 2021;10(277):1-12. DOI:10.3390/plants10020277
9. Kartha KK, Leung NL, Mroginski LA. In vitro growth responses and plant regeneration from cryopreserved meristems of cassava (*Manihot esculenta* Crantz). Z. Pflanzen. Bd. 1982;107:133-140.
10. Leunufna S, Keller ERJ. Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). Plant Cell Rep. 2003;21(12):1159-1166. DOI:10.1007/s00299.003.0652.3
11. Towill LE, Bonnart R. Cracking in a vitrification solution during cooling or warming does not effect Growth of cryopreserved mint shoot tips. CryoLetters. 2003;24(6):342-346.
12. Kim HH, Lee YG, Shin DJ, Ko HC, Gwang JG, Cho EG, Engelmann F. Development of alternative plant vitrification solutions in droplet-vitrification procedures. CryoLetters. 2009;30(5):320-334. DOI:10.17660/ActaHortic.2011.908.20
13. Panis B, Piette B, André E, van den Houwe I, Swennem R. Droplet-vitrification: the first generic cryopreservation protocol for organized plant tissues. Acta Hortic. 2011;908:157-164. DOI: 10.17660/ActaHortic.2011.908.17
14. Halmagyi A, Pinker I. Plant regeneration from Rosa shoot tips cryopreserved by a combined droplet-vitrification method. Plant Cell Tissue and Organ Cult. 2006;84:145-153. DOI:10.1007/s11240.005.9012.z
15. Sant R, Panis B, Taylor M, Tyagi A. Cryopreservation of shoot tips by droplet-vitrification applicable to all taro (*Colocasia esculenta* var. *esculenta*) accessions. Plant Cell Tiss Org Cult. 2008;1:107-111. DOI:10.1007/s11240-007-9302-8
16. Chen XL, Li JH, Xin X, Zhang ZE, Xin PP, Lu XX. Cryopreservation of in vitro-grown apical meristems of *Lilium* by droplet-vitrification. South African J of Bot. 2011;77(2):397-403. DOI:10.1016/j.sajb.2010.10.005
17. Oliveira DV, Santos IRI, Martins IS, Salomão AN. Cryopreservation of shoot tips of "Brazilian Ginseng" (*Pfaffia glomerata* Spreng. Pedersen) by vitrification. J Agric Sci (Tor). 2019;11(11):146-155. DOI:10.5539/jas.v11n11p146
18. Murashige T, Skoog F. A Revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15:473-497. DOI:10.1111/j.1399-3054.1962.tb08052.x
19. Cruz, CD. Programa GENES: Estatística experimental e matrizes. 2013. UFV, Viçosa.
20. Bi WL, Hao XY, Cui ZH, Volk GM, Wang QC. Droplet-vitrification cryopreservation of in vitro-grown shoot tips of grapevine (Vitis spp.). In Vitro Cell Devel Biol – Plant. 2018;54:590–599. DOI:10.1007/s11627-018-9931-0
21. Ruzic D, Vujovic T, Cerovic R. Cryopreservation of cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) shoot tips by droplet-vitrification technique. 2013;21(2):79-85. DOI:10.2478/johr-2013-0025
22. Wang B, Li JW, Zhang ZB, Wang RR, Ma YL, Blystad DR, Joachim Keller ER, Wang QC. Three vitrification-based cryopreservation procedures cause different cryo-injuries to potato shoot tips while all maintain genetic integrity in regenerants. J of Biotech. 2014;184:47-55. DOI:10.1016/j.jbiotec.2014.04.021
23. Maslanka M, Panis B, Bach A. Cryopreservation of *Galanthus elwesii* Hook. apical meristems by droplet vitrification. CryoLetters. 2013;34:1-9.

24. Silva LC, et al. Shoot-Tip Cryopreservation by droplet-vitrification of *Byrsonima Intermedia* A. Juss.: A Woody Tropical and Medicinal Plant Species from Brazilian Cerrado. CryoLetters. 2013;34(4):338-348.
25. Pawlowska B, Szewczyk-taranek B. Efficient cryopreservation by droplet - vitrification of pentaploid roses and the phenotype of regenerated plants. Acta Soc Bot Pol. 2015;84(4):439–442. DOI: 10.5586/asbp.2015.038
26. Barraco G, Sylvestre I, Engelmann F. Comparing encapsulation-dehydration and droplet-vitrification for cryopreservation of sugarcane (*Saccharum* spp.) shoot tips. Sci Hortic. 2011;130:320-324. DOI: 10.1016/j.scienta.2011.07.003
27. Vujovic T, Sylvestre I, Ruzic D, Engelmann F. Droplet-vitrification of apical shoot tips of *Rubus fruticosus* L. and *Prunus cerasifera* Ehrh. Sci. Hortic. 2011;130: 222–228.
28. Engelmann F. Use of biotechnology for the conservation of plant biodiversity. In vitro Develop Biol. 2011;47:5-16.
29. Streczynski R, Clark H, Whelehan LM, Ang ST, Hardstaff LK, Funnekotter B, Bunn E, Offord CA, Sommerville KD, Mancera RL. Current issues in plant cryopreservation and importance for ex situ conservation of threatened Australian native species. Australian J Bot, 2019;67:1–15. DOI:10.1071/BT1
30. Suranthran P, Gantait S, Sinniah UR, Subramaniam S, Alwee Ssrs, Roowi SH. Effect of loading and vitrification solutions on survival of cryopreserved oil palm polyembryoids. Plant Growth Regul. 2012;66:101-109. DOI:10.1007/s10725-011-9633-7
31. Fuller BJ. Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo Letters. 2004;25;375-388.

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