Article

Developing a Cryopreservation Protocol for Embryonic Axes of Six South American Peanut Genotypes (*Arachis hypogaea* L.) Using Desiccation–Vitrification

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Abstract: The present study investigates the cryopreservation of embryonic axes from the peanut genotype ECU-12466, demonstrating a successful protocol involving a 1 h desiccation on silica gel followed by a 1 h cryoprotection with Plant Vitrificatin Solution 2 (PVS2). The average dimensions of the excised embryonic axes were 5.6 mm in length and 3.5 mm in width, with plumule lengths averaging 2.2 mm. Notably, germination rates for cryopreserved axes ranged from 71.4% to 85.7%, showing resilience to varying desiccation and PVS2 treatment times, particularly at 1 h. Shoot length was significantly enhanced by a 1 h PVS2 exposure, while longer durations resulted in phytotoxic effects. Rooting rates were higher for samples treated with shorter desiccation periods, with 54% rooting achieved at 1 h of PVS2 exposure, contrasting sharply with just 16% at 2 h. The moisture content of the embryonic axes remained stable between 9.3% and 9.5%, indicating no detrimental impact from the applied treatments. To evaluate the protocol's broader applicability, five additional peanut genotypes (ECU-11401, ECU-11418, ECU-11448, ECU-11469, and ECU-11494) were tested. While cryopreserved samples demonstrated high germination rates of up to 95.4% after 15 days, the rooting success was significantly lower (25.2%) compared to the control group's 90.9%. Following transplantation, the growth performance varied among genotypes, with a success rate of 93% for ECU-11494. Overall, this study elucidates the critical parameters for optimizing cryopreservation protocols in peanut embryonic axes, contributing to more effective long-term conservation strategies.

Keywords: groundnut; cryoconservation; peanut; dehydration; silica gel; PVS2

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1. Introduction

Peanut (*Arachis hypogaea* L.), also known as groundnut, plays a crucial role in global agriculture, particularly in developing countries where it serves as a vital source of protein and oil. In 2023, the world's peanut production was approximately 50.46 million metric tons, with China and India accounting for nearly 58% of this production [1]. Peanuts are rich in protein (about 25–30%), healthy fats (40–50%), and carbohydrates (10–20%), along with essential vitamins and minerals such as vitamin E, magnesium, and potassium, making them a key food source for protein and energy [2].

For the preservation and distribution of germplasm, collections of peanut cultivars and their related wild species are frequently preserved in seed banks. Although *A. hypogaea*

seeds have been classified as orthodox [3], viability losses occur frequently even under optimal storage conditions [4]. Their storage tissues have a high lipid content [5], which makes them susceptible to auto-oxidation and the production of free radicals, which can harm proteins and nucleic acids [6]. Therefore, *Arachis* seeds can be classified as sub-orthodox, which can be stored under the same conditions as orthodox seeds but for shorter periods. Due to thin seed coats or high lipid content [7], the loss of seed viability occurs even under adequate storage conditions [8]. Ex situ conservation of *Arachis* germplasm is typically implemented through living plants in field collections or seed banks, but living collections are vulnerable to diseases, pests, fire, drought, human damage, and genetic erosion [9].

Although in vitro conservation is an alternative for conserving peanut cultivars under slow-growth conditions, this involves the risk of somaclonal variation, which is considered a severe threat to conservation programs. Therefore, it is preferable to store germplasm in cryopreservation at ultra-low temperatures in liquid nitrogen (LN; −196 °C), as it enables preservation under reduced cost and space requirements for indefinite time periods, ensuring that plant material stays genetically stable over time [10].

Over the last decade, new rapid freezing techniques were developed that do not require controlled cooling devices. The vitrification technique, where explants are treated with highly concentrated cryoprotectant solutions prior to exposure to LN, has been successfully applied with protoplasts, cells, shoot tips [11–13], somatic embryos [14,15], and embryonic axes [16–18] of several genera. Alternatively, desiccation procedures can be used to cryopreserve plant structures without the need to treat the explants with cryoprotectant agents (chemicals). This is achieved by exposing the samples to an air stream (in a laminar flow chamber), by desiccating them on silica gel or exposing them to elevated temperatures (e.g., 40 °C) for a prolonged period (e.g., 14 days) prior rapid freezing in LN [19–21]. Desiccation procedures have been successfully used to cryopreserve orthodox embryonic axes from a variety of plant species [22,23]. Beyond this, cryopreservation has been successfully applied to numerous species with recalcitrant seeds, vegetatively propagated plants, cereals, fruit trees, legumes, oilseed species, and medicinal and aromatic plants [24–26]. In the case of ground nut (*A. hypogaea* L.) and its wild relatives (*Arachis* sp.) the successful cryopreservation of embryonic axes was reported by vitrification (PVS2) and desiccation. Embryonic axes that were desiccated with an air stream in laminar flow chambers for 1 to 5 h (prior freezing in LN), produced high germination rates of 75–96% [27–30]. Common air-desiccation of embryonic axes of *Arachis pintoi* at 40 °C for 14 days prior cryopreservation, also showed good results (germination rate of 90%) [21]. Only limited success (germination rate of 25–30%) was obtained when the embryonic axes were subcultured on sucrose-rich medium (0.5–1.0 M), followed by desiccation on silica gel and cryopreservation using the encapsulation method (+LN) [31]. However, successful cryopreservation of embryonic peanut axes was reported in previous studies for the PVS2 vitrification method, showing the highest germination rates (27–93%) with PVS2 treatment times of 1 or 2 h [27,32]. Depending on the genotype, different survival rates of 40–90% were obtained by cryopreserving the peanut embryonic axes using controlled freezing and glycerol as a cryoprotectant agent [33]. As an alternative, shoot tips were used as an explant type for the cryopreservation of *Arachis* sp. [34,35]. However, to the best knowledge of the authors, there are no studies published on the combined and sequential applications of desiccation on silica gel and vitrification (PVS2) for the cryopreservation of embryonic axes of *A. hypogaea* L.

The objective of the present study is to develop a cryopreservation protocol for the long-term conservation of embryonic axes of peanuts using a combined approach of desiccation and vitrification. The developed protocol will be applied to cryopreserve five peanut accessions, preserved in the Genebank of the National Institute of Agricultural Research of Ecuador (INIAP).

