Effects of accelerated ageing and cryopreservation on seeds and embryonic axes of *Phaseolus vulgaris* L and *Arachis hypogaea* L. Germination and seedlings vigor

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The effects of artificial aging and cryopreservation methods on the germination of seeds and embryonic axes of *Phaseolus vulgaris* and *Arachis hypogaea* , were studied. Aging and cryopreservation treatments in bean seeds not affect the germination percentage, reaching values not significantly different from control. Germination percentage was higher when isolated embryonic axes were studied. Aging treatments gave rise to larger bean seedlings than the controls and cryopreservation treatments affected the size depending on the explant used. In groundnut seeds, aging treatments and cryopreservation positively affected germination and vigor of seedlings, but embryonic axes from not rehydrated aging seeds not germinate. Undried groundnut seeds not survive cryopreservation, while the dried ones showed a germination behavior superior to the control. Embryonic axes tolerate immersion in liquid nitrogen but when droplet-vitrification was used, formed callus instead of seedlings. Electrolyte leakage was always higher in embryonic axes than in seeds, both in beans and groundnut, and not to have a significant effect, under the conditions studied, on germination and vigor of seedlings. The present study evidences the possibility of cryopreserving, by simple procedures, germplasm of beans and groundnut, and to verify the aging tolerance of the seeds and embryonic axes of these important species.

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Abstract: The effects of artificial aging and cryopreservation methods on the germination of seeds and embryonic axes of *Phaseolus vulgaris* and *Arachis hypogaea*, were studied. Aging and cryopreservation treatments in bean seeds not affect the germination percentage, reaching values not significantly different from control. Germination percentage was higher when isolated embryonic axes were studied. Aging treatments gave rise to larger bean seedlings than the controls and cryopreservation treatments affected the size depending on the explant used. In groundnut seeds, aging treatments and cryopreservation positively affected germination and vigor of seedlings, but embryonic axes from not rehydrated aging seeds not germinate. Undried groundnut seeds not survive cryopreservation, while the dried ones showed a germination behavior superior to the control. Embryonic axes tolerate immersion in liquid nitrogen but when droplet-vitrification was used, formed callus instead of seedlings. Electrolyte leakage was always higher in embryonic axes than in seeds, both in beans and groundnut, and not to have a significant effect, under the

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Keywords: Leguminoseae, *Phaseolus vulgaris*, *Arachis hypogaea*, artificial aging, cryopreservation, germination, seedlings vigor, electrolyte leakage.

INTRODUCTION

Legumes are critically important in agriculture as they are the main contributors to human and animal nutrition and the maintenance of soil fertility. The protein content in the seeds of grain legumes can be up to three times higher than in cereal grains and are a major supplier to human nutritional protein requirements (Gepts et al., 2005).

Common bean (*Phaseolus vulgaris* L.) is the most important legume in human nutrition. Annually, about 12 million metric tons of common beans are produced. Latin America is the largest producer, with 5.5 million metric tons (FAOSTAT 2014). Groundnut (*Arachis hypogaea* L.) is also an important crop throughout the world. It is grown in more than 100 countries, of which China (37.6%), India (14.9%) and Nigeria (7.7%) are the largest producers. World production is about 43.9 million metric tons (FAOSTAT, 2014).

Conservation of germplasm banks is essential for all crops, including *P. vulgaris* to reduce the significant genetic erosion suffered and to have adequate genetic resources for improvement (Cejas et al., 2012). *P. vulgaris* seeds have an orthodox storage behavior (Walters et al., 2004) and are

tolerant to desiccation and conservation in LN, being an excellent model for studies of tolerance to different *ex situ* conservation conditions (Cejas et al., 2012, 2013, Stanwood 1985).

Groundnut seeds are conventionally stored in gene banks, however, because of their high lipid content and thin seminal covers, they not tolerate storage conditions for such long periods as true orthodox seeds. As consequence, groundnut is now considered a suborthodox species (Vázquez-Yanes and Arechiga, 1996; Gagliardi et al., 2000). There are studies that show that viability of groundnut seeds loses viability after two years of storage, in practically all lines evaluated (Abdulmalik et al., 2014). Therefore, the long-term conservation of *A. hypogaea* seeds by conventional techniques is not a viable option. There are previous studies on cryopreservation of groundnut embryonic axes using the vitrification technique (Abdulmalik et al., 2014, Gaglardi et al., 2002, Ozudogru et al., 2009) and by air drying in a laminar flow cabinet (Abdulmalik et al., 2013).

Cryopreservation is recommended not only for the long-term storage of unorthodox seed species, but also for orthodox seeds by lowering costs and increasing safety (Engelmann & Rao, 2012; Harding, 2004). Pritchard (1995) suggested that the longevity of the seeds conserved at -196 ° C could be, approximately, 175 times greater than those stored at -18 ° C, i.e. the temperature used for seed storage in the base collections.

A better understanding of the mechanisms of aging and physiological deterioration of seeds during storage is essential to save large amounts of time, efforts and material in germplasm banks. Studies to investigate the metabolic deterioration during seeds aging were done, mainly, with techniques of artificial aging (McDonald, 1999), under conditions of high temperature and humidity. Aging seeds tolerance varies between the families and the species and physiological mechanisms on which it depends are not fully established. Deterioration of seeds during aging is usually associated

with loss of membrane integrity (Priestley 1986) and, as a consequence, electrolyte leakage, which increases the electrical conductivity of seed leaching (Ratzaczak and Pukacka, 2005).

The present work aims to contribute to a better understanding of aspects related to the long - term conservation of germplasm, such as seed aging and cryopreservation tolerance, of two important legumes, with orthodox (common bean) and sub - orthodox (groundnut) storage behavior by comparing the results. At the same time, simple, safe and economical alternatives for the cryopreservation of both species are evidenced.

MATERIAL AND METHODS

Plant material

Seeds of *P. vulgaris* cv Yunguilla and *A. hypogaea* cv Caramelo were provided by the National Department of Plant Genetic Reources (INIAP, Ecuador) germplasm bank.

Seeds were randomly selected from seed lots stored at 5^{o}C until utilization. Seeds were introduced 5 min in 70% alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 to 3 drops of tween 20 and rinsed twice with sterile distilled water. Thereafter, seeds were soaked in sterile distilled water for 3 h.

Embryonic axes were aseptically excised from surface sterilized seeds.

Treatments

Bean seeds (BS), groundnut seeds (GS), bean embryonic axes (BEA) and groundnut embryonic axes (GEA) were subjected to the following treatments, prior to germination and seedling vigor tests:

Control (C): Untreated

Treatment 1: 45 ° C for 72 hours and rehydration 24 h in sterile water

Treatment 2: 45 ° C for 96 hours and rehydration 24 h in sterile water

Treatment 3: 45 ° C for 120 hours and rehydration 24 h in sterile water

Treatment 4: 45 ° C for 72 hours without rehydration

Treatment 5: 45 ° C for 96 hours without rehydration

Treatment 6: 45 ° C for 120 hours without rