Article

Cryopreservation of *Arachis hypogaea* L. varieties, from the INIAP-Ecuador Germplasm Bank.

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Abstract: The peanut (*Arachis hypogaea* L.) is recognized as one of the most important legume crops globally for its use in human food; it is widely distributed and cultivated in tropical and subtropical regions. The purpose of this study was to evaluate the cryopreservation of five peanut varieties conserved in the INIAP Germplasm Bank, testing cryopreservation methods, evaluating the germination percentage of whole seeds and embryonic shoots. Subsequently, two quantitative variables, shoot length and root, were evaluated. The average germination percentage of varieties and treatments was higher when embryonic axes were isolated with 99.31% than 86.06% seeds. The best germination percentage of the five varieties for seeds and embryonic shoots was obtained by the Peruvian variety with 88.13% and 92.50%. The best treatments by variety for the germination of whole seeds and embryonic axes were obtained by the treatment (desiccation and NL) for whole seeds (GS2) with 95.42% and embryonic axes with 92.83%. Ageing and cryopreservation treatments positively affected germination and seedling vigor in whole seeds and embryonic axes. The two quantitative variables, shoot and root length showed variability between the five varieties; significant differences were observed between the four treatments evaluated for whole seeds and embryonic axes. The three treatments for whole seeds (GS1, GS2 GS3) and the non-cryopreserved control treatment (GSC), as well as the treatments for embryonic axes (GEA1, GEA2 GEA3) and the non-cryopreserved control treatment (GEAC), obtained good survival. They germinate whole seeds and embryonic axes with sprout development (aerial part) and root formation. With the most effective treatments for whole seeds (GS2) and embryonic axes (GEA2), the cryopreservation of the national peanut collection of the INIAP Germplasm Bank could be started.

Keywords: Varieties, seeds, embryonic shoot.

1. Introduction

The peanut (*Arachis hypogaea* L.) is native to South America, where the *Arachis* genus is widely distributed [1] and is currently cultivated in tropical and subtropical regions [2]. This herbaceous species with erect, decumbent, or creeping vegetative growth presents two branching patterns: sequential with bushy and compact growth or alternate with prostrate or decumbent vegetative growth [3].

Based on the morphological characteristics, the cultivated peanut comprises six varieties grouped into two subspecies: *A. hypogaea* subsp. *hypogaea* Waldron and *A. hypogaea*

subsp. *fastigiata* Waldron. The subspecies *A. hypogaea* subsp. *hypogaea* includes the varieties *hypogaea* and *hirsuta* Köhler, while *A. hypogaea* subsp. *fastigiata* C. Harz includes *fastigiata* C. Harz, *vulgaris*, *peruviana* Krapov & W.C. Gregory varieties. and Ecuadorian Krapov. & W.C. Gregory [4]. Of the existing varieties, only *hypogaea*, *fastigiata* and *vulgaris* are widely cultivated globally [5].

Although *A. hypogaea* seeds have been classified as orthodox by Hanson et al. [6], viability losses frequently occur even under optimal storage conditions [7]. This behavior may result from the high-fat content of their storage tissues [8,9] that can undergo autooxidation and generate free radicals, which damage proteins and nucleic acids [10]. Therefore, *Arachis* seeds can be more appropriately classified as sub-Orthodox, a subdivision of seeds, including species whose seeds can be stored under the same conditions as Orthodox, but for shorter periods [8,9,11].

Many crop varieties are in danger of extinction due to habitat loss and global changes. For example, climate change will be responsible for the 50% loss in the range of distribution of the wild populations of groundnut (*Arachis* sp.), potato (*Solanum* sp.) and cowpea (*Vigna* sp.) Moreover, due to its effects, 16–22% of these species will be extinct by 2055 [12]. Therefore the conservation of some species in germplasm banks such as seeds, field collections or in vitro is promoted [13]. However, in vitro storage can lead to somaclonal variation, which is a drawback in conservation programs. For this reason, cryopreservation at low temperatures for germplasm storage is considered the best method for conservation for indefinite periods, with lower risks of inducing genetic alterations [14,15] and fewer periodic tests of viability, reducing conservation costs [8].

There are several cryopreservation techniques; one of them is the desiccation technique that is based on exposing the tissues to a stream of air, compressed air stream or silica gel before rapid freezing by direct immersion in liquid nitrogen [16–18]. This approach has been used for orthodox seed embryonic axes [19–24] and recalcitrant seeds [23,25–28]. On the other hand, the vitrification technique, in which the explants are pretreated with high concentrations of cryoprotectants, has been successfully applied to embryonic axes [29–31] of genera like orchids, peas and jack fruit. Furthermore, Runthala et al. [32] and Abdulmalik et al. [18] published protocols to preserve the embryonic axes of peanuts using cryoprotectants, with which they obtained variable levels of survival (40-90%) according to the genotype. Several methods have been developed for the cryopreservation of peanuts, such as desiccation in a laminar flow chamber [18] or the vitrification technique in embryonic axes [33], or both techniques in cultivated and wild species [34–36].

In Tacán et al. [37], the effects of accelerated ageing and cryopreservation were measured in seeds and embryonic axes of *Phaseolus vulgaris* L and *Arachis hypogaea* L. On the germination and vigor of the seedlings, they were using whole seeds and embryonic axes of a commercial variety of peanuts. It was observed that the ageing and cryopreservation treatments positively affected the germination and vigor of the peanut seedlings. In another study [38], our results show the important differences, attributable to the genotype, concerning the content of proline and the rest of amino acids, about the stress conditions related to desiccation tolerance and cryopreservation. The present research aimed to determine the effect of cryopreservation with three treatments and control for five varieties existing in the peanut collection conserved at the BG-INIAP to evaluate the method's usefulness for the conservation of the peanut *Arachis hypogaea* collection.

2. Materials and Methods

2.1 Plant Material

The INIAP germplasm bank provided the seeds of *A. hypogaea*. The selection of eight morphotypes that are detailed in Table 1 was made based on the taxonomic and morphological characterization carried out in the National Department of Plant Genetic Resources (DENAREF) of the INIAP, considering the following parameters: groups, species, subspecies, province (geographic distribution) and altitude. (Table 1).

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Table 1. Selected morphotypes of *A. hypogaea* from the National Peanut Collection of the Germplasm Bank. INIAP, 2021.

