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Research Article

Cryopreservation of *Arachis hypogaea* L. Varieties at the Gene Bank of INIAP-Ecuador

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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 Gene Bank, testing cryopreservation methods, and evaluating the germination percentage of whole seeds and embryonic shoots. Subsequently, two quantitative variables, shoot length and root, were evaluated. The average germination/elongation percentages of varieties and treatments ware higher when embryonic axes were isolated with 99.31% than 86.06% seeds. The best germination and elongation percentage of the five varieties for seeds and embryonic shoots was obtained by the *Peruvian* variety with 88.13% and 92.50%. only for whole seeds statistical differences were observed between the *fastigiata* and *hypogaea* subspecies in NL and for embryonic axes they were not observed. observed statistical differences in the treatments evaluated for the two subspecies. The best treatments by variety for the germination and elongation of whole seeds and embryonic axes were obtained by the treatment (desiccation and LN) for whole seeds with 95.42% and embryonic axes with 92.83%. Ageing and cryopreservation treatments positively affected germination and elongation in whole seeds and embryonic axes. The two quantitative variables, shoot and root length showed variability between the five varieties; significant differences (≤ 0.05) were observed between the four treatments evaluated for whole seeds and embryonic axes. The three treatments for whole seeds and embryonic axes the non-cryopreserved control treatment, obtained good survival, then whole seeds germinated, and embryonic axes produced sprout development (aerial parts) and root formation. With the most effective treatments (desiccation and LN) for whole seeds and embryonic axes, the cryopreservation of the national peanut collection of the INIAP Germplasm Bank could be started.

Keywords: Varieties; Seeds; Embryonic Shoot

Introduction

The peanut (*Arachis hypogaea* L.) is native to South America, where the genus *Ara-chis* is widely distributed [1] in tropical and subtropical regions [2]. Cultivated peanuts comprise six varieties grouped into two subspecies: *A. hypogaea* subsp. *hypogaea* L. and *A. hypogaea* subsp. *fastigiata* Waldron. The subspecies *A. hypogaea* subsp. *hypogaea* includes the varieties *hypogaea* L. and *hirsuta* J. Köhler., while *A. hypogaea* subsp. *fastigiata* includes *fastigiata* (Waldron) Krapov. and W.C. Greg., *vulgaris* Harz, *peruviana* Krapov. and W.C. Greg. and *aequatoriana* Krapov. and W.C. Greg. [3]. The National Institute of Agricultural Research (INIAP) collection includes the *fastigiata*, *peruviana* and *aequatoriana* varieties [4]. For the *fastigiata* variety, the most important centers of diversity are found in Peru, Brazil, and Paraguay; the *peruviana* variety, is found in Peru and Ecuador, and the *aequatoriana* variety, is almost exclusive to southern Ecuador and northern Peru [4,5].

Climate change will be responsible for the loss of 50% in the range of distribution of the wild populations of peanuts, potatoes (*Solanum tuberosum*) and cowpeas (*Vigna unguiculata*). Furthermore, due to its effects, 16–22% of these species will become extinct by 2055 [6]. Due to the drastic process of genetic erosion, a series of strategies have been generated by gene banks [7], such as the conservation of orthodox seeds in cold rooms, field or *in vitro* collections [8]. However, *in vitro* storage can lead to somaclonal variation, which is a drawback in conservation programs.

A method that allows the conservation of this type of seeds in the long term is the cryogenic technology [9,10]. For this reason, cryopreservation at low temperatures (-196 °C) for storage is considered the most appropriate method for indefinite periods, with lower risks of genetic alterations [11,12] and less periodic viability tests [13] reducing conservation costs [11,12]. Advanced cryopreservation methods can be used to avoid lethal damage from freezing [14,15].

Runthala., *et al*. [16] and Abdulmalik., *et al*. [17] published protocols for preserving peanut embryonic axes using cryoprotectants, with which they obtained variable survival levels (40-90%) depending on the genotype. Several methods have been developed for the cryopreservation of peanuts, such as desiccation in a laminar flow chamber [17] vitrification technique in embryonic axes [18], or both techniques in cultivated and wild species [19,20].

Tacan., *et al*. [21], measured the effects of accelerated aging and cryopreservation in seeds and embryonic axes of *Phaseolus vulgaris* L and *Arachis hypogaea* L. It was observed that aging and cryopreservation treatments positively affected germination and elongation. Another study [22] shows the differences, attributable to the genotype, in terms of proline content and the rest of amino acids, on stress conditions related to tolerance to desiccation and cryopreservation. Gagliardi., *et al*. [8], studied the effects of two cryopreservation methods (chemical vitrification and air drying) on embryonic axes isolated from *A. hypogaea*, in which both methods resulted in high levels (70–90 %) of recovery of the whole plant after liquid nitrogen treatment. The objective of this research was to determine the effect of cryopreservation with three treatments and control for five existing varieties in the peanut collection preserved in the gene bank of the National Institute of Agricultural Research (INIAP) of Ecuador, to evaluate the usefulness of the method for conservation of the peanut collection.

Materials and Methods

Sampling, experimental settings

The seeds of *A. hypogaea* were provided by the gene bank of INIAP. The selection of the eight morphotypes was made based on the morphological and taxonomic characterization carried out at the National Department of Plant Genetic Resources (DENAREF) of INIAP. Table 1 shows the two subspecies, the five varieties and the eight accessions with their respective codification and geographic distribution.

Subspecies (subsp)	Variety (var)	Accessions
Arachis hypogaea subspecies. *fastigiata*	var. *aequatoriana* Krapov. and W.C. Greg.	ECU-11418 ECU-11428 ECU-11449
	var. *fastigiata* Harz	ECU-11448
	var. *peruviana* Krapov. and W.C. Greg.	ECU-11494
Arachis hypogaea. subspecies *hypogaea*	var. *hypogaea* Kohler	ECU-11484 ECU-11469
	var. *hirsuta* Kohler	ECU-11405

Table 1: Subspecies, varieties, and codes of peanut accessions from INIAP´s Gene Bank.

The previous process to this research was seed multiplication to have an adequate number of seeds per treatment and repetitions. Then the seed conditioning was carried out, considering the protocols of Monteros-Altamirano., *et al*. [23].