2. Materials and Methods

2.1. PlantMaterial

The *A. hypogaea* accessions were provided by the Germplasm Bank of the National Institute of Agricultural Research (INIAP), Table 1. Prior to conducting the experiment, the peanut seeds were maintained for 1 year in a seed storage chamber at 15 °C and 30–40% of RH [36]. The accession ECU-12466 was used for protocol development, and then the optimum treatment was evaluated on a set of five diverse peanut accessions (ECU-11448, ECU-11418, ECU-11494, ECU-11469, and ECU-11401).

Table 1. *Arachis hypogaea* L. accessions used for the experiments.

Genotype	Taxonomic Classification		Geographic Origin (Province, City)
	Current [1]	Former	
ECU-12466	*A. hypogaea* L.	*Arachis hypogaea* L.	Guayas, Naranjal
ECU-11448	*A. hypogaea* L.	*A. hypogaea* subsp. *fastigiata* var. *fastigiata* Harz	Loja, Paltas
ECU-11418	*A. hypogaea* L.	*A. hypogaea* subsp. *fastigiata* var. *aequatoriana* Krapov. & W.C. Greg.	Napo, Tena
ECU-11494	*A. hypogaea* L.	*A. hypogaea* subsp. *fastigiata* var. *peruviana* Krapov. & W.C. Greg.	Esmeraldas, Quininde
ECU-11469	*A. hypogaea* L.	*A. hypogaea* subsp. *hypogaea* var. *hypogaea* Köhler	Manabí, Sucre
ECU-11401	*A. hypogaea* L.	*A. hypogaea* subsp. *hypogaea* var. *hirsuta* Köhler	Pichincha, Quito

[1] According to World Flora Online (WFO) [37] and Plants of the World Online of the Royal Botanic Gardens of Kew [38], all infraspecific taxa listed in the former taxonomic classification are currently considered conspecific with the species *Arachis hypogaea* L.

2.2. Disinfection and Conditioning of Seeds

Working in a laminar flow chamber, the peanut seeds were immersed for 5 min in 70% alcohol, followed by 20 min in NaOCl at 10% plus 2 to 3 drops of Tween®-20. The drops of Tween®-20 had a volume of 30–35 µL each and were added at room temperature (20–25 °C). During the disinfection process, the seeds were maintained under constant agitation and then rinsed three times with sterile water [32].

2.3. Excision of Embryonic Axes

The embryonic axes were extracted from the disinfected seeds using sterile tweezers and a No. 11 scalpel. To separate the two cotyledons with the use of a scalpel, one of the cotyledons was maintained with tweezers at the opposite side of the embryo. Using the tip of the scalpel, the embryo was carefully levered out from the plumule to the radicle without damaging the plumule or embryonic root (Figure 1). The embryonic axes were placed on a sheet of sterile paper and exposed to laminar flow (0.55–0.58 m/s) for 1–3 min prior to desiccation with silica gel. The length and width of the embryonic axes, as well as the length of the plumule, were measured on a separate set of 20 embryonic axes (ZEN 3.0 Blue edition software).

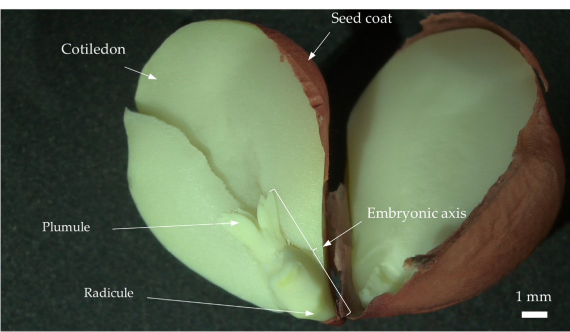


Figure 1. Schematic view of peanut seed.

2.4. Desiccation of Embryonic Axes and Determination of Moisture Content

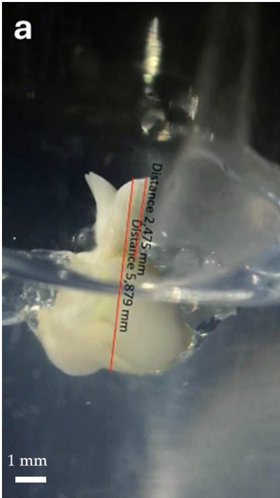
Embryonic axes (genotype ECU-12466) were placed in standard Petri dishes (Ø: 9.0 cm; depth: 0.8 cm) on top of two sheets of sterile filter paper supported on a layer of 25 g of dehydrated silica gel (SiO_2). Each Petri dish contained 30 embryonic axes. The embryonic axes were dehydrated for different time periods (0, 1, 2, and 3 h) and then cryoprotected and frozen in LN as described below. Additionally, a separate sample of 10 embryonic axes was dehydrated for each time period and used for calculating the axes' moisture content. Before and after each desiccation interval, the moisture content was determined gravitationally by weighing the axis (in groups of 10) on an analytical balance. The moisture content (%) was expressed as: [(fresh weight − dry weight)/fresh weight] × 100%.

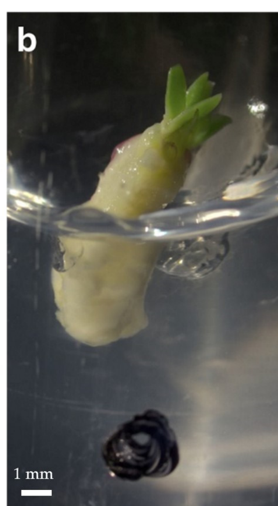
2.5. Treatment with Cryoprotectant Solutions and Freezing in LN

At the end of the desiccation process, 10 embryonic axes were introduced in a 2.0 mL cryovial and filled up with Loading Solution (LS) up to a volume of 2.0 mL. LS was composed of liquid Murashige Skoog (MS) medium with vitamins [39], supplemented with glycerol (2.0 M) and sucrose (0.4 M) [pH: 5.80 ± 0.02] [12]. The embryonic axes were treated with LS at room temperature (20–25 °C) for 20 min. Using a syringe (5 mL), LS was removed from the cryovial and replaced by Plant Vitrification Solution 2 (PVS2), filling the cryovial with PVS2 up to a volume of 2.0 mL. PVS2 was composed of liquid MS medium with vitamins, supplemented with sucrose (0.4 M), glycerol (3.28 M), ethylene glycol (2.42 M), and dimethyl sulfoxide (1.9 M) [pH: 5.80 ± 0.02]. The closed cryovials were placed in crushed ice (0 °C), and the embryonic axes were treated with PVS2 for different time periods (0, 1, and 2 h). The cryovials were capped, located in aluminium canes for cryovials, and submersed in a LN tank (−196 °C) for minimum 1 h. Preliminary experiments using the genotype ECU-12466 showed that when the embryonic axes were not exposed to liquid nitrogen (−LN), treatment with silica gel and PVS2 had no detrimental impact on their development. Considering this, only frozen samples (+LN) were used in the experiment with ECU-12466. The screening experiment with ECU-11448, ECU-11418, ECU-11494, ECU-11469, and ECU-11401 was performed both with control (−LN) and frozen samples (+LN).