Group	Genus	Species	Subspecies	Variety	Code	No. ECU
1	*Arachis*	*hypogaea*	*hypogaea*	*hypogaea* Kohler	var. 3	11484
				hirsuta Kohler	var. 4	11405
			fastigiata	*aequatoriana* Krapov. & W. C. Gregory	var. 1	11418
				peruviana Krapov. & W. C. Gregory	var. 5	11494
2			*fastigiata*	*aequatoriana* Krapov. & W. C. Gregory	var. 1	11449
3			*fastigiata*	*fastigiata* C. Harz	var. 2	11448
				aequatoriana Krapov. & W. C. Gregory	var. 1	11428
4			*hypogaea*	*hypogaea* Kohler	var. 3	11469
Total			2	5		8

The previous process for this research was refreshing and multiplication to have an adequate number of seeds per treatment and repetition. Then, conditioning was carried out, considering the protocols of Monteros-Altamirano et al. [39].

The already conditioned materials remained in seed lots stored at 5 °C until use. In a laminar flow chamber, the embryonic axes of the previously disinfected seeds were extracted. For the disinfection process, the seeds were placed 5 min in 70% alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 to 3 drops of tween 20, rinsed three times with sterile distilled water and immersed in 5 NaOCl % plus 2 to 3 drops of tween 20 for 10 min. With occasional shaking and rinsing twice with sterile distilled water. Subsequently, the seeds were soaked in sterile distilled water for 3 h.

2.2 Seedling germination and growth tests

The whole seed germination tests were carried out by placing four replicates of 10 seeds in 9 cm Petri dishes on two sheets of filter paper (previously moistened with 3.5 ml of distilled water, which was added periodically) in the dark 25 0C. The criterion for germination was the protuberance of the radicle of 1 mm, and it was quantified on day 10. The results are expressed as the germination percentages (%) and the number of days necessary to reach 50% of germination final (T50). In addition, the length of the radicle (mm) and the length of the shoots (mm) were also determined.

The embryonic axes were cultured in sterile glass containers with MS medium [40] supplemented with 0.3 M sucrose. The germination temperature was 25 °C with illumination and a light / dark photoperiod of 16: 8 h. cold white fluorescent light (40 μmol m^{-2} s^{-1}). Four 10-axis replicas per bottle were studied, quantified at ten days, and the percentage of germinated embryos, the T50, the length of the radicles (mm) and the length of (mm) were measured.

For whole seeds, it was carried out in Treatment 2 (direct immersion in NL) and Treatment 4 (desiccation and immersion in NL); the seeds were introduced into cryovials and were introduced directly into liquid nitrogen. NL was removed from the cryovials after 1 h at room temperature (20 °C), and they were immediately placed in Petri dishes under the germination conditions indicated above. For the embryonic axes, the whole peanut seeds were immersed in liquid nitrogen for 5 minutes, then they were placed in a Petri dish at

room temperature 180 °C, for one hour. After dissecting the embryonic axes under sterile conditions in a laminar flow chamber, ten embryonic axes were placed in each container; in total, there were four containers and 40 embryonic axes; each container contains a solid MS base culture medium supplemented with 20 g l^{-1} of sucrose.

2.3 Experimental Unit

The experimental unit for whole seeds (GS) consisted of a 10 cm × 6 cm petri dish containing ten whole seeds. For the embryonic axes (GEA), the experimental unit consisted of a 10 cm × 2 cm flask containing 30 mL of culture medium with ten embryonic axes.

2.4 Treatments

The GS and GEA were subjected to the following treatments: Control (C): untreated, Treatment 1: direct immersion in liquid Nitrogen (NL), Treatment 2: desiccation, Treatment 3: desiccation and immersion in NL.

2.5 Experimental Design

A completely randomized experimental design (DCA) with ten observations was used. For each treatment, four replicas of 10 whole seeds (GS) and embryonic axes (GEA) were made.

2.6 Data Analysis

The statistical program InfoStat version 2008 was used [41]. An analysis of variance and the Tukey test was performed to compare means ($\alpha = 0.05$).

2.7 Handling the experiment

2.7.1 Desiccation: silica gel

For the drying of the seeds, fifty seeds were placed in Petri dishes (9 cm in diameter) containing a layer of dehydrated silica gel of approximately 5 g and covered by a filter paper disk in which the seeds were placed, which, once sealed, were kept for 3 hours at 20 °C.

2.7.2 Cryoconservation

The embryonic axes were cultured in sterile glass containers with MS medium supplemented with 0.3 M sucrose. Germination temperature was 25 °C with a light / dark photoperiod of 16: 8 h with cold white fluorescent light illumination (40 µmol m^{-2} s^{-1}). Four 10-axis replicas per bottle were studied, quantified at ten days, and the percentage of germinated embryos, the T50, the length of the radicles (mm) and the length of (mm) were measured.

For whole seeds, it was carried out in Treatment 2 (direct immersion in NL) and Treatment 4 (desiccation and immersion in NL); the seeds were introduced into cryovials and were introduced directly into liquid nitrogen. NL was removed from the cryovials after 1 h at room temperature (20 °C), and they were immediately placed in Petri dishes under the germination conditions indicated above. For the embryonic axes, the whole peanut seeds were immersed in liquid nitrogen for 5 minutes, then they were placed in a Petri dish at room temperature 18 °C, for one hour. After dissecting the embryonic axes under sterile conditions in a laminar flow chamber, ten embryonic axes were placed in each container; in total, there were four containers and 40 embryonic axes; each container contains a solid MS base culture medium supplemented with 20 g l^{-1} of sucrose.

3. Results

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The seeds of all the studied peanut varieties tolerated desiccation and cryopreservation treatments, both for whole seeds and for embryonic axes, since, in all cases, the treated seeds germinated. The four treatments (GSC, GS1, GS2 and GS3,) on the germination of whole seeds (at ten days) for the five varieties were evaluated. For the *hypogaea* variety (Figure 1-black bar, Appendix 1), it was observed that the GSC germination percentages were higher than 87.50% with the Ecuadorian variety, and differences were detected with the other varieties. In GS1, the germination was higher than 62.50% of the Peruvian variety, and differences with the other varieties were also detected. The GS2 presented a germination superior to 88.75% with the *hypogaea* variety and presented differences between varieties. For GS3, the germination percentages were higher than 70.00% with the *fastigiata* variety. The GS1 and GS3 treatments delay germination start, although T50 values do not differ significantly from the control and each of the varieties.

In the same way, the effect of the four treatments on the germination of embryonic axes of the five varieties of *A. hypogaea* was analyzed (Figure 1-gray bar, Appendix 2). In the four treatments (GEAC, GEA1, GEA2 and GEA3), it was observed that, in all treatments, the germination percentages of the embryonic axes at ten days. GEAC were higher than 95.00% with the *fastigiata* variety, and differences were detected with the other varieties. In GEA1, germination was higher than 84.17% of the Ecuadorian variety and differences were detected with the other varieties. The GEA2 presented a germination superior to 87.50% with the hirsute variety and presented differences between varieties. For GEA3, the germination percentages were higher than 77.50% with the *fastigiata* variety. The T50 values do not differ significantly from the treatments and each of the varieties.