Materials remained in seed lots stored at 15 °C and 30–40% RH for three months 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 for 5 min in 70 % alcohol, 20 min in 10 % NaOCl (commercial bleach) plus two to three drops of Tween®-20, rinsed three times with a distilled-sterile water solution with 5% NaOCl plus two to three drops of Tween®-20 for 10 min. Shake occasionally and rinse twice with sterile distilled water.

Description of the applied experimental methods

Whole peanut seeds (GS) and peanut embryonic axes (GEA) were subjected to the treatments detailed in Table 2, before germination tests. For each treatment, four replicates of 10 complete seeds and 10 embryonic axes were prepared. All experiments were repeated twice.

Treatments	Whole seeds coding	Embryonic axes coding
T1: Control	GS	GEA
T2: Direct immersion in LN	GS1	GEA1
T3: Dessication with silica gel	GS2	GEA2
T4: Desiccation with silica gel and immersion in LN	GS3	GEA3

Table 2: Treatments for whole seeds and embryonic axes of *A. hypogaea*. INIAP, 2023.

Germination tests

Whole seed germination tests were performed 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 and moistened periodically further) in the dark at 25 °C; they were quantified at 10 days; the germination percentage of whole seeds and the number of days necessary to reach 50% germination of whole germinated seeds (T50) were measured [24]. In addition, the radicle length (mm) and shoot length (mm) were also registered.

Embryonic axes were cultured in sterile glass containers with MS medium [25] supplemented with 0.3 M sucrose at 25 °C with illumination and a light/dark photoperiod of 16:8 h cool white fluorescent light (40 µmol m^{-2} s^{-1}). Four repetitions of 10 axes per glass container were set up and quantified after ten days. The percentage of elongation of the embryonic axes, the number of days necessary to reach 50% of germinated embryonic axes (T50), the length of the radicles (mm) and the length of the aerial parts (mm) were measured.

Desiccation: silica gel

For seed desiccation (T3), forty seeds were placed in Petri dishes (9 cm in diameter) containing a layer of dehydrated silica gel of approximately 5 g and covered with a filter paper disc on which the seeds were placed; once sealed, they were kept for 3 h at 20 °C (Table 2).

Whole seeds cryopreservation

For whole seeds, Treatment 2 and Treatment 4 were carried out (Table 2); the seeds were submerged in cryovials and introduced directly into LN. The seeds were removed from the cryovials after 1 h at room temperature (20 °C) and were immediately placed in Petri dishes under the germination conditions indicated above.

For the T2 and T4 treatments of the embryonic axes, the whole peanut seeds were immersed in LN for 5 minutes, then 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, 10 embryonic axes were placed in each container. In total there were four containers and 40 embryonic axes: each container with a solid MS base culture medium supplemented with 20 g l-1 of sucrose.

Data analysis

Sugarcane 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.

A completely randomized experimental design (DCA) with ten observations was used. For each treatment, four replicates of 10 whole seeds and embryonic axes were made for the five varieties

(Tables 1, and 2). For the analysis, the statistical program InfoStat version 2008 [26] was used. An analysis of variance and Tukey's test were performed to compare means (α = 0.05).

Results and Discussion

Results and discussion must illustrate and interpret the reliable results of the study.

Germination

The seeds of all the peanut varieties studied tolerated the desiccation and cryopreservation treatments, both for whole seeds and for embryonic axes, since, in all cases, the treated seeds germinated (Figure 1).

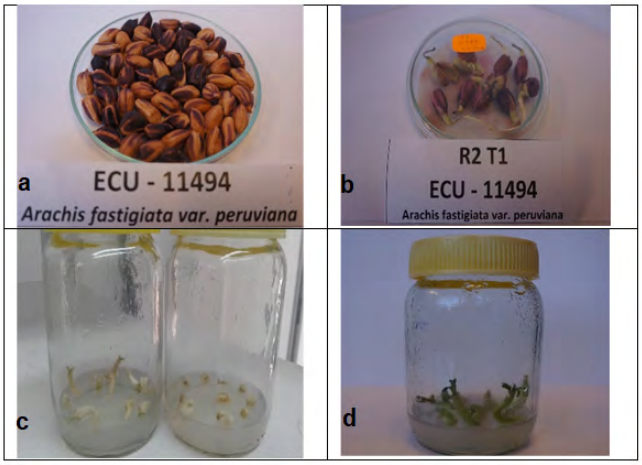

Figure 1: Recovery of plants after cryopreservation of *A. hypogaea* of the *peruvian* variety. a) Seeds selected to carry out the treatments of both whole seeds and embryonic axes; b) Whole cryopreserved seeds; c) Germination of cryopreserved embryonic axes; d) Cryopreserved embryonic axes.

Study of the effect of cryopreservation on the germination of the subspecies: A. hypogaea subsp. fastigiata *and A. hypogaea subsp. Hypogaea*.

Germination data of whole seeds and the elongation of embryonic axes of *A. hypogaea* subsp. *fastigiata* and *A. hypogaea* subsp. *hypogaea* for 4 treatments is summarized in (Table 1).

The average germination for whole seeds for the *fastigiata* and *hypogaea* subspecies was 85.6%. (Figure 2 – *fastigiata*: black bar, *hypogaea*: gray bar, Appendix 5). For the treatments with applied NL, the average germination for T2 fluctuated between 76.0% (subsp. *fastigiata*) to 80.0% (subsp. *hypogaea*) and no statistical differences were detected between the two subspecies. For T4, the germination percentages were between 78.5% (subsp. *fastigiata*) to 78.3% (subsp. *hypogaea*), there was no statistical significance between the two subspecies. Only the T50 values for T1 obtained statistical significance between the two subspecies and an average of 3.5 days for the four treatments.

The mean elongation percentage for embryonic axes for *fastigiata* and *hypogaea* subspecies was 90.1%. (Figure 2 – *fastigiata*: black bar, *hypogaea*: gray bar, Appendix 6). For the treatments that were applied NL, the average germination was: for T2, germination ranged from 87.5% (subsp. *hypogaea*) to 88.5% (subsp. *fastigiata*). On T4, the germination percentages were between 82.0% (subsp. *fastigiata*) to 82.5% (subsp. *hypogaea*), in none of the treatments there was statistical significance between subspecies. The T50 values for the four treatments did not show significant differences between the two subspecies and an average of 3.6 days for the four treatments.