2.6. Thawing, Recovery, and Assessment of Embryonic Axes

The cryovials were removed from LN and rewarmed at their bases in a water bath (38–40 °C) for 2–3 min [32]. Using a sterile syringe (5 mL), PVS2 was removed from the cryovial and replaced by 2.0 mL of rewarming solution (RS) containing liquid MS with vitamins and 1.2 M of sucrose [pH: 5.80 ± 0.02]. The embryonic axes were rewarmed in RS for 15 min at room temperature (20–25 °C). RS was removed with a syringe (5.0 mL), and the embryonic axes were placed in sterile 18 mm × 150 mm glass test tubes containing 5 mL of solid MS medium with vitamins, supplemented with sucrose (25 g L^{-1}) and phytagel (3.0 g L^{-1}) [pH: 5.60 ± 0.02], and incubated at 28 °C for 30 days, under cold white light, fluorescent tubes (light intensity of 40 µmol m^{-2} s^{-1}), with a 16 h photoperiod. No additional medium changes were performed prior to regrowth evaluation. A seedling originating from the plumule of the embryonic axis was considered germinated when shoot growth and root swelling were observed 10 days after thawing without signs of deformation, oxidation, or necrosis. Recovered in vitro seedlings showed normal root formation when they were further subcultured. The shoot length (mm) and germination and rooting rates (%) were assessed 10, 15, and 30 days after thawing (Figure 2).





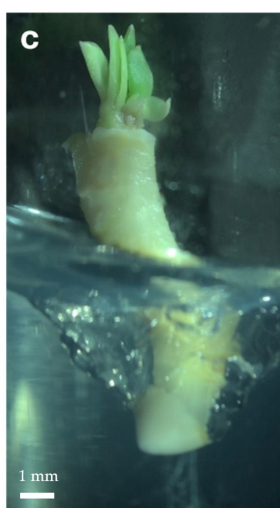


Figure 2. Germination and shoot formation of cryopreserved embryonic axes (+LN) of *A. hypogaea* (ECU-12466), 0 days (**a**), 10 days (**b**), 15 days (**c**), and 30 days (**d**) after rewarming.

2.7. Assessment of Five Peanut Accessions with an Optimized Cryopreservation Protocol

The optimized cryopreservation protocol was evaluated on a set of five different *A. hypogaea* genotypes (ECU-11448, ECU-11418, ECU-11494, ECU-11469, and ECU-11401). Seed disinfection and excision of the embryonic axes were performed as previously described. The embryonic axes were desiccated for 1 h on silica gel, followed by cryoprotectant treatments of 20 and 60 min with LS and PVS2, respectively (optimized treatment). Sets of 15 embryonic axes were processed for the non-frozen (−LN) and frozen samples (+LN) of each of the five genotypes. An additional set of 10 axes was processed separately to determine the embryonic axes' moisture content prior to and after desiccation on silica gel for 1 h. Freezing, thawing in RS, and recovery of the embryonic axes were conducted as described before. A subsample of recovered seedlings was transplanted to the greenhouse, as outlined below.

2.8. Transplant of Recovered Seedlings to the Greenhouse

A subsample of 30 plants of each genotype (ECU-11448, ECU-11418, ECU-11494, ECU-11469, and ECU-11401) was transplanted to the greenhouse to confirm adequate ex vitro adaptation, development, and growth. Phytagel residues were removed from the roots of the 45-day-old in vitro seedlings (originating from the embryonic axes) by washing them with distilled water (at 28 ± 2 °C) for 2 min. The seedlings were planted in white plastic cups (12 ounces) containing sterilized substrate composed of white peat and perlite (Florapeat, Plus 90/100 with perlite). A clear plastic cup (10 ounces) was placed over the pot cup to establish an adequate transpiration rate. The seedlings were incubated at 28 ± 2° C under laboratory conditions for 15 days. Seedlings were irrigated with distilled water every 4–5 days to maintain the substrate at field capacity. The clear lid cup was removed from the base cup, and the seedlings that showed no signs of fungal contamination and good development were then moved to a greenhouse for an additional 15 days (day: 35 °C; night: 6 °C). Finally, the seedlings were transplanted into 1 L polyethylene plant bags containing a substrate of dark loamy soil (60%), pumice (20%), and peat (20%). Every 4 to 5 days, the plants were irrigated with tap water to keep the substrate at field capacity.

After 50 days, a subsample of five plants per genotype was transplanted individually into pots (Ø of 21 cm; height of 17 cm; volume of 4.3 L) on the same substrate mixture as used for the plant bags. The plants were watered every 4 to 5 days, and plant development and flowering were assessed after 45 days (Figure 3).

Figure 3. Seedlings grown from cryopreserved embryonic axes (+LN) of *A. hypogaea.* The two plants belong to the genotype ECU-12466, with the presence of flowering 3 months after transplanting. The flowers are highlighted with arrows.

2.9. Experimental Design and Statistical Analysis

The experiment for developing the cryopreservation protocol of peanuts was performed as a completely random design (CRD) with a factorial arrangement (4 × 3). The effect of four dehydration times on silica gel (0 h, 1 h, 2 h, and 3 h) combined with three PVS2 exposure times (0, 1 h, and 2 h) was assessed with the peanut genotype ECU-12466. The experiment was replicated three times, processing 10 embryonic axes per treatment. The cryopreservation protocol showing the best results was tested under a CRD on five different peanut genotypes (ECU-11448, ECU-11418, ECU-11494, ECU-11469, and ECU-11401). The later experiment (screening) was replicated six times with a sample size of 15 embryonic axes per replicate. The germination rate of the embryonic axes (%), shoot length (mm), and root formation (%) were assessed 10, 15, and 30 days after thawing. Furthermore, the number of days when 50% of the embryonic axes germinated was recorded (T50).

The experiments for the determination of the embryonic axes' moisture content, prior to and after their desiccation on silica gel, were performed with 10 embryonic axes per treatment. The experiment was performed with one single repetition, both for protocol development with ECU-12466 (with four different desiccation times) and for the screening experiment with five diverse peanut genotypes (dehydration on silica gel for 1 h).