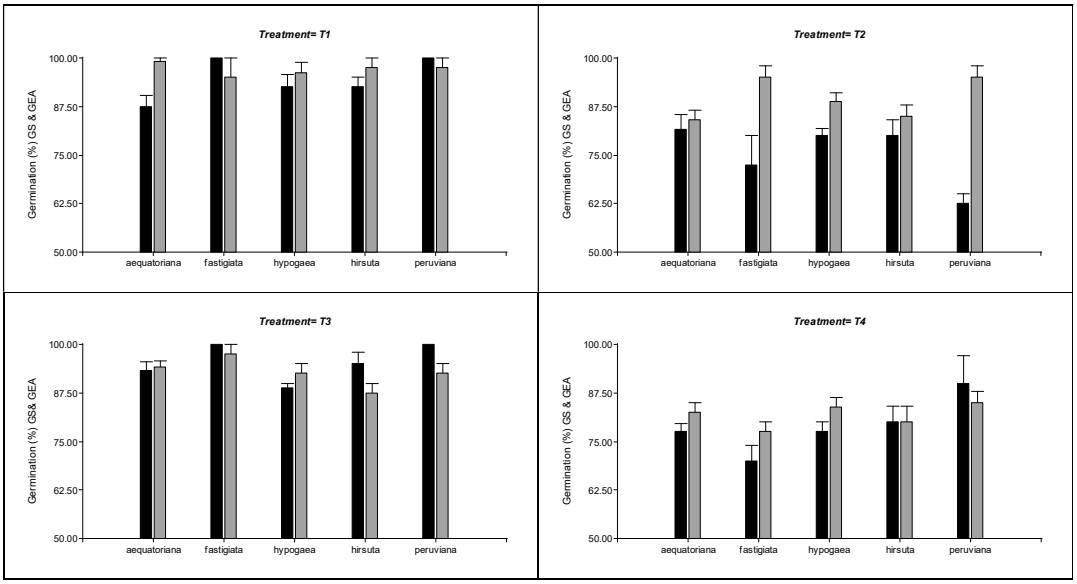

Figure 1. Germination percentages (mean value + standard error), for whole seeds and embryonic axes at ten days of onset, in five varieties of *A. hypogaea*, subjected to the following pretreatments: T1: Untreated control; T2: direct immersion in LN; T3: drying and, T4: drying and immersion in LN. The black bar is data from evaluating whole peanut seeds, and the grey bar is data from embryonic axes.

Figures 2, 3 and Appendices 3, 4 summarize the morphological variability in whole seeds and embryonic axes for the four treatments and the variables of root length (mm) and aerial length (mm) for each of the *A. hypogaea* varieties.

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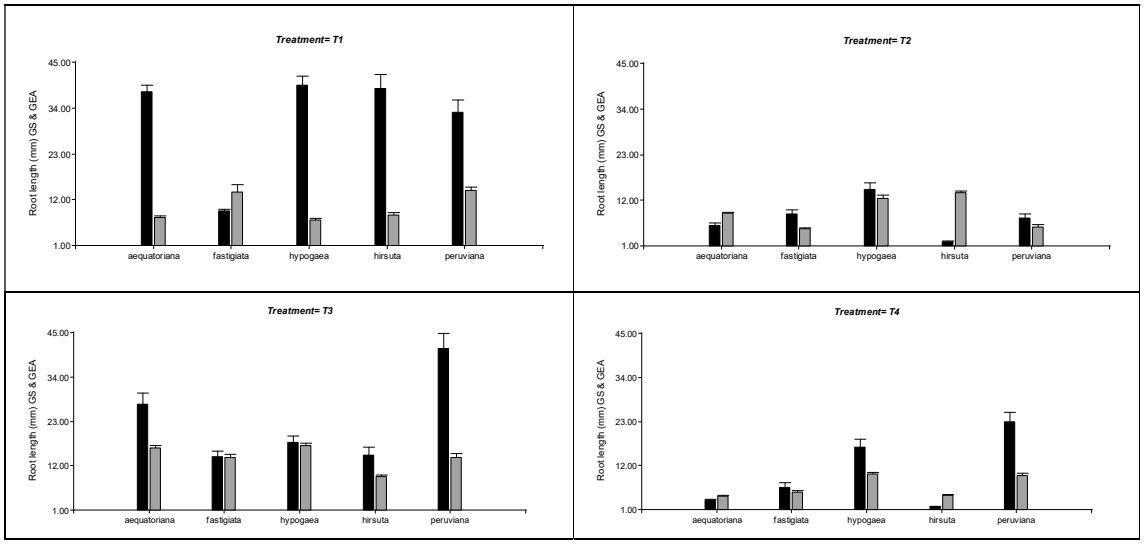

Figure 2. Morphological variability in whole seeds and embryonic axes for the quantitative character roots length (mean value + standard error) in 10-day seedlings is also shown. In five varieties of the Ecuadorian collection of *A. hypogaea*, submitted to the following pretreatments: (C) Untreated control; (1) Direct immersion in LN; (2) Desiccation and, (3) Desiccation and immersion in LN. The black bar is data from evaluating whole peanut seeds, and the grey bar is data from embryonic axes.

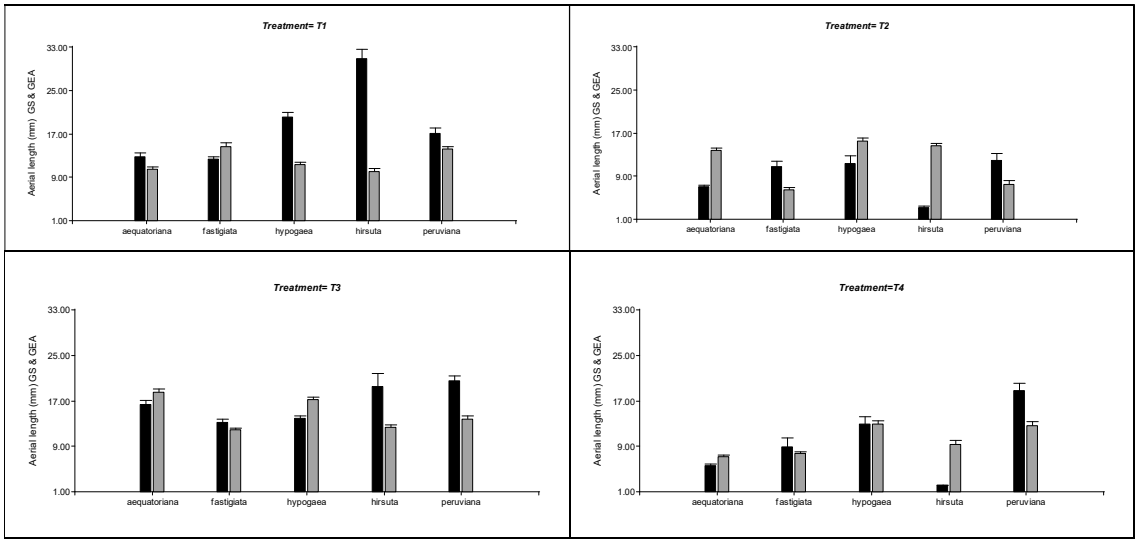

Figure 3. Morphological variability in whole seeds and embryonic axes for the quantitative character shoots length (mean value + standard error) in 10-day seedlings is also shown. In five varieties of the Ecuadorian collection of *A. hypogaea*, submitted to the following pretreatments: (C) Untreated control; (1) Direct immersion in LN; (2) Desiccation and, (3) Desiccation and immersion in LN. The black bar is data from evaluating whole peanut seeds, and the grey bar is data from embryonic axes.

A comparison was made between whole seeds and embryonic axes for root length. For whole seeds and embryonic axes, a significant effect was observed in the treatments used ($p \leq 0.05$), whereas for treatment 1 of whole seeds (GSC), there was a greater root length of var. 3 with 39.42 mm, followed by var. 4 with 38.74 mm. In embryonic axes (GEAC), for root length, the var. 5 with 14.22 mm, followed by var. 2 with 13.87 mm. For root length in whole seeds treatment 2 (GS1), var. 3 had the most extended length at 14.59 mm. For embryonic axes (GEA1), var. 4 and var. 3's root length is 13.73 mm and 12.41 mm. For treatment 3 (GS2), the variety obtained the most extended root length in whole seeds

was var. 5 with 41.05 mm. The var. 3 and var. 1 obtained the highest root length values (GEA2) with 16.95 mm, and 16.36 mm, respectively, for embryonic axes. Finally, for treatment 4 (GS3), var. 5 reached the highest value for root length with 22.91 mm. For embryonic axes (GEA3), the var. 3 and var. 5 obtained the highest root length with 9.86 mm., And 9.48 mm., Respectively. (Fig. 2, Appendix 3).

Also, a comparison was made between whole seeds and embryonic axes for aerial length. For whole seeds and embryonic axes, a significant effect was observed in the treatments used ($p \leq 0.05$), where for treatment 1 of whole seeds (GSC), there was a greater aerial length in var. 4 with 30.82 mm. In embryonic axes (GEAC), var. 2 with 14.57 mm, followed by var. 5 with 14.27 mm. For aerial length in whole seeds treatment 2 (GS1), var. 5 had the most extended length with 11.85 mm, Followed by var. 3 of 11.40 mm. For embryonic axes (GEA1), the aerial length of the var. 3 and var. 4, with 15.49 mm and 14.62 mm, respectively. For treatment 3 (GS2), the variety obtained the greatest aerial length in whole seeds was var. 5 with 20.45 mm, Followed by var. 4 with 19.56 mm. The var. 1 and var. 3 obtained the highest values of aerial length (GEA2) with 18.50 mm, and 17.26 mm, respectively, for embryonic axes. Finally, for treatment 4 (GS3), var. 5 reached the highest value for air length with 18.86 mm. For embryonic axes (GEA3), the var. 3 and var. 5 obtained the highest values for aerial length with 12.97 mm., And 12. mm., respectively (Fig. 2, Appendix 4).

4. Discussion

Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

The seeds of the five varieties of *A. hypogaea* provided by the INIAP-DENAREF seed bank were refreshed and multiplied to avoid problems due to storage times for the recovery of whole seeds and embryonic axes. As a result, all the shoots developed roots and aerial parts, confirming the results obtained by Tacán et al. [37]. Gagliardi et al. [13] mention that the age of the seed does not influence the recovery of the plant from its embryonic axes.

The whole seeds and embryonic axes of the *Arachis* varieties showed high survival (germination) after ten days after sowing. The explant was not interfered with by the growth or presence of callus. This is confirmed by Ishikawa et al. [29] and Kuranuki et al. [42] in that the vitrification procedure allows the direct development of meristem shoots without callus formation).

The whole seeds of the peanut varieties showed average germination between treatments of 85.6% and an average moisture content of the seed of 6.4%. The embryonic axes showed average germination of 90.3% with a moisture content of the seeds at the beginning of each of the treatments of 6.4%, which confirms the results of previous work about the germination percentages and moisture content of whole seeds and embryonic axes [38]. In the case of embryonic axes, it agrees with the results obtained by Gagliardi et al. [35], where it indicates that embryonic axes dried for 1 hour (up to a moisture content of 18%) and submerged in NL for 24 h produced outbreaks in 80%. In wild *Arachis* species in species of the *Arachis*, Triseminatae and Erectoid Sections, the regenerative response of the embryonic axes was similar to our study and ranged between 78 and 100% [43].

The lowest germination percentages occurred in treatments 2 and 4, both for whole seeds (GS1 and GS3) and embryonic axes (GEA1 and GEA3). These results agree with those obtained by Tacán et al. [38], Radhamani et al. [24] and Chaudhuri et al. [19].

The minimum differences of the peanut varieties depend on the morphological characteristics, such as the size of the leaflets, plant height, and periods of emergence and ripening of the fruits [44]. However, the differences found between the varieties in this study

revealed a similarity between the data obtained between whole seeds and embryonic axes for aerial and root length. Therefore, it can be indicated that these differences found between the varieties could have been due to the characteristics of the seeds concerning color and size, which are characteristics of the domestication of *A. hypogaea* [45].

Recovery after freezing was influenced by the treatments for whole seeds and embryonic axes, not the culture medium. This confirms the results of other authors about the culture medium [38,46–49]. All BV, with whole seeds and embryonic axes, developed aerial parts and roots. The results confirm that the cryopreservation of whole seeds and embryonic axes of the *A. hypogaea* varieties have the tolerance to the osmotic stress that is required for successful cryopreservation, which is related to the amino acid composition of peanut embryonic axes [23,37]. Cryopreservation of organized tissues such as shoot apices and embryonic axes is often appropriate for conserving genetic resources. In vitro plants derived from these tissues can ensure a reduced genetic change, although these structures could be recovered after cryopreservation [46].

In summary, we demonstrate and confirm the possibility of cryopreserving whole seeds and embryonic axes of the five peanut varieties studied using desiccation and rapid cooling with NL. This methodology is advantageous compared to the previously described methods for the cryopreservation of *A. hypogaea*, providing recovery rates significantly like those obtained by Tacán et al. [38] and Bajaj et al. [50]. Furthermore, unlike the method reported by Runthala et al. [32], this protocol does not require cryoprotectants or programmable freezers.

5. Conclusions

This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

The variables aerial and root length showed significant variations between treatments and varieties. These differences indicate that the five varieties conserved within the peanut collection in the INIAP Germplasm Bank can be cryopreserved. Furthermore, these results would indicate that cryopreservation could be successfully used in other intermediate (semi-orthodox) species.

This study used two cryopreservation strategies according to the type of explant (whole seed and embryonic axes) for each of the five genotypes. The behavior of the five varieties of *Arachis* suggests that characteristics related to each have been physiologically conserved.

All varieties showed excellent results with GS2 treatments for whole seeds and GEA2 in embryonic axes. In addition, an important point to consider is that none of the treatments existed in the presence of the callus phase, and therefore, the risk of somaclonal variation is reduced.

In this study, a cryopreservation and regeneration protocol for explants has been generated and developed for five varieties of *Arachis*, using whole seeds and embryonic axes, which can be replicated in other germplasm banks desiccation and rapid cooling with NL. The five varieties of peanuts present in the INIAP Germplasm Bank showed excellent results with the GS2 treatments for whole seeds and GEA2 in embryonic axes. In addition, an important point to keep in mind is that none of the treatments had the presence of the callus phase, and therefore, the risk of somaclonal variation is reduced.

Author Contributions: Conceptualization, C.P. and M.T.; design of the study and supervision. C.P., C.T. and M.T.; a collection of materials and maintenance of the field experiment E.Z., A.M. and M.T.; data acquisition M.T; data curation and statistical analysis M.T., C.T., Á.M.-A. and E.Z.; interpretation of results and drafting the first manuscript M.T., C.P. C.T. and Á.M.-A.; Writing, review and final editing M.T., C.P., C.T., Á.M.-A and M.S.; All authors have read and agreed to the published version of the manuscript.

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Appendix 1. Percentages of germination in complete seeds (mean value + standard error) at 10 days of germination tests, T50, minimum and maximum values in *A. hypogaea*, submitted to the following pre-treatments: (GSC) Untreated control; (GS1) Direct immersion in LN and; (GS2) Desiccation, (GS3) Desiccation and immersion in LN.

Variety	ECU	Plant material	Water content (%)	Germination (%) day 10 Mean±SE	T50 days	Min	Max
aequatoriana	11418 11428 11449	GSC	6.0	87.5±2.8	3	70.0	100.0
		GS1		81.7±3.7	3	70.0	100.0
		GS2		93.3±2.5	3	80.0	100.0
		GS3		77.5±.2.2	4	70.0	90.0
fastigiata	11448	GSC	6.6	100	5	100.0	100.0
		GS1		72.5±7.5	5	60.0	90.0
		GS2		100	4	100.0	100.0
		GS3		70.0±4.1	5	60.0	80.0
hirsuta	11405	GSC	6.6	92.5±2.5	2	90.0	100.0
		GS1		80.0±4.1	3	70.0	90.0
		GS2		95.0±2.9	3	90.0	100.0
		GS3		80.0±4.1	3	70.0	90.0
hypogaea	11469 11484	GSC	6.6	92.5±3.1	3	80.0	100.0
		GS1		80.0±1.9	4	70.0	90.0
		GS2		88.8±1.2	4	80.0	90.0
		GS3		77.5±2.5	4	70.0	90.0
peruviana	11494	GSC	6.9	100	3	100.0	100.0
		GS1		62.5±2.5	4	60.0	70.0
		GS2		100	2	100.0	100.0
		GS3		90.0±7.1	3	70.0	100

Appendix 2. Percentages of germination in embryonic axes (mean value + standard error) 10 days after starting the germination tests, T50, minimum and maximum values in *A. hypogaea*, submitted to the following pre-treatments: (GEAC) Untreated control; (GEA1) Direct immersion in LN and; (GEA2) Desiccation, (GEA3) Desiccation and immersion in LN.

Subspecies	ECU	Plant material	Water content (%)	Germination (%) day 10 Mean±SE	T50 days Mean±SE	Min	Max
aequatoriana	11418	GEAC	6.0	99.2±0.8	3	90.0	100.0
		GEA1		84.2±2.3	4	70.0	100.0

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		GEA2		94.2±1.5	3	90.0	100.0
		GEA3		82.5±2.5	3	70.0	90.0
fastigiata	11448	GEAC	6.6	95.0±5.0	4	80.0	100.0
		GEA1		95.0±2.9	4	90.0	100.0
		GEA2		97.5±2.5	4	90.0	100.0
		GEA3		77.5±2.5	4	70.0	80.0
hirsuta	11405	GEAC	6.6	97.5±2.5	3	90.0	100.0
		GEA1		85.0±2.9	4	80.0	90.0
		GEA2		87.5±2.5	3	80.0	90.0
		GEA3		80.0±4.1	3	70.0	90.0
hypogaea	11469	GEAC	6.6	96.2±2.6	3	80.0	100.0
		GEA1		88.8±2.3	4	80.0	100.0
		GEA2		92.5±2.5	3	80.0	100.0
		GEA3		83.8±2.6	4	70.0	90.0
peruviana	11494	GEAC	6.9	97.5±2.5	4	90.0	100.0
		GEA1		95.0±2.9	4	90.0	100.0
		GEA2		92.5±2.5	4	90.0	100.0
		GEA3		85.0±2.9	3	80.0	90.0

Appendix 3. Morphological variability in complete seeds with two quantitative characters: shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In Botanical Varieties of the Ecuadorian of peanut collection, submitted to the following pre-treatments: (GSC) Untreated Control; (GS1) Direct immersion in LN; (GS2) Desiccation and (GS3) Desiccation and immersion in LN. In each column, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the Tukey test.

Plant material	Variety	Shoot length (mm)				Root length (mm)			
		CV	Min	Max	Mean±SE	CV	Min	Max	Mean±SE
GSC	*aequatoriana*	56.99	4.03	32.33	12.79±0.67 a	49.29	7.40	99.10	37.85±1.70 b
	fastigiata	20.10	6.94	17.28	12.32±2.48 a	33.14	4.48	18.14	9.21±3.05 a
	hirsuta	35.49	16.56	52.72	30.82±10.94 c	53.03	12.76	82.47	38.74±20.55 b
	hypogaea	41.40	10.95	44.32	20.03±0.93 b	50.73	10.53	104.79	39.42±2.24 b
	peruviana	36.78	6.92	34.34	17.10±6.29 b	55.19	11.62	86.63	32.95±18.18 b
GS1	*aequatoriana*	52.23	1.95	15.43	7.04±0.34 ab	81.29	1.09	26.36	5.99±0.44 b
	fastigiata	59.81	2.70	27.05	10.72±6.41 bc	80.64	2.93	27.82	8.63±6.96 b
	hirsuta	43.07	1.63	6.50	3.18±1.37 a	42.94	1.14	4.18	2.07±0.89 a
	hypogaea	110.54	2.16	79.83	11.40±1.41 c	102.70	1.45	60.96	14.59±1.68 b
	peruviana	70.63	2.88	46.20	11.85±8.37 c	73.23	2.07	34.11	7.72±5.65 b
GS2	*aequatoriana*	46.68	3.82	39.17	16.41±0.70 ab	105.64	1.84	129.37	27.33±2.64 b
	fastigiata	31.68	4.63	24.16	13.13±4.16 a	54.55	2.30	32.49	14.27±7.79 a
	hirsuta	72.63	3.79	53.66	19.56±14.21 bc	84.79	2.07	57.21	14.59±12.37 a