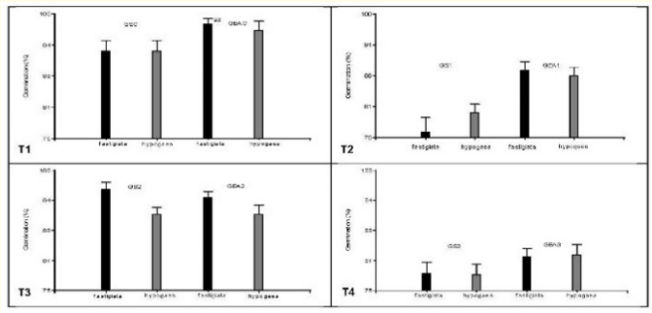

Figure 2: Germination and elongation percentages of whole seeds and embryonic axes at ten days, respectively, of the subspecies *hypogaea* and *fastigiata* (mean value + standard error), subjected to the following pretreatments: (T1) Untreated control; (T2) Direct immersion in LN; (T3) Desiccation and, (T4) Desiccation and immersion in LN. The black bar is data from evaluating whole peanut seeds (GS), and the grey bar is data from embryonic axes (GEA).

Study of the effect of cryopreservation on the elongation of embryonic axes and germination of whole seeds of five varieties of *A. hypogaea*

The germination for the treatments with whole seeds with NL application were as follow: for T2 the average germination for the five varieties (*aequatoriana*, *fastigiata*, *hypogaea*, *hirsuta* and *peruviana*) was 75.3% (Figure 3 - black bar, Appendix 1); germination ranged from 62.5% (var. *peruviana*) to 81.7% (var. *aequatoriana*) and statistical differences were detected with the other varieties. For T4, the average germination of the five varieties was 79.0%, the germination percentages ranged from 70.0% (var. *fastigiata*) to 90.0% (var. *peruviana*), statistical significance was also observed between varieties. The T50 values for the four treatments had statistical significance between varieties and an average of 3.6 days. The average moisture content of the seeds before applying the treatments was 6.5%.

Appendix 1: Percentages of germination in complete seeds (mean value + standard error) at 10 days of germination tests, T_{50}, 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. In each column, values followed by the sameletter are not significantly different at $p \leq 0.05$ as determined by the Tukey test.

Variety	Plant material	Water content (%)	Germination(%) day 10 Mean ± SE	T50 days	Min	Max
T1 (GSC)	*aequatoriana*	6.0	87.5 ± 2.8	2.9 b	70.0	100.0
	fastigiata	6.6	100	5.0 a	100.0	100.0
	hypogaea	6.6	92.5 ± 3.1	2.6 b	80.0	100.0
	hirsuta	6.6	92.5 ± 2.5	2.2 b	90.0	100.0
	peruviana	6.9	100	2.8 b	100.0	100.0
T2 (GS1)	*aequatoriana*	6.0	81.7 ± 3.7b	3.5 bc	70.0	100.0
	fastigiata	6.6	72.5 ± 7.5ab	5.5 a	60.0	90.0
	hypogaea	6.6	80.0 ± 1.9ab	3.8 bc	70.0	90.0
	hirsuta	6.6	80.0 ± 4.1ab	3.0 c	70.0	90.0
	peruviana	6.9	62.5 ± 2.5a	4.2 b	60.0	70.0
T3 (GS2)	*aequatoriana*	6.0	93.3 ± 2.5ab	3.1 bc	80.0	100.0
	fastigiata	6.6	100 b	4.5 a	100.0	100.0
	hypogaea	6.6	88.8 ± 1.2ab	4.1 ab	80.0	90.0
	hirsuta	6.6	95.0 ± 2.9a	2.8 c	90.0	100.0
	peruviana	6.9	100b	2.2 c	100.0	100.0
T4 (GS3)	*aequatoriana*	6.0	77.5 ± .2.2ab	3.8 b	70.0	90.0
	fastigiata	6.6	70.0 ± 4.1a	5.0 a	60.0	80.0
	hypogaea	6.6	77.5 ± 2.5ab	4.0 b	70.0	90.0
	hirsuta	6.6	80.0 ± 4.1ab	3.2 bc	70.0	90.0
	peruviana	6.9	90.0 ± 7.1b	2.8 c	70.0	100.0

* Different letters indicate significant difference ($p \leq 0.05$).

The percentage of embryonic axes that elongated after the application of NL were for T2 the average of the five varieties (*aequatoriana*, *fastigiata*, *hypogaea*, *hirsuta* and *peruviana*) was 89.6% (Figure 3 – gray bar, Appendix 2), which ranged from 84.2% (var. *aequatoriana*) to 95.0% (var. *fastigiata* and var. *peruviana*) and no statistical differences were detected with the other varieties. For T4, the average percentage of the five varieties was 81.8%, the germination percentages were between 77.5% (var. *fastigiata*) and 85.0% (var. *peruviana*), they did not present statistical significance. between varieties. The T50 values of T4 showed significant differences and an average for the four treatments of 3.8 days. The average moisture content of the seeds before applying the treatments was 6.5%.

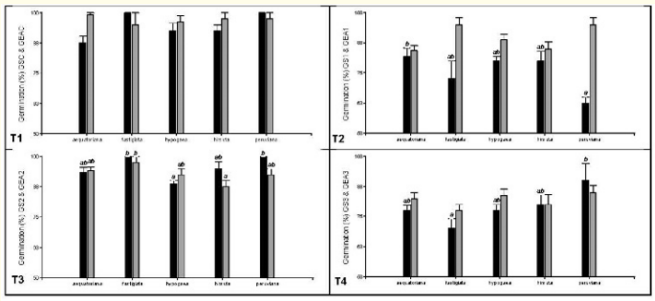

Figure 3: Whole seed germination and embryonic axes elongation percentages (mean value + standard error), for whole seeds and embryonic axes at ten days of onset. Five varieties of *A. hypogaea* were, subjected to the following pretreatments: (T1) Untreated control; (T2) Direct immersion in LN; (T3) Desiccation and (T4) Desiccation and immersion in LN. The black bar is data from evaluating whole peanut seeds (GS), and the grey bar is data from embryonic axes (GEA). Different letters in the bars indicate significant differences (Tukey, $p \leq 0.05$).