Means of the response variable showing normal distribution (shoot length) were compared using the analysis of variance procedure (ANOVA) and Tukey's multiple comparison test ($\alpha = 0.05$). When required, shoot length data were transformed using the Johnson Transformation. As germination and rooting rates showed a significant skewness from a normal distribution, their medians were analyzed using a Kruskal–Wallis multiple comparison test ($p < 0.05$). The Info Stat (Version 2008) and Minitab software (Version 22.1) were used for statistical analysis [38], and data presentation (graphs, tables) was conducted with the Minitab (Version 22.1) and Excel software (Version 2406).

3. Results

The length and width of the excised embryonic axes of the peanut genotype ECU-12466 ranged from 4.3–6.7 mm and 2.4–4.2 mm, respectively. Its average length and width measured 5.6 mm and 3.5 mm, respectively. The plumules' lengths ranged from 1.5 to 2.8 mm, with a mean value of 2.2 mm (Figure 4).

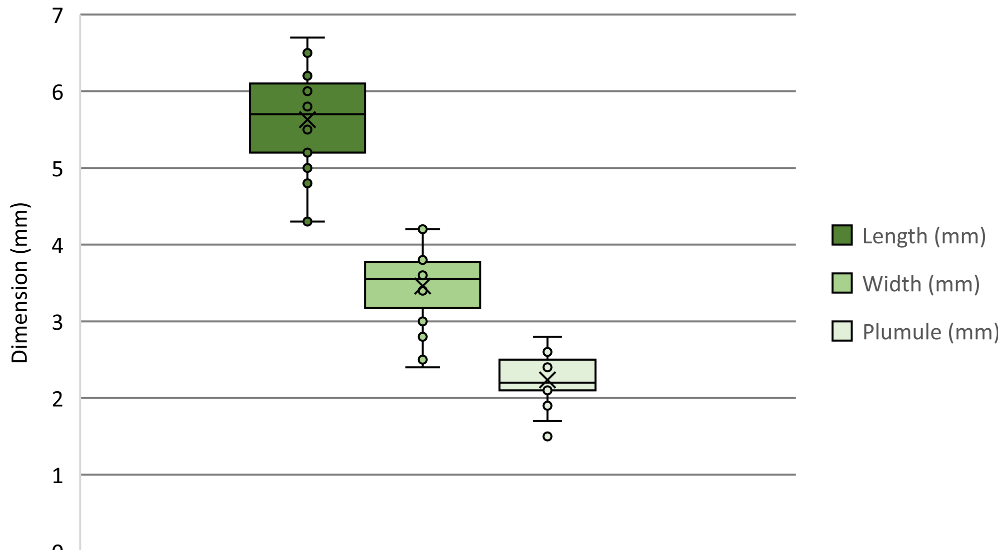


Figure 4. Length and width of 20 embryonic axes of A. hypogaea (ECU-12466), together with the dimensions of the plumule. Cross: media value. Empty circles: Individual measurements.

The germination rate of cryopreserved (+LN) embryonic axes of *A. hypogaea* (genotype ECU-12466) was not significantly affected by desiccation with silica gel (0–3 h) or cryoprotection with PVS2 (0–2 h). The median values of germination rates were high (71.4–85.7%) 10 days after rewarming, even in cases where the embryonic axes were not cryoprotected with PVS2 and desiccated with silica gel (S). The germination rate showed no significant interactions for S and PVS2 (Table 2). The desiccation period with silica gel did not impact the shoot length of the germinated peanut embryonic axis (+LN) (21.7–33.7 mm). In contrast, compared to the embryonic axis that were not exposed to PVS2 (30.0 mm) or exposed to PVS2 for 2 h (13.0 mm), the 1 h treatment with PVS2 significantly increased the shoot length to 45.0 mm. It is assumed that PVS2 induced phytotoxic effects on the embryonic axes when they were treated for 2 h with PVS2 since the average shoot length of the embryonic axes was 2.3 to 3.5 times lower than for the shorter treatments (0 and 1 h). The interaction of S x PVS2 showed significant differences in the shoot length, leading to multiple combinations of S x PVS2 with shoot lengths that did not significantly differ from one another. For example, the embryonic axes developed long shoots of 55.7 mm, 48.3 mm, and 48.0 mm under S/PVS2 combinations of 0 h/1 h, 1 h/1 h, and 3 h/1 h, respectively (Table 2).

The treatment with PVS2 and silica gel had a notable impact on the median values of the rooting rate. Embryonic axes that were not desiccated with silica gel (41%) or desiccated for 1 h (34%) or 3 h (28%), had a significantly higher median root rate compared to a desiccation time of 2 h (20%). Significantly superior rooting (54%) was obtained by treating the embryonic axes with PVS2 for one (54%) instead of 2 h (16%), which further suggests that 2 h of PVS2 exposure is harmful to the embryonic axes. Significant rooting differences were noted for the S x PVS2 interaction, with various S/PVS2 time combinations (e.g., 0 h/1 h, 1 h/1 h, or 3 h/1 h) resulting in similar root formation (Table 2).

The treatment with a 1 h desiccation on silica gel followed by a 1 h exposure to PVS2 was chosen for the screening experiment with five different genotypes of peanuts based on the results of shoot length and rooting rate for the principal factors of S and PVS2, as well as the fact that a 1 h desiccation time with silica gel resulted in a 10% higher germination rate compared to 0 h of desiccation.

Remarkably, compared to exposure intervals of 0 and 2 h, a 1 h treatment with PVS2 induced a rise in germination and rooting rates between days 10 and 15 after rewarming. Rooting accelerated in a brief time between day 10 and 15 (higher slope) and subsequently slowed down from day 15 to day 30 (lower slope), while shoot length showed a close to linear behavior between days 10 and 30 [Figure 5].

Table 2. Effect of silica gel desiccation and cryoprotection with PVS2 on germination (%), rooting (%), and shoot formation (mm) of cryopreserved peanut embryonic axes (genotype ECU-12466) [+LN]. Germination/rooting rates and shoot length are shown as median and mean values, respectively.

Treatment	Germination Rate (After 10 Days) [1]	Shoot Length ± SE (mm) [After 30 Days] [1]	Rooting Rate (After 30 Days) [1]
Desiccation with silica gel (S)			
0 h	71.4% a	32.36 ± 7.60 a	41.0% a
1 h	83.3% a	33.69 ± 5.72 a	34.1% a b
2 h	85.7% a	21.69 ± 4.47 a	19.8% b
3 h	85.7% a	29.54 ± 5.86 a	28.0% a b
Cryoprotection (PVS2)			
0 h	73.2% a	30.04 ± 3.13 b	22.7% a b
1 h	79.2% a	44.96 ± 4.56 a	53.6% a
2 h	85.7% a	12.96 ± 2.77 c	16.1% b
S × PVS2			
0 h × 0 h	N.S.	29.60 ± 13.38 a,b	20.0% a,b,c
0 h × 1 h	N.S.	55.70 ± 3.61 a	80.0% a
0 h × 2 h	N.S.	11.77 ± 3.60 b	50.0% a,b,c
1 h × 0 h	N.S.	28.40 ± 4.67 a,b	16.7% a,b,c
1 h × 1 h	N.S.	48.30 ± 12.83 a	71.4% a,b
1 h × 2 h	N.S.	24.37± 6.56 a,b	14.3% a,b,c
2 h × 0 h	N.S.	31.30 ± 2.92 a,b	42.9% a,b,c
2 h × 1 h	N.S.	27.90 ± 6.32 a,b	16.7% a,b,c
2 h × 2 h	N.S.	5.87 ± 0.68 b	0.0% c
3 h × 0 h	N.S.	30.87 ± 1.94 a,b	12.5% a,b,c
3 h × 1 h	N.S.	47.93 ± 5.20 a	71.4% a,b
3 h × 2 h	N.S.	9.83 ± 4.13 b	0.0% b,c

[1] Different lowercase letters indicate significant differences for the mean values of shoot length (Tukey test; $p \leq 0.05$) and median values of the germination and rooting rates (Kruskal–Wallis multiple comparison test; $p \leq 0.05$). N.S.: not significant; SE: standard error.

The moisture content of the embryonic axes oscillated from 9.3 to 9.5%, without showing a relationship with the applied desiccation and PVS2 treatment times. None of the treatments were able to reduce the moisture content of the embryonic axis below 9.3%. In fact, the treatment with PVS2 even caused a rise to 9.4–9.5%. The treatments had no effect on the number of days needed to reach 50% of germination rate (T50), which varied between 3 and 4 days.

To assess the general applicability of the developed protocol, a diverse set of five genotypes of *A. hypogaea* (ECU-11401, ECU-11418, ECU-11448, ECU-11469, and ECU-11494) was cryopreserved with the selected testament combination of 1 h desiccation on silica gel followed by 1 h cryoprotection with PVS2. Ten days after rewarming, the control samples (−LN) showed high germination rates of 60.7 to 94.0%, while the cryopreserved embryonic axis (+LN) required fifteen days before reaching a similar range (61.2 to 95.4%).

In particular, the genotypes ECU-11418 had a low germination rate (22.4%), 10 days after rewarming. Root formation was high for the control treatments (80.8–95.4%) but not for the samples exposed to liquid nitrogen. Hence, the average rooting rate of the cryopreserved samples (+LN) was significantly lower (25.2%) than the one of the control samples (90.9%). In particular, the genotypes ECU-11448 and ECU-11418 showed poor rooting (3.0–3.4%) [Table 3]. Nevertheless, it was possible to stimulate normal rooting through subsequent in vitro subculture cycles.

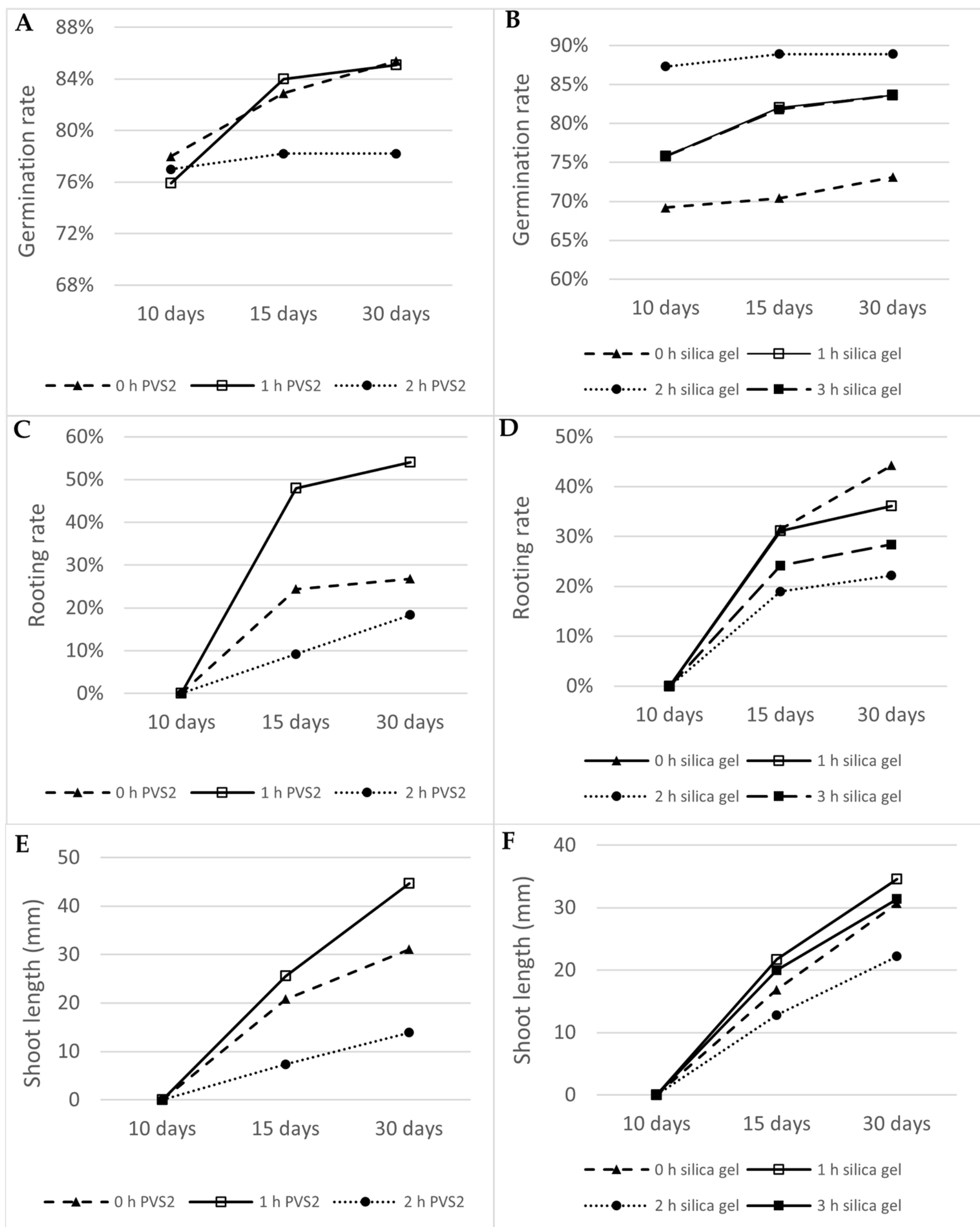


Figure 5. Effect of desiccation with silica gel and cryoprotection with PVS2 on the germination rate (**A**,**B**), rooting rate (**C**,**D**), and shoot length (**E**,**F**) of cryopreserved embryonic axes (+LN) of *A. hypogaea* (genotype ECU-12466), 10, 15, and 30 days after thawing.