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	hypogaea	29.67	5.73	26.04	13.86±0.46 a	77.74	3.08	64.96	17.86±1.55 ab
	peruviana	29.41	9.91	37.76	20.45±6.01 c	57.90	7.75	99.78	41.05±23.77 c
GS3	*aequatoriana*	50.69	2.09	16.84	5.62±0.26 b	85.73	1.11	23.24	3.33±0.26 ab
	fastigiata	114.56	2.04	47.59	8.87±10.16 c	111.81	1.62	27.78	6.54±7.31 bc
	hirsuta	19.78	1.18	3.09	2.15±0.42 a	29.54	1.10	4.40	1.77±0.52 a
	hypogaea	88.30	1.84	38.22	12.98±1.28 bc	102.99	1.40	61.65	16.57±1.91 c
	peruviana	41.70	6.87	40.78	18.86±7.87 d	64.50	4.12	62.09	22.91±14.77 d

Appendix 4. Morphological variability in embryonic axes with two quantitative characters: shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In Botanical Varieties of the Ecuadorian of peanut collection, submitted to the following pre-treatments: (GSC) Untreated Control; (GS1) Direct immersion in LN; (GS2) Desiccation and, (GS3) Desiccation and immersion in LN. In each column, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the Tukey test.

Plant material	Variety	Shoot length (mm)				Root length (mm)			
		CV	Min	Max	Mean±SE	CV	Min	Max	Mean±SE
GEAC	*aequatoriana*	42.22	4.21	21.47	10.46±040 a	60.51	3.08	22.83	7.63±0.42 a
	fastigiata	30.81	8.34	24.64	14.57±4.49 b	76.31	3.80	48.33	13.87±10.58 b
	hirsuta	30.77	5.23	19.01	10.11±3.11 a	36.07	3.98	18.02	8.35±3.01 a
	hypogaea	26.77	5.57	18.33	11.39±034 a	35.14	3.51	14.43	7.21±0.28 a
	peruviana	15.60	7.66	17.78	14.27±2.23 b	31.23	7.99	27.66	14.22±4.44 b
GEA1	*aequatoriana*	34.86	4.57	27.18	13.82±0.44 b	39.17	3.78	22.66	8.83±0.32 b
	fastigiata	40.49	3.10	12.75	6.41±2.59 a	23.68	3.35	8.83	5.17±1.23 a
	hirsuta	17.93	9.59	23.09	14.62±2.62 b	26.81	6.06	21.40	13.73±3.68 c
	hypogaea	31.15	9.24	32.66	15.49±0.54 b	65.62	5.03	43.79	12.41±0.91 c
	peruviana	61.16	3.32	19.69	7.43±4.54 a	55.84	2.03	15.80	5.55±3.10 a
GEA2	*aequatoriana*	30.68	7.04	35.41	18.50±0.52 b	45.45	3.35	38.63	16.36±0.68 b
	fastigiata	18.66	7.10	15.90	11.86±2.21 a	39.67	4.50	28.70	14.02±5.56 b
	hirsuta	19.24	7.74	17.33	12.36±2.38 a	33.38	5.03	17.11	9.24±3.08 a
	hypogaea	22.07	9.16	29.75	17.26±0.43 b	35.40	6.73	36.47	16.95±0.67 b
	peruviana	22.09	4.12	20.68	13.81±3.05 a	40.96	4.83	26.79	14.04±5.75 b
GEA3	*aequatoriana*	54.72	3.09	20.79	7.16±0.36 a	50.39	2.08	16.48	4.41±0.20 a
	fastigiata	28.13	4.20	12.95	7.69±2.16 ab	47.05	2.30	15.30	5.27±2.48 a
	hirsuta	49.66	3.14	22.97	9.29±4.62 b	34.23	2.53	7.97	4.59±1.57 a
	hypogaea	31.26	5.49	25.55	12.97±0.45 c	46.28	4.01	31.49	9.86±0.51 b
	peruviana	33.00	3.80	22.58	12.67±4.18 c	40.14	2.69	18.61	9.48±3.81 b

References

1. Knauft, D.; Norden, A.; Gorbet, D. Principles of Cultivar Development: Crop Species. In *Agronomy Books*; Fehr, W., Ed.; Macmillan Publishing Company, New York, USA, 1987; Volumen 2; pp. 346-385. https://lib.dr.iastate.edu/agron_books/2
2. Halward, T.M.; Stalker, H.T.; LaRue, E.A.; Kochert, G. Genetic variation detectable with molecular markers among unadapted germ-plasm resources of cultivated peanut and related wild species. *Genome*, **1991**, *34*(6), 1013–1020. https://doi.org/10.1139/g91-156
3. León, J. Botánica de los cultivos tropicales. *Instituto Interamericano de Cooperación Para La Agricultura*, **1987**, 462. http://repositorio.iica.int/bitstream/11324/7228/1/ BVE18040317e.pdf
4. Krapovickas, A.; Vanni, R.O. El Maní de Llullaillaco. *Bonplandia*, **2009**, *18*(1), 51. https://doi.org/10.30972/bon.1811347
5. Holbrook, C.C.; Stalker, H.T. Peanut Breeding and Genetic Resources. *Plant Breed. Rev.* **2010**, *22*, 297–356. https://doi.org/10.1002/9780470650202.ch6
6. Hanson, J.; Williams, J.T.; Freund, R. *Institutes conserving crop germplasm: The IBPGR (International Board for Plant Genetic Resources) global network of genebanks*. IBPGR, Rome, Italy. 1984. 25 p. https://cgspace.cgiar.org/handle/10568/105344
7. Morris, J.B.; Dunn, S.; Pittman, R.N. Plant Recovery from Embryonic Axes of Deteriorated Peanut Seed for Germplasm Renewal1. *Peanut Sci.* **1995**, *22*(1), 66–70. https://doi.org/10.3146/pnut.22.1.0015
8. Pence, V.C. Cryostorage of embryo axes of several large-seeded temperate tree species. *Cryobiology*, **1990**, *27*(2), 212–218. https://doi.org/10.1016/0011-2240(90)90013-T
9. Vázquez-Yanes, C.; Aréchiga, M.R. Ex situ conservation of tropical rain forest seed: Problems and perspectives. *Interciencia*, **1978**, *21*(5), 293–298. https://www.researchgate.net/profile/Mariana-Rojas-2/publication/ 340132970_Ex_situ_conservation_of_tropical_species/links/5e7a6822a6fdcc57b7bba656/Ex-situ-conservation-of-tropical-species.pdf
10. Roos, E.E. Physiological, biochemical, and genetic changes in seed quality during storage. *HortScience*, **1980**, *15*(6), 19–22). https://agris.fao.org/agris-search/ search.do?recordID=US19810654977
11. Bonner, F. Storage of seeds: Potential and limitations for germplasm conservation. *Forest Ecol. Manag.* **1990**, *35*(1–2), 35–43. https://doi.org/10.1016/0378-1127(90)90230-9
12. IUCN. Guidelines for Using the IUCN Red List Categories and Criteria. IUCN, Gland, Switzerland, 2010, 5 p. http://intranet.iucn.org/webfiles/doc/SSC/RedList/ RedListGuidelines.pdf
13. Gagliardi, R.; Pacheco, G.; Coculilo, S.; Valls, J.; Mansur, E. In vitro plant regeneration from seed explants of wild groundnut species (genus *Arachis*, section Extranervosae). *Biodivers. Conserv.* **2000**, *9*(7), 943–951. https://doi.org/10.1023/A:1008960608543
14. Reed, B.M.; Normah, M.N.; Yu, X. Stratification is necessary for successful cryopreservation of axes from stored hazelnut seed. *Cryo-Letters*, **1994**, *15*(6), 377–384.
15. Stushnoff, C. Cryopreservation of Fruit Crop Genetic Resources— Implications for Maintenance and Diversity during Conservation. *HortScience*, **1991**, *26*(5), 518–522. https://doi.org/10.21273/hortsci.26.5.518
16. Benson, E.E. Chapter 6. Cryopreservation. In *Plant conservation Biotechnology*. Taylor & Francis, London; 1999, pp. 83–96. ISBN: 0748407464 9780748407460. https://books.google.dk/books?hl=da&lr=&id=SUZZDwAAQBAJ&oi=fnd&pg=PP1&dq=Benson,+E.+(2020).+%22Cryopreservation+In+Plant+Conservation%22+Biotechnology+(pp.+109%E2%80%93122)&ots=ZdKTrahupm&sig=6qEsPMyzGyJ7jZUFxJHNwdPCiOc&redir_esc=y#v=onepage&q&f=false
17. Withers, L.A.; Engelmann, F. In vitro Conservation of Plant Genetic Resources. In *Agricultural biotechnology*; Altman, A., Colwell, R.R., First, N.L., Schell, J., Vasil, I.K., Eds; M. Dekker, New York, USA, 1997, pp. 58-88. ISBN 0585157472. https://horizon.documentation.ird.fr/exl-doc/pleins_textes/divers19-09/010056428.pdf
18. Abdulmalik, M.; Usman, I.; Olarewaju, J.; Aba, D. Influence of Desiccation Time on Survival and Regeneration of Embryonic Axes of Groundnut (*Arachis hypogaea* L.) Immersed in Liquid Nitrogen. *Am. J. Plant Sci.* **2013**, *4*(09), 1725–1730. https://doi.org/10.4236/ ajps.2013.49211
19. Chaudhury, R.; Chandel, K. Studies on germination and cryopreservation of Cardamom (Elettaria cardamomum Maton) seeds. *Seed Sci. Technol.* **1995**, *23*(1), 235–240. https://agris.fao.org/agris-search/search.do?recordID=CH9600084
20. González-Benito, M.E.; Carvalho, J.M.F.C.; Perez, C. Effect of desiccation and cryopreservation on the germination of embryonic axes and seeds of cotton. *Pesqui. Agropecu. Bras.* **1998**, *33*(1), 17–20. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/44658/1/Effect-of-desiccation-and-cryopreservation-on-the-germination.pdf
21. Hong, T.D.; Ellis, R.H. The survival of germinating orthodox seeds after desiccation and hermetic storage. *J. Exp. Bot.* **1992**, *43*(2), 239–247. https://doi.org/10.1093/jxb/43.2.239
22. Marin, M.; Mafla, G.; Roca, W.; Withers, L. Cryopreservation of cassava zygotic embryos and whole seeds in liquid nitrogen. *Cryo-Letters*, **1990**, *11*, 257–264. https://cgspace.cgiar.org/handle/10568/83253
23. Pritchard, H.W.; Tompset, P.B.; Manger, K.; Smidt, W.J. The effect of moisture content on the low temperature responses of Araucaria hunsteinii seed and embryos. *Ann. Bot.* **1995**, *76*(1), 79–88. https://doi.org/10.1006/anbo.1995.1081
24. Radhamani, J.; Chandel, K.P.S. Cryopreservation of embryonic axes of trifoliate orange (Poncirus trifoliata [L.] RAF.). *Plant Cell Rep.* **1992**, *11*(7), 372–374. https://doi.org/ 10.1007/BF00233369
25. Berjak, P.; Dumet, D. Cryopreservation of seeds and isolated embryonic axes of neem (*Azadirachta indica*). *Cryo-Letters*, **1996**, *17*(2), 99–104. https://agris.fao.org/agris-search/search.do?recordID=US201301515078
26. Berjak, P.; Walker, M.; Watt, M.; Mycock, D. Experimental parameters underlying failure or success in plant germplasm cryopreservation: A case study on zygotic axes of *Quercus robur* L. *Cryo-Letters*, **1999**, *20*(4), 251–262. https://agris.fao.org/agris-search/search.do?recordID=US201302939183
27. Sun, W.Q. State and phase transition behaviors of *Quercus rubra* seed axes and cotyledonary tissues: Relevance to the desiccation sensitivity and cryopreservation of recalcitrant seeds. *Cryobiology*, **1999**, *38*(4), 372–385. https://doi.org/10.1006/ cryo.1999.2180