Appendix 2: Percentages of elongation of embryonic axes (mean value + standard error) 10 days after starting the elongation tests, T_{50}, minimum and maximum values in *A. hypogaea*, subjected to the following pre-treatments: (GEAC) Untreated control; (GEA1) Direct immersion in LN and; (GEA2) Desiccation, (GEA3) Desiccation and immersion in LN. In each column, valuesfollowed by the same letter are not significantly different at $p \leq 0.05$ as determined by the Tukey test.

Variety	Plant material	Water content (%)	Germination (%) day 10 Mean ± SE	T50 days	Min	Max
T1 GEAC)	*aequatoriana*	6.0	99.2 ± 0.8	3.6 ab	90.0	100.0
	fastigiata	6.6	95.0 ± 5.0	4.5 a	80.0	100.0
	hypogaea	6.6	96.2 ± 2.6	3.5 b	80.0	100.0
	hirsuta	6.6	97.5 ± 2.5	3.5 b	90.0	100.0
	peruviana	6.9	97.5 ± 2.5	4.0 ab	90.0	100.0
T2 GEAC)	*aequatoriana*	6.0	84.2 ± 2.3	3.9	70.0	100.0
	fastigiata	6.6	95.0 ± 2.9	4.5	90.0	100.0
	hypogaea	6.6	88.8 ± 2.3	3.8	80.0	100.0
	hirsuta	6.6	85.0 ± 2.9	3.8	80.0	90.0
	peruviana	6.9	95.0 ± 2.9	4.0	90.0	100.0

T3 (GEAC)	*aequatoriana*	6.0	94.2 ± 1.5ab	3.2	90.0	100.0
	fastigiata	6.6	97.5 ± 2.5b	4.0	90.0	100.0
	hypogaea	6.6	92.5 ± 2.5a	3.4	80.0	100.0
	hirsuta	6.6	87.5 ± 2.5ab	3.2	80.0	90.0
	peruviana	6.9	92.5 ± 2.5ab	3.8	90.0	100.0
T4 (GEAC)	*aequatoriana*	6.0	82.5 ± 2.5	3.6 ab	70.0	90.0
	fastigiata	6.6	77.5 ± 2.5	4.5 a	70.0	80.0
	hypogaea	6.6	83.8 ± 2.6	3.5 b	70.0	90.0
	hirsuta	6.6	80.0 ± 4.1	3.5 b	70.0	90.0
	peruviana	6.9	85.0 ± 2.9	3.5 b	80.0	90.0

Different letters indicate significant difference ($p \leq 0.05$).

Effect of cryopreservation on the length of roots of whole seeds and embryonic axes

According to the statistical analysis of the data from the experiment (n = 320 whole seeds and n = 320 embryonic axes), a significant effect was observed in the four treatments used for both whole seeds and embryonic axes. For the length of the shoots in whole seeds T2, var. *peruviana* had the most extended length with 11.8 mm. For T2 embryonic axes, stem length between var. *hypogaea* with 15.5 mm. For whole seeds T4, var. *peruviana* reached the highest value for stem length with 18.9 mm. For T4 embryonic axes, var. *hypogaea* and var. *peruviana* obtained the highest values of stem length with 13.0 mm and 12.7 mm, respectively (Figure 5 and Appendices 3, 4).

Appendix 3: Morphological variability in complete seeds with two quantitative characters: aerial 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 $p \leq 0.05$ as determined by the Tukey test.

* Different letters indicate significant difference ($p \leq 0.05$).

Plant material	Variety	Length of aerial part (mm)				Root length (mm)			
		CV	Min	Max	Mean±SE	CV	Min	Max	Mean±SE
T1 (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
	hypogaea	41.40	10.95	44.32	20.03±0.93 b	50.73	10.53	104.79	39.42±2.24 b
	hirsuta	35.49	16.56	52.72	30.82±10.94 c	53.03	12.76	82.47	38.74±20.55 b
	peruviana	36.78	6.92	34.34	17.10±6.29 b	55.19	11.62	86.63	32.95±18.18 b
T2 (GS1)	*aequatoriana*	52.23	1.95	15.43	7.04±0.34 ab	81.29	1.09	26.36	5.99±0.44ab
	fastigiata	59.81	2.70	27.05	10.72±6.41 bc	80.64	2.93	27.82	8.63±6.96 b
	hypogaea	110.54	2.16	79.83	11.40±1.41 c	102.70	1.45	60.96	14.59±1.68 c
	hirsuta	43.07	1.63	6.50	3.18±1.37 a	42.94	1.14	4.18	2.07±0.89 a
	peruviana	70.63	2.88	46.20	11.85±8.37 c	73.23	2.07	34.11	7.72±5.65 b
T3 (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
	hypogaea	29.67	5.73	26.04	13.86±0.46 a	77.74	3.08	64.96	17.86±1.55 ab
	hirsuta	72.63	3.79	53.66	19.56±14.21 bc	84.79	2.07	57.21	14.59±12.37 a
	peruviana	29.41	9.91	37.76	20.45±6.01 c	57.90	7.75	99.78	41.05±23.77 c
T4 (GS3)	*aequatoriana*	50.69	2.09	16.84	5.62±0.26 ab	85.73	1.11	23.24	3.33±0.26 a
	fastigiata	114.56	2.04	47.59	8.87±10.16 b	111.81	1.62	27.78	6.54±7.31 a
	hypogaea	88.30	1.84	38.22	12.98±1.28 c	102.99	1.40	61.65	16.57±1.91 b
	hirsuta	19.78	1.18	3.09	2.15±0.42 a	29.54	1.10	4.40	1.77±0.52 a
	peruviana	41.70	6.87	40.78	18.86±7.87 d	64.50	4.12	62.09	22.91±14.77 c

Appendix 4: Morphological variability in embryonic axes with two quantitative characters: aerial 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: (T1) Untreated Control; (T2) Direct immersion in LN; (T3) Desiccation and (T4) Desiccation and immersion in LN. In each column, values followed by the same letter are not significantly different at $p \leq 0.05$ as determined by the Tukey test.

* Different letters indicate significant difference ($p \leq 0.05$).

Plant material	Variety	Length of aerial part (mm)				Root length (mm)			
		CV	Min	Max	Mean±SE	CV	Min	Max	Mean±SE
T1 (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
	hypogaea	26.77	5.57	18.33	11.39±034 a	35.14	3.51	14.43	7.21±0.28 a
	hirsuta	30.77	5.23	19.01	10.11±3.11 a	36.07	3.98	18.02	8.35±3.01 a
	peruviana	15.60	7.66	17.78	14.27±2.23 b	31.23	7.99	27.66	14.22±4.44 b
T2 (GEAC)	*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
	hypogaea	31.15	9.24	32.66	15.49±0.54 b	65.62	5.03	43.79	12.41±0.91 c
	hirsuta	17.93	9.59	23.09	14.62±2.62 b	26.81	6.06	21.40	13.73±3.68 c
	peruviana	61.16	3.32	19.69	7.43±4.54 a	55.84	2.03	15.80	5.55±3.10 a
T3 (GEAC)	*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
	hypogaea	22.07	9.16	29.75	17.26±0.43 b	35.40	6.73	36.47	16.95±0.67 b
	hirsuta	19.24	7.74	17.33	12.36±2.38 a	33.38	5.03	17.11	9.24±3.08 a
	peruviana	22.09	4.12	20.68	13.81±3.05 a	40.96	4.83	26.79	14.04±5.75 b
T4 (GEAC)	*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
	hypogaea	31.26	5.49	25.55	12.97±0.45 c	46.28	4.01	31.49	9.86±0.51 b
	hirsuta	49.66	3.14	22.97	9.29±4.62 b	34.23	2.53	7.97	4.59±1.57 a
	peruviana	33.00	3.80	22.58	12.67±4.18 c	40.14	2.69	18.61	9.48±3.81 b

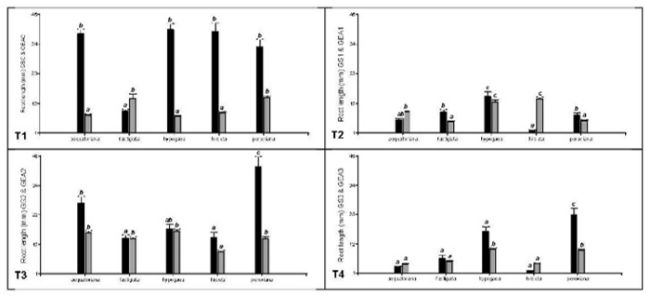

Figure 4: Root length (mm) (mean value + standard error) in whole seeds and embryonic axes in 10-day seedlings. Five varieties of the Ecuadorian collection of *A. hypogaea* were, subjected to the following pretreatments: (T1) Untreated control; (T2) Direct immersion in LN; (T3) Desiccation and, (T4) Desiccation and immersion in LN. The black bar is data from whole peanut seeds (GS), and the grey bar is data from embryonic axes (GEA).

Effect of cryopreservation on the length of shoots obtained from whole seeds and embryonic axes

According to the statistical analysis of the data from the experiment (n = 320 whole seeds and n = 320 embryonic axes), a significant effect was observed in the four treatments used for both whole seeds and embryonic axes. For the length of the shoots in whole seeds T2, var. *peruviana* had the most extended length with 11.8 mm. For T2 embryonic axes, stem length between var. *hypogaea* with 15.5 mm. For whole seeds T4, var. *peruviana* reached the highest value for stem length with 18.9 mm. For T4 embryonic axes, var. *hypogaea* and var. *peruviana* obtained the highest values of stem length with 13.0 mm and 12.7 mm, respectively (Figure 5 and Appendices 3, 4).

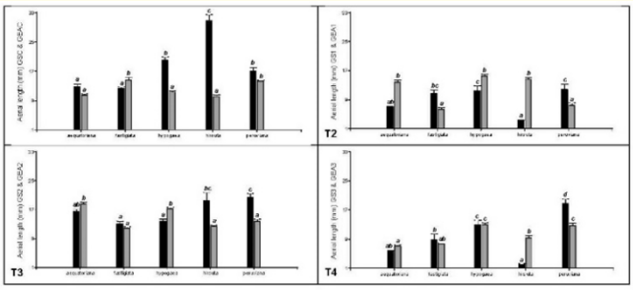

Figure 5: Aerial length (mm) (mean value + standard error), from whole seeds and embryonic axes in 10-day seedlings. Five varieties of the Ecuadorian collection of *A. hypogaea* were, subjected to the following pretreatments: (T1) Untreated control; (T2) Direct immersion in LN; (T3) Desiccation and, (T4) Desiccation and immersion in LN.

Appendix 5: Percentages of germination in complete seeds (mean value + standard error) at 10 days of germination tests, T_{50}, minimum and maximum values of the *fastigiata* and *hypogaea* subspecies, submitted to the following pre-treatments: (T1) Untreated control; (T2) Direct immersion in LN and (T3) Desiccation, (T4) Desiccation and immersion in LN. In each column, values followed by the same letter are not significantly different at $p \leq 0.05$ as determined by the Tukey test.

Plant material	Subspecie	Water content (%)	Germination (%) day 10 Mean±SE	T_{50} days	Min	Max
T1 (GSC)	*fastigiata*	6.5	92.50±2.16	2.5 a	70.0	100.0
	hypogaea	6.6	92.50±2.18	3.3 b	80.0	100.0
T2 (GS1)	*fastigiata*	6.5	76.00±3.11	3.5	60.0	100.0
	hypogaea	6.6	80.00±1.74	4.0	70.0	90.0
T3 (GS2)	*fastigiata*	6.5	96.00±1.52 a	3.2	80.0	100.0
	hypogaea	6.6	90.83±1.49 b	3.7	80.0	100.0
T4 (GS3)	*fastigiata*	6.5	78.50±2.44	3.8	60.0	100.0
	hypogaea	6.6	78.33±2.07	3.8	70.0	90.0

Appendix 6: Percentages of elongation of embryonic axes (mean value + standard error) 10 days after starting the elongation tests, T_{50}, minimum and maximum values in the subespecies *fastigiata* and *hypogaea*, submitted to the following pre-treatments: (T1) Untreated control; (T2) Direct immersion in LN and; (T3) Desiccation, (T4) Desiccation and immersion in LN. In each column, values followed by the same letter are not significantly different at $p \leq 0.05$ as determined by the Tukey test.

Plant material	Subspecies	Water Content (%)	Germination (%)			
			day 10 Mean±SE	T_{50} days	Min	Max
T1 (GEAC)	*fastigiata*	6.5	98.00±1.17	3.5	80.0	100.0
	hypogaea	6.6	96.67±1.88	3.8	80.0	100.0
T2 (GEAC)	*fastigiata*	6.5	88.50±1.96	3.8	70.0	100.0
	hypogaea	6.6	87.50±1.79	4.0	80.0	100.0
T3 (GEAC)	*fastigiata*	6.5	94.50±1.14	3.3	90.0	100.0
	hypogaea	6.6	90.83±1.93	3.5	80.0	100.0
T4 (GEAC)	*fastigiata*	6.5	82.00±1.72	3.5	70.0	90.0
	hypogaea	6.6	82.50±2.18	3.8	70.0	90.0

The black bar is data from whole peanut seeds (GS), and the grey bar is data from embryonic axes (GEA). Different letters in the bars indicate significant differences (Tukey, $p \leq 0.05$).

Cryopreservation and its effect on germination

Effect of cryopreservation at the subspecies level

In the present study, for whole seeds, only statistical differences were observed between the *fastigiata* and *hypogaea* subspecies in the GS2 of the evaluated treatments. For embryonic axes, no statistical differences were observed in the treatments evaluated for the two subspecies, results similar to those obtained in seeds of leguminous species [27]. Different growth habit of the two subspecies did not influence the germination percentages, the subspecies *hypogaea* presents creeping or decumbent growth habit, [28,29] while *fastigiata* erect growth habit [30].

The whole seeds of the subspecies *fastigiata* and *hypogaea* presented an average germination between treatments of 86% and 85% respectively. The germination of the embryonic axes of the *fastigiata* and *hypogaea* subspecies presented germination between 91% and 89% respectively. The moisture content for whole seeds embryonic axes for the two subspecies was 6%. The lowest germination percentages for whole seeds and embryonic axes are the GS1 (LN) and GS3 (dried and LN) treatments for both subsp. *fastigiata* and subsp. *fastigiata*, in agreement with the results obtained by Tacán., *et al*. [22], where they average germination percentages of 80%.

Effect of cryopreservation on the germination of the five botanical varieties

The best germplasm conservation protocol, after cryopreservation, should be the one that allows the full expression of the germinative potential of the seed sample under study [31]. As can be seen in the results, all the whole seed sprouts and embryonic axes developed roots and aerial part; these results are like Tacán., *et al*. [21] and Gagliardi., *et al*. [8,20], who obtained the best results using peanut embryonic axes.

Factors such as the origin, quality and integrity of the seeds can affect their germination capacity [32,33]. In this study, the five botanical varieties of peanuts come from seven provinces of Ecuador with different adaptation characteristics or oil content and with limited intravarietal genetic diversity [34]. However, the results indicate that the origin and genetic diversity of the peanut varieties do not influence the cryopreservation processes used.

The whole seeds and the embryonic axes of the *Arachis* varieties showed high elongation ten days after sowing, with explants without the presence of calluses, like what was observed by Ishikawa., *et al*. [35] and Kuranuki., *et al*. [36].

The whole seeds of the peanut varieties presented germination and average seed moisture content between treatments of 86% and 6%, respectively, and the embryonic axes presented an average elongation of 90% with a seed moisture content of 6%. A similar study in peanuts presented percentage germination of whole seeds (79%), embryonic axes (92%), and a moisture content of 7% [21]. The lowest percentages of germination and elongation occurred in the treatments with immersion in LN and desiccation plus LN, both for whole seed (GS1 and GS3) and for embryonic axes (GEA1 and GEA3), in agreement with the results obtained. by Tacan., *et al*. [22], Radhamani., *et al*. [37] and Chaudhuri., *et al*. [9], were they average germination percentages of 80% and elongation of 82%. The oleaginous seed of *Jatropha curcas* L. presented similar percentages for cryopreservation in whole seeds and embryonic axes with a moisture content of 6% and after exposure to liquid nitrogen (LN), the germination percentages were 48 % (whole seeds) and 57% (embryonic axes) [31].

Almeida., *et al*. (2010) indicate that germination losses were not recorded for cryopreserved peanut seeds, but losses were recorded with these seeds in a natural environment. Similarly, Araujo., *et al*. (2015) indicate that the germination of cryopreserved peanuts is low because the seeds are kept with low moisture content (5%).

In the case of embryonic axes, this study agrees with the results obtained by Gagliardi., *et al*. [20], where they indicate that peanut embryonic axes dried for 1 hour (at a moisture content of 18%) and submerged in LN for 24 hours produced 80% sprouts. In wild species of *Arachis* from sections *Arachis*, *Triseminatae* and *Erectoid*, the regenerative response of embryonic axes was like this study and ranged from 70% to 100% [38].

In relation to moisture content, the percentages obtained could be attributed to adequate dehydration of the seeds and embryonic axes (water content 6.5%), which did not allow vitrification,

ensuring high survival [20]. Pritchard (1995) indicates that for peanuts the moisture content ranges from 6.7% to 7.4%, which agrees with the moisture content of this study. Reducing water content of tissues to a critical level seems to be a necessary requirement for successful cryopreservation of any type of material [41,42].

A characteristic of intermediate seeds of tropical origin, such as peanuts, is the fact that the longevity of dry seeds (moisture content from 7% to 10% is reduced with the drop in storage temperature below 10 ^{0}C, such as coffee (*Coffea* spp), papaya (*Carica papaya* L.) and African oil palm crops (*Elaeis guineensis* Jacq.) [43-46]. Cryopreservation of embryonic axes with a moisture content of (7% to 10%), may have a better chance of surviving cryopreservation in liquid nitrogen than in the case of recalcitrant whole seeds such as *Citrus aurantiifolia (*Christm.) Swingle, *C. halimii* B.C. Stone [47], *Coffea arabica* L. [48], *Corylus avellana* L. [49,50] and *Elaeis guineensis* [51,52].

Cryopreservation and its effect on root length and shoot length of whole seeds and embryonic axes

The minimal differences found in the length of the root of whole seeds and embryonic axes between the varieties could be due to the differences between the peanut varieties for morphological characteristics such as: the size of the leaflet, the height of the plant and the periods emergence and fruit maturation [53], variables used for the identification of botanical varieties of *A. hypogaea* [54].

Recovery after freezing was influenced by the treatments for whole seeds and embryonic axes and not by the culture medium. This confirms other results that indicate that the culture medium does not influence regeneration but is related to the characteristics of the genetic resource used, that is, it depends on the type of seed: orthodox, sub-orthodox (intermediate) and recalcitrant [8,55-57]. Cryopreservation of whole seeds and embryonic axes of varieties of *A. hypogaea* have the tolerance to osmotic stress necessary for successful cryopreservation.

Conclusion

All the peanut varieties germinated and showed excellent results with the GS1 and GS3 treatments for whole seed and GEA1 and GEA3 in embryonic axes, considering that none of the treatments had the presence of callus and, therefore, reduces the risk of somaclonal variation.

The similarity of behavior to the treatments of the single variety of *A. hypogaea* subsp. *hypogaea* and the four varieties of *A. hypogaea* subsp. *fastigiata*, without losing the morphological characteristics, both of whole seeds and embryonic axes, has shown that cryopreservation is a good tool for the long-term conservation of this species.

The effect of each treatment on the survival of whole seeds and embryonic axes of subsp. *fastigiata* and *hypogaea*, constitutes an important starting point to continue with the implementation of cryopreservation and to continue applying methodologies to maintain or increase the survival percentages achieved after NL and seek to promote their regeneration.

The variables aerial and root length for whole seeds and embryonic axes presented significant variations between treatments and varieties. These differences indicate that this protocol could be used successfully in other intermediate species.

The vitrification technique for the cryopreservation of whole seeds and embryonic axes showed promise, since it allowed obtaining high percentages of germination and regeneration, using silica gel and NL, which will allow long-term conservation in the Gene Bank of INIAP for the simplicity and practicality of the methodology for the cryopreservation of sub-orthodox seeds.

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., Á.M.-A. 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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Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Bibliography

1. Knauft D., *et al*. "Principles of Cultivar Development: Crop Species". In Agronomy Books; Fehr, W., Ed.; Macmillan Publishing Company, New York, USA, 2 (1987): 346-385.

2. Halward TM., *et al*. "Genetic variation detectable with molecular markers among unadapted germ-plasm resources of cultivated peanut and related wild species". *Genome* 34.6 (1991): 1013-1020.

3. Krapovickas A and Vanni RO. "El Maní de Llullaillaco". *Bonplandia* 18.1 (2009): 51.

4. Mendoza ZH., *et al*. "El Maní. Tecnología de manejo y usos. Instituto Nacional de Investiga ciones Agropecuarias (INIAP)". *Quito. Bol. Div. No.* 315 (2005): 32.

5. Chávez-Servia JL., *et al*. Fundamentos Genéticos y Socioeconómicos para Analizar la Agrobiodiversidad en la Región de Ucayali, 16 de enero de 2003, Pucallpa, Perú. 1st ed., Publisher: Bioversity International (2006): 93.

6. IUCN. Guidelines for Using the IUCN Red List Categories and Criteria. IUCN, Gland, Switzerland (2010): 5.

7. E Pennisi. *Science* 329 (2010): 1274.

8. Gagliardi R., *et al*. "Cryopreservation of Arachis species by vitrification of in vitro-grown shoot apices and genetic stability of recovered plants". *Cryo-Letters* 24.2 (2003): 103-110.

9. Chaudhury R and Chandel K. "Studies on germination and cryopreservation of Cardamom (Elettaria cardamomum Maton) seeds". *Seed Science and Technology* 23.1 (1995): 235-240.

10. Hong TD and Ellis RH. "The survival of germinating orthodox seeds after desiccation and hermetic storage". *Journal of Experimental Botany* 43.2 (1992): 239-247.

11. Marin M., *et al*. "Cryopreservation of cassava zygotic embryos and whole seeds in liquid nitrogen". *Cryo-Letters* 11 (1990): 257-264.

12. Pritchard HW., *et al*. "The effect of moisture content on the low temperature responses of Araucaria hunsteinii seed and embryos". *Annals of Botany* 76.1 (1995): 79-88.

13. Pence VC., *et al*. "Cryobiotechnologies: Tools for expanding long-term ex situ conservation to all plant species". *Biological Conservation* 250 (2020): 108736.

14. Reed BM., *et al*. "Stratification is necessary for successful cryopreservation of axes from stored hazelnut seed". *Cryo-Letters* 15.6 (1994): 377-384.

15. Stushnoff C. "Cryopreservation of Fruit Crop Genetic Resources— Implications for Maintenance and Diversity during Conservation". *HortScience* 26.5 (1991): 518-522.

16. Runthala P., *et al*. "Cryopreservation of groundnut (*Arachis hypogaea* L.) embryonic axes for germplasm conservation". *Cryo-Letters* 14.6 (1993): 337-340.

17. Abdulmalik M M., *et al*. "Influence of Desiccation Time on Survival and Regeneration of Embryonic Axes of Groundnut (*Arachis hypogaea* L.) Immersed in Liquid Nitrogen". *American Journal of Plant Sciences* 4 (2013): 1725-1730.

18. Abdulmalik M., *et al*. "Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by vitrification". *African Journal of Biotechnology* 13.2 (2014): 280-285.

19. Ozudogru EA., *et al*. "Development of a cryopreservation procedure for peanut (*Arachis hypogaea* L.) embryonic axes and its application to local Turkish germplasm". *Advances in Horticultural Science* 23.1 (2009): 41-48.

20. Gagliardi R F., *et al*. "Cryopreservation of cultivated and wild Arachis species embryonic axes using desiccation and vitrification methods". *Cryo-Letters* 23 (2002): 61-68.

21. Tacán M., *et al*. "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* 5 (2017): e3203v1.

22. Thammasiri K. "Cryopreservation of embryonic axes of jackfruit". *Cryo-Letters* 20 (1999): 21-28.

23. Monteros-Altamirano Á., *et al*. "Guía para el manejo de los recursos fitogenéticos en Ecuador. Protocolos". INIAP; Estación Experimental Santa Catalina. Departamento Nacional de Recursos Fitogenéticos; Mejía; Ecuador. Publicación Miscelánea 432 (2018): 1-104.

24. Sobrevilla J., *et al*. Evaluación de diferentes tratamientos pregerminativos y osmóticos en la germinación de semillas Prosopis laevigata (Humb. & Bonpl. ex Willd.) M.C. Johnston. Estudios Científicos En El Estado de Hidalgo y Zonas Aledañas (2013): 12.

25. Murashige T., *et al*. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures". *Physiologia Plantarum* 15.3 (1962): 473-497.

26. Di Rienzo JA., *et al*. "InfoStat, versión 2008". Manual del Usuario. Grupo InfoStat, FCA, Universidad Nacional de Córdoba. 1st Ed., Editorial Brujas Argentina (2008): 335.

27. CARDOSO F., *et al*. "Efecto de la crioconservación sobre la germinación de semillas de leguminosas. Campiña Grande". *Revista Brasileira de Produtos Agroindustriais* 2 (2000): 67-71.

28. Krapovickas A and Gregory W.C. "Taxonomía del género Arachis (Leguminosae)". *Bonplandia* 8 (1994): 1-186

29. Harch BD., *et al*. "The analysis of large scale data taken from the world groundnut (*Arachis hypogaea* L.) germplasm collection. II. Two-way data with mixed data types". *Euphytica* 105 (1999): 73-82.

30. Ferguson, M., *et al*. "Gene diversity among botanical varieties in peanut (*Arachis hypogaea* L.)". *Crop Science* 44.5 (2004): 1847-1854.

31. Salomão AN., *et al*. "Methods to assess the viability of cryopreserved Jatropha curcas L. seed germplasm". *Revista Brasileira de Plantas Medicinais* 18.2 (2016): 391-398.

32. Brenha J., *et al*. "Teste de tetrazólio em sementes de pinhão manso". *Visão Acad* 13 (2012): 63-79.

33. Loureiro MB., *et al*. "Caracterização morfoanatomica e fisiológica de sementes e plântulas de Jatropha curcas L. (Euphorbiaceae)". *Rev. Árvore* 37.6 (2013): 1093-1101.

34. Kochert G., *et al*. "RFLP variability in peanut (*Arachis hypogaea* L.) cultivars and wild species". *Theoretical and Applied Genetics* 81.5 (1991): 565-570.

35. Ishikawa K., *et al*. "Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (Bletilla striata) by vitrification". *Plant Cell Reports* 16 (1997): 754-757.

36. Kuranuki Y and Sakai A. *Cryo-Letters* 16 (1995): 345-352.

37. Radhamani J and Chandel KPS. "Cryopreservation of embryonic axes of trifoliate orange (Poncirus trifoliata [L.] RAF.)". *Plant Cell Report* 11.7 (1992): 372-374.

38. Almeida FAC., *et al*. "Estudo de técnicas para o armazenamento de cinco oleaginosas em condições ambientais e criogênicas". *Revista Brasileira de Produtos Agroindustriais* 12 (2010): 189-202.

39. Dumet D., *et al*. "Cryopreservation of oil palm (Elaeis guineensis Jacq) somatic embryos involving a desiccation step". *Plant Cell Reports* 12 (1993): 352-355.

40. Dumet D., *et al*. "Replacement of cold aclimatization with high socrose pretreatment in black currant cryopreservation". En: Cryopreservation of tropical plant germplasm. Current Research Progress and Aplications. F. Engelmann, H. Takagi (eds.). Japan International Center for Agricultural Sciences, Tsukuba, Japan/ International Plant Genetic Resources Institute, Rome, Italy. pp: 385-387.

41. Roos EE. "Physiological, biochemical, and genetic changes in seed quality during storage". *HortScience* 15.6 (1980): 19-22.

42. Vázquez-Yanes C and Aréchiga MR. "Ex situ conservation of tropical rain forest seed: Problems and perspectives." *Interciencia* 21.5 (1978): 293-298.

43. Ellis R H., *et al*. "An intermediate category of seed storage behaviour? II. Effects of provenance, immaturity, and imbibition on desiccation tolerance in coffee". *Journal of Experimental Botany* 42 (1991a): 653-657.

44. Ellis R H., *et al*. "Effect of storage temperature and moisture on the germination of papaya seeds". *Seed Science Research* 1 (1991b): 69-72.

45. Ellis R H., *et al*. "Seed storage behaviour in *Elaeis guineensis*". *Seed Science Research* 1.2 (1991c): 99-104.

46. Hong T D., *et al*. "Optimum air-dry seed storage environments for arabica coffee". *Seed Science and Technology* 20 (1991): 547-560.

47. Normah M N and Serimala M NSD. "Cryopreservation of seeds and embryonic axes of several Citrus species". In: Ellis, R.H.; Black, M.; Murdoch, A.J.; Hong, T.D., eds. Basic and applied aspects of seed biology. Proceedings of the fifth international workshop on seeds; 1995; [Place of meeting unknown]. Dordrecht, The Netherlands: Kluwer Academic Publishers (1995): 817-823.

48. Abdelnour A., *et al*. "Cryopreservation of zygotic embryos of *Coffea* spp". *Cryo-Letters* 13 (1992): 297-302.

49. González-Benito ME and Pérez C. "Cryopreservation of embryonic axes of two cultivars of hazelnut (Corylus avellana L.)". *Cryo-Letters* 15 (1994): 41-46.

50. González-Benito ME and Perez-Ruiz, C. "Cryopreservation of Quercus faginea embryonic axes". *Cryobiology* 29 (1992): 685-690.

51. Engelmann F., *et al*. "Cryopreservation of zygotic embryos and kernels of oil palm (Elaeis guineensis Jacq.)". *Seed Science Research* 5 (1995a): 81-86.

52. Engelmann F., *et al*. "Factors affecting the cryopreservation of coffee, coconut and oil palm embryos". *Plant Genetic Resources Newsletter* 103 (1995b): 27-31.

53. Grabiele M., *et al*. "Genetic and geographic origin of domesticated peanut as evidenced by 5S rDNA and chloroplast DNA sequences". *Plant Systematics and Evolution* 298.6 (2012): 1151-1165.

54. Chiyembekeza AJ., *et al*. "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.

55. Chetverikova EP. "Role of abscisic acid in frost tolerance of plants and cryopreservation of cultured tissues". *Russian Journal of Plant Physiology* 46.5 (1999): 721-727.

56. Hitmi A., *et al*. "Cryopreservation of Chrysanthemum cinerariaefolium shoot tips". *Journal of Plant Physiology* 156.3 (2000): 408-412.

57. Na HY and Kondo, K. "Cryopreservation of tissue-cultured shoot primordia from shoot apices of cultured protocorms in Vanda pumila following ABA preculture and desiccation". *Plant Science* 118.2 (1996): 195-201.