The embryonic axes' shoot growth of the five genotypes was significantly affected by exposure to liquid nitrogen. The control samples (−LN) developed significantly longer shoots (37.0 mm) compared to the cryopreserved axes (21.6 mm) 30 days after rewarming. As expected, the shoot length of all five peanut genotypes (ECU-11401, ECU-11418, ECU-11448, ECU-11469, and ECU-11494) was higher when they were not exposed to LN (26.1–61.0 mm), compared to the samples treated with LN (11.2–48.7 mm).

On the genotype level, ECU-11494 showed a vigorous growth pattern and developed significantly longer shoots (−LN: 61.0 mm; +LN: 48.7 mm) compared to the other four genotypes. Also, ECU-11469 showed good shoot growth (−LN: 41.4 mm; +LN: 21.7 mm), developing significantly longer shoots than ECU-11401, ECU-11418, and ECU-11448. No significant differences were observed for shoot length between the genotypes with slow growth (−LN: 26.1–29.7 mm; +LN: 11.2–12.8 mm) [Figure 6].

Table 3. Germination (−LN: after 10 days; +LN: after 10, 15, and 21 days) and rooting rates (−/+LN: after 30 days) of cryopreserved embryonic axes of five peanut accessions (genotypes ECU-11401, ECU-11418, ECU-11448, ECU-11469, and ECU-11494). The embryonic axes were desiccated on silica gel for 1 h and treated for 1 h with PVS2 prior to exposure to LN.

Genotype	Germination Rate (±SE) [1]				Rooting Rate [1]	
	−LN	+LN			−LN	+LN
	10 Days	10 Days	15 Days	21 Days	30 Days	
ECU-11448	93.7 ± 1.3%	90.8 ± 3.1%	89.7 ± 3.3%	94.3 ± 2.5%	93.1 ± 2.7%	3.4 ± 2.0%
ECU-11418	60.7 ± 2.5%	22.4 ± 4.2%	61.2 ± 4.9%	70.4 ± 4.6%	80.8 ± 4.0%	3.0 ± 1.7%
ECU-11494	96.6 ± 1.0%	95.4 ± 2.3%	97.7 ± 1.6%	93.1 ± 2.7%	94.3± 2.5%	59.8 ± 5.3%
ECU-11469	94.0 ± 1.3%	88.5 ± 3.4%	95.4 ± 2.3%	97.7 ± 1.6%	95.4 ± 2.3%	40.2 ± 5.3%
ECU-11401	93.2 ± 1.4%	91.3 ± 3.2%	88.9 ± 3.2%	92.6 ± 2.9%	92.6 ± 2.9%	22.2 ± 4.6%
Average	87.0 ± 1.0% a	76.1 ± 2.0% b	85.9 ± 1.7% a	89.1 ± 1.5% a	90.9 ± 1.4% A	25.2 ± 2.1% B

[1] Different lower- and uppercase letters indicate significant differences for the medians of the germination and rooting rates, respectively (Kruskal–Wallis multiple comparison test; $p \leq 0.05$). SE: standard error.

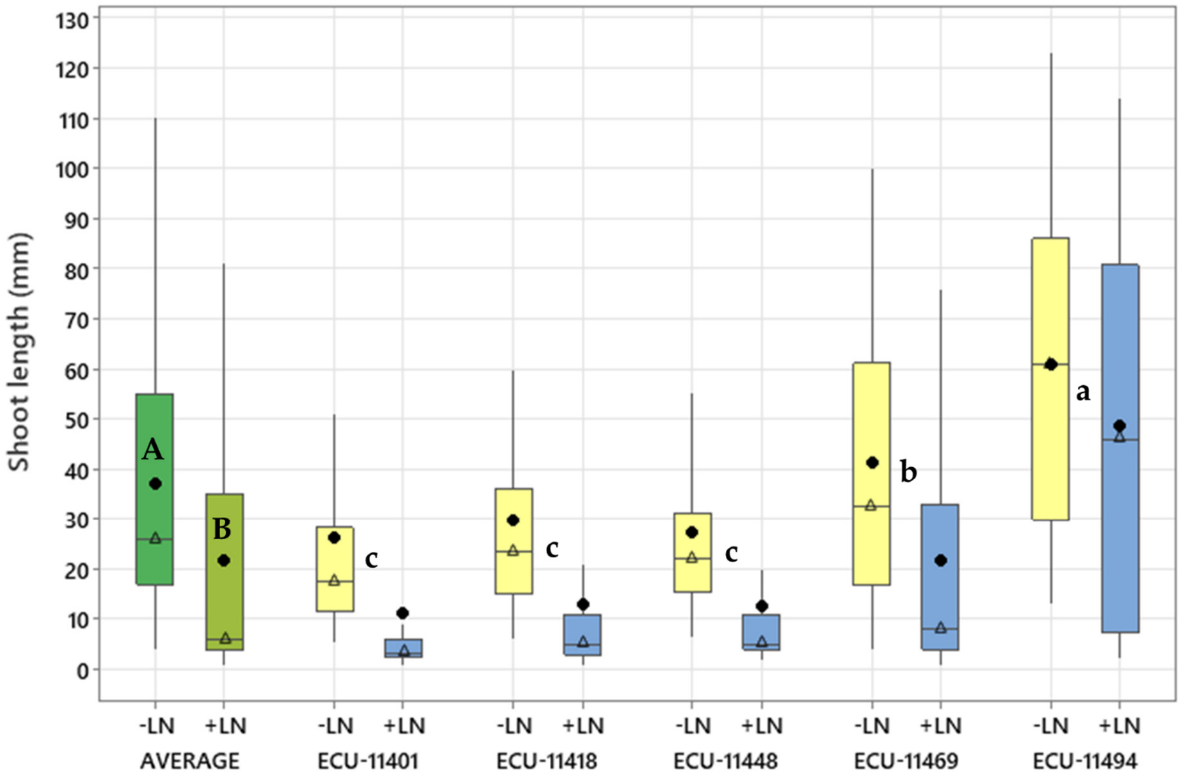


Figure 6. Shoot length (after 30 days) of cryopreserved embryonic axes (+LN) of five peanut accessions (genotypes ECU-11401, ECU-11418, ECU-11448, ECU-11469, and ECU-11494) together with their control treatments (−LN). The embryonic axes were desiccated on silica gel for 1 h and treated for 1 h with PVS2 prior to exposure to LN. Different lower- and uppercase letters indicate significant differences in the average shoot length of the accessions and the effect of liquid nitrogen (−LN vs. +LN), respectively. Comparison was performed using the Tukey multiple comparison test ($p < 0.05$). Black circle: mean; black triangle: median. Average bars are highlighted in green.

The number of days passed until 50% of the embryonic axes germinated (T50) varied significantly between the control (−LN; 3.8 days) and liquid nitrogen exposed samples (+LN; 6.2 days). The moisture content of the embryonic axes of the five peanut genotypes, posterior to a 1 h desiccation on silica gel and 1 h treatment with PVS2 (−LN), ranged from 7.9 to 10.8% (Table 4).

Table 4. Period until 50% (T50) of the embryonic axes of five peanut genotypes have germinated. The table shows the T50s for cryopreserved axes (+LN) and control samples (−LN). The embryonic axes were desiccated on silica gel for 1 h, followed by cryoprotection with PVS2 for 1 h. The water content was determined gravimetrically on a separate set of control samples (−LN). Different lowercase letters indicate significant differences for the medians of T50 (−LN vs. +LN) [Kruskal–Wallis multiple comparison test; $p \leq 0.05$]. SE: standard error.

Genotype	Moisture Content of Embryonic Axes	T50 ± SE	
		−LN	+LN
ECU-11418	9.0%	3.6 ± 0.2	6.0 ± 0.3
ECU-11448	10.8%	4.4 ± 0.1	6.8 ± 0.2
ECU-11494	8.9%	3.8 ± 0.1	6.3 ± 0.2
ECU-11469	7.9%	3.6 ± 0.2	6.3 ± 0.3
ECU-11401	9.2%	3.5 ± 0.1	5.9 ± 0.2
AVERAGE	9.2%	3.8 ± 0.1 b	6.2 ± 0.1 a

A sample of 30 plants per genotype was transplanted into the greenhouse to evaluate the growth and development of peanut plants derived from cryopreserved embryonic axes (+LN). Thirty days after transplantation, ECU-11448 and ECU-11494 had the lowest success rate (53%) and maximum success rate (93%), respectively, with 70% of the transplanted plants growing into full plants on average. Forty-five days after transplantation, a subsample of five plants was selected, and their physiological conditions were evaluated categorically. For each genotype, four out of five plants had normal or good physiological conditions. All genotypes showed signs of flowering after 90 days, with one to three flower-setting plants per genotype. The seedlings grown from cryopreserved embryonic axes had the same morphological characteristics as plants from non-cryopreserved seeds, using the original morphological descriptors of the five genotypes as a reference for comparison (Table 5, Figure 7).

Table 5. Recovery, physiological condition, and flowering of greenhouse plants of five genotypes of A. hypogaea coming from cryopreserved embryonic axes (+LN) 30 to 90 days after transplanting.

Peanut Genotype	Number of Plants Selected for Transplanting, 50 Days After Rewarming (+LN)	Condition of Plants, 30 Days After Transplanting				Classification of Plants (+LN) According to Their Physiological Condition, 45 Days After Transplanting			Number of Plants Flowering 90 Days After Transplanting
		Complete Plants	%	Dead Plants	%	Good	Regular	Weak	
ECU-11418	30	20	67%	10	33%	2	2	1	2
ECU-11448	30	16	53%	14	47%	1	3	1	1
ECU-11401	30	19	63%	11	37%	2	2	1	1
ECU-11494	30	28	93%	2	7%	3	1	1	3
ECU-11469	30	22	73%	8	27%	2	2	1	2
AVERAGE	30	21	70%	9	30%	2	2	1	1.8

Figure 7. Seedlings grown from cryopreserved embryonic axes (+LN) of five accessions of the Ecuadorian peanut collection: (**a**) ECU-11418, (**b**) ECU-11448, (**c**) ECU-11494, (**d**) ECU-11469, and (**e**) ECU-11401. The photos show the plants 3 months after having been transplanted to pots.

4. Discussion

In the present study, it was shown that the sequential application of a 1 h desiccation on silica gel, followed by a 1 h cryoprotection with PVS2, enables the successful cryopreservation (+LN) of embryonic axes of *A. hypogaea* and its subsequent full-plant recovery after rewarming. Moreover, it was evidenced that embryonic axes of peanut seeds have a relatively high tolerance to desiccation with silica gel and PVS2 exposure, as none of the here assessed treatment combinations negatively affected the germination rate, contrary to the variables of shoot length and rooting rate.

Similarly, in a previous study performed with embryonic axes of *A. hypogaea* cv Tatu, the highest recovery rate (+LN; 80 ± 10%) was observed for a 1 h treatment with PVS2 (without desiccation on silica gel). Longer PVS2 treatment times of 2 to 5 h caused a decrease in the germination rate and stimulated callus formation [27]. Within the same study, the optimum moisture content for cryopreservation of embryonic axes was determined by desiccating them for 0–5 h in the laminar flow chamber prior to submersion in LN (without a PVS2 treatment). In contrast to our study, the highest recovery rates (+LN) were observed at a moisture content of 14–18% (germination rate of 78–80%), while lower moisture contents of 8–9% (as measured in the present study) resulted in a decrease of the germination rate to 53–57%. It is important to highlight that this experiment was performed with *A. hypogaea* seeds cv Tatu obtained from a local market and not with seeds coming from cold storage (e.g., 10 °C). The 1 h PVS2 treatment was then assessed on six wild *Arachis* species, resulting in high recovery rates of 75–90%. However, the six wild *Arachis* species were obtained from the germplasm bank, where they were previously stored for 2 to 13 years at 10 °C and 25% relative humidity. The moisture content was 9–10% for three of four species and 17% for the fourth species (the moisture content was not determined for the other two species). These moisture contents are similar to the values measured in our study (7.9–10.8%), suggesting that the pre-conditioning of seeds at 10 °C and low relative humidity may be favorable for their subsequent cryopreservation. On the other side, the mentioned study also reported high recovery rates for embryonic axes with moisture contents of >17% (*A. hypogaea* cv Tau and *A. prostrata*), which either suggests that the optimum moisture content is highly variable between *A. hypogaea* genotypes and *Arachis* species, as reported for other species and structures [40–43], or that the embryonic axes of *A. hypogaea* have a relatively high range of moisture content for which successful cryopreservation is possible (e.g., 10–17%). To clarify this question, additional studies are required with a wide range of *A. hypogaea* genotypes and *Arachis* wild species to correlate the different moisture content data points with their recovery rates after cryopreservation (+LN and −LN).

Contradictory results were observed in a similar study when four peanut genotypes were desiccated in the laminar flow chamber for a 0, 1, 2, 3, 4, or 5 h period prior to exposure to LN [28]. Also, the desiccation method was the same as reported previously [27]. The moisture content decreased from 27.3% to 16.7% after 5 h of desiccation, showing the highest recovery rate of 92–97% (+LN) with desiccation times of 4 to 5 h when the embryonic axes

reached a moisture content of 16–17% [28]. Although the optimum moisture content was similar for subsets of these two studies, for the first study, only a 1 h desiccation period was required to reduce the moisture content from 39% to 18% [27]. In another study, embryonic axes *A. hypogaea* cv. Virginia decreased their moisture content from 25% to 8.5% when they were desiccated for 2.5 h in the sterile air flow of a laminar flow cabinet. Applying this dehydration method a high post-rewarming (+LN) recovery rate of 75–92% was reported for four peanut genotypes [29]. In contrast to all the former reports, a high germination rate of 90% was obtained when embryonic axes of *A. pintoi* were desiccated at 40 °C for 14 days to a moisture content of only 4% [21]. Slower desiccation may permit the embryonic axes to survive lower moisture contents, adapting their metabolic pathways. For the embryonic axes of *Aesculus*, *Castanea*, and *Quercus*, it was stated that there is a window of desiccation during which survival through freezing was maximized [44].

Nevertheless, we suggested not to use the air flow of laminar flow chambers for the desiccation of embryonic axes, as the drying rates are highly variable (as shown), depending on the velocity of the air flow, the model of the flow chamber, the position of the samples within the chamber, and other variables that are difficult to control. We recommend desiccating the embryonic axes on silica gel, as done in this and other studies [45], which helps to control the conditions of the desiccation process and increase the precision and repeatability of the method. Silica gel is one of the most widely used solid desiccant agents due to its numerous desiccant characteristics, low price, and availability [46].

Moisture contents of the axes close to 20% or higher result in a significant decrease in the recovery rate, as the remaining water may form larger ice crystals, which damage the cell structure and membranes [47]. Sequential desiccation of embryonic axes of *Arachis pintoi* with sucrose (0.5 to 1.0 M) and silica gel to a moisture content of 20%, followed by cryopreservation (+LN) with an encapsulation protocol, resulted in a low germination rate of 25–30% [31].

When using a 2 h PVS2 treatment for the cryoprotection of embryonic peanut axes, callus formation, and poor root development were reported [29]. Likewise, in our study, we also observed a significant reduction of the rooting rate to 16.1% for a 2 h exposure to PVS2, compared to the 1 h treatment (rooting rate of 53.6%). This highlights the importance of careful control of the chemical toxicity, as it increases with prolonged treatment times with PVS2 [32,33,48]. Regarding the role of PVS2, three principal cryoprotection mechanisms were postulated: (a) replacement of cellular water, (b) change of the freezing behavior of water remaining in cells, and (c) prevention of water loss during air drying [49]. Beyond this, cryoprotectants can impart protection against cryoinjuries, stabilizing proteins and membranes and acting as antioxidants [50].

However, when the 1 h silica gel/1 h PVS2 treatment was tested with more genotypes, 2 of 5 accessions (ECU-11448 and ECU-11418) had a very low rooting rate of 3.0–3.4%. On the other hand, ECU-12466 showed better rooting (+LN) for a 1 h treatment with PVS2, compared to pure desiccation with silica gel. This could be an indicator for PVS2 being able to vitrify and protect root-generating cells when an ideal PVS2 exposure time is reached. Additional fundamental studies should be performed to determine the distribution of the cryoprotectants in the complete embryonic axes. Cell turgor and size, water potential, and the characteristics and quantity of water channels may play an important role in this context. Although rooting can be induced through subsequent subculture cycles of the recovered in vitro seedlings, it is preferable to achieve direct root formation in the recovered samples.

Based on our results, we recommend further investigating the combined application of desiccation on silica gel and PVS2, especially for those genotypes that require a higher moisture content for survival, as the lack of desiccation tolerance of their embryonic axes may be compensated by chemical vitrification. As the recovery process of embryonic axes requires in vitro culture in the post-rewarming phase, even a lower germination rate permits efficient long-term conservation through cryopreservation because the obtained in vitro plants can be further multiplied until the required plant quantity is obtained. In contrast to previous studies, shoot length was also considered a variable for selecting the

optimum treatment combination. Based on our results, shoot length was identified as a valuable and sensible variable for measuring the toxic effect of PVS2 with increasing exposure time.

Other than embryonic axes, shoot tips have also been successfully used for cryopreserving *A. hypogaea* [34,35]. The use of shoot tips has the advantage that the originating in vitro plant material (grown from the embryonic axes) can be passed through a pathogen-elimination process in the case a specific virus infection is a limiting factor for its future use and distribution. Oppositely, the use of shoot tips is more work and space-demanding than direct cryopreservation of embryonic axes.

5. Conclusions

Our study demonstrates that a 1 h desiccation on silica gel followed using a 1 h cryoprotection with PVS2 successfully cryopreserves embryonic axes of *Arachis hypogaea*, enabling full-plant recovery after rewarming. It highlights the high tolerance of these axes to desiccation and PVS2 exposure, with no negative impact on germination rates from the treatment combinations tested. However, prolonged PVS2 treatment times lead to reduced rooting rates, underscoring the importance of controlling chemical toxicity. The study suggests further investigation into the combined effects of desiccation and cryoprotection, especially for genotypes needing higher moisture content. The significance of shoot length as an assessment variable for treatment toxicity is also emphasized. The authors recommend using silica gel for desiccation due to its reliability over laminar flow chambers, which can produce variable drying conditions.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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