28. Vertucci, C.W.; Berjak, P.; Pammenter, N.W.; Crane, J. Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. *CryoLetters*, **1991**, *12*, 339–350. https://agris.fao.org/agris-search/search.do?recordID=US201301743939
29. Ishikawa, K.; Harata, K.; Mii, M.; Sakai, A.; Yoshimatsu, K.; Shimomura, K. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. *Plant Cell Rep.* **1997**, *16*(11), 754–757. https://doi.org/10.1007/s002990050314
30. Mycock, D.J.; Watt, M.P.; Berjak, P. A Simple Procedure for the Cryopreservation of Hydrated Embryonic Axes of *Pisum sativum*. *J. Plant Physiol.* **1991**, *138*(6), 728–733. https://doi.org/10.1016/S0176-1617(11)81323-4
31. Thammasiri, K. Preservation of seeds of some Thai orchid species by vitrification. In *Proceedings of The 16th World Orchid Conference* (April 28 – May 2, 1999). The Vancouver Orchid Society, 1st Ed. January 1, 2002, Vancouver, Canada, pp. 248–251. ISBN-10: 0973015209
32. Runthala, P.; Jana, M.K.; Mohanan, K. Cryopreservation of groundnut (Arachis hypogaea L.) embryonic axes for germplasm conservation. *Cryo-Letters*, **1993**, *14*(6), 337–340. https://doi.org/10.3/JQUERY-UI.JS
33. Abdulmalik, M.; Usman, I.; Olarewaju, J.; Aba, D. Cryopreservation of embryonic axes of groundnut (Arachis hypogaea L.) by vitrification. *Afr. J. Biotechnol.* **2014**, *13*(2), 280–285. https://doi.org/10.5897/ajb2013.13452
34. Ozudogru, E.A.; Ozden-Tokatli, Y.; Gumusel, F.; Benelli, C.; Lambardi, M. Development of a cryopreservation procedure for peanut (Arachis hypogaea L.) embryonic axes and its application to local Turkish germplasm. *Adv. Hortic. Sci.* **2009**, *23*(1), 41–48. https://doi.org/10.1400/109127
35. Gagliardi, R.; Pacheco, G.; Valls, J.; Mansur, E. Cryopreservation of cultivated and wild Arachis species embryonic axes using desiccation and vitrification methods. *Cryo-Letters*, **2002**, *23*(1), 61–68. https://www.ingentaconnect.com/content/cryo/cryo/2002/00000023/00000001/art00009#
36. Hoyt, E.. Conservando los parientes silvestres de las plantas cultivadas. *Consejo Internacional de Recurso Fitogenéticos*, IBPGR, UICN, WWF. Addison-Wesley, Wilmington, Delaware, USA; 1992, 60 p. ISBN: 0-201-51830-3. https://portals.iucn.org/library/sites/ library/files/documents/1992-010-Es.pdf
37. Tacán, M.; Tapia, C.; Pérez, C. Effects of accelerated ageing and cryopreservation on seeds and embryonic axes of *Phaseolus vulgaris* L. and *Arachis hypogaea* L. Germination and seedlings vigor. *PeerJ Preprints* **2017**, 5:e3203v1 https://doi.org/10.7287/peerj.preprints.3203v1
38. Tacán, M.; Tapia, C.; Pérez Ruiz, C. Desiccation method and cryoconservation affects amino acids composition of embryonic axes of *Phaseolus vulgaris* L and *Arachis hypogaea* L. *IJCIAR*, **2017**, *3*(9), 783–788. http://journalijcir.com/sites/default/files/issue-files/00538-A-2017_0.pdf
39. Monteros-Altamirano, Á. On-farm conservation of potato landraces in Ecuador. *Agron. Colomb.* **2018**. *36*(3), 189–200. https://doi.org/10.15446/agron.colomb.v36n3.66640
40. Murashige, T.; Skoog, F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant.* **1962**, *15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
41. Di Rienzo, J.A.; Balzarini, M.G.; Gonzalez, L.A.; Tablada, E.M.; Casanoves, F.; Robledo, C.W. InfoStat (2008). InfoStat, versión 2008. Manual del Usuario. Grupo InfoStat, FCA, Universidad Nacional de Córdoba. 1st Ed., Editorial Brujas Argentina. 2008, 335 p. https://www.researchgate.net/profile/Fernando-Casanoves/publication/ 319875343_Manual_del_usuario/links/5e2ee26992851c9af7280cfa/Manual-del-usuario.pdf
42. Kuranuki, Y.; Sakai, A. Cryopreservation of in vitro-grown shoot tips of tea (Camellia sinensis) by vitrification. *Cryo-Letters*, **1995**, *16*(6), 345–352. https://eurekamag.com/ research/002/791/002791415.php
43. Pacheco, G.; Gagliardi, R.F.; Carneiro, L.A.; Valls, J.F.M.; Mansur, E. In vitro regeneration and conservation of wild species of *Arachis*. *J. Hortic. Sci. Biotechnol.* **2007**, *82*(2), 311–315. https://doi.org/10.1080/14620316.2007.11512234
44. Chiyembekeza, A.J.; Subrahmanyam, P.; van der Merwe, P.J.A. *Rural Prosperity is Nation's Economic Stability: A Partnership Approach to Attain Sustainable Production of Groundnut and Pigeonpea in Smallholder Agriculture for Quality Diet, Household Food Security, and Poverty Alleviation in Malawi*. Quarterly Report (January to March 2001). Lilongwe: USAID, ICRISAT in partnership with The Ministry of Agriculture and Irrigation, Malawi; 2001.
45. Grabiele, M.; Chalup, L.; Robledo, G.; Seijo, G. Genetic and geographic origin of domesticated peanut as evidenced by 5S rDNA and chloroplast DNA sequences. *Plant Syst. Evol.* **2012**, *298*(6), 1151–1165. https://doi.org/10.1007/s00606-012-0627-3
46. Gagliardi, R.; Pacheco, G.; Carneiro, L.; Valls, J.; Vieira, M.; Mansur, E. Cryopreservation of *Arachis* species by vitrification of in vitro-grown shoot apices and genetic stability of recovered plants. *Cryo-Letters*, **2003**, *24*(2), 103–110. https://www.researchgate.net/ publication/10696446
47. Chetverikova, E.P. Role of abscisic acid in frost tolerance of plants and cryopreservation of cultured tissues. *Russ. J. Plant Physiol.* **1999**, *46*(5), 721–727. https://elibrary.ru/ item.asp?id=13317302
48. Hitmi, A.; Barthomeuf, C.; Sallanon, H. Cryopreservation of *Chrysanthemum cinerariaefolium* shoot tips. *J. Plant Physiol.* **2000**, *156*(3), 408–412. https://doi.org/ 10.1016/S0176-1617(00)80081-4
49. Na, H.Y.; Kondo, K. Cryopreservation of tissue-cultured shoot primordia from shoot apices of cultured protocorms in *Vanda pumila* following ABA preculture and desiccation. *Plant Sci.* **1996**, *118*(2), 195–201. https://doi.org/10.1016/0168-9452(96)04438-X
50. Bajaj, Y. Technology and prospects of cryopreservation of germplasm. *Euphytica*, **1979**, *28*(2), 267–285. https://doi.org/10.1007/BF00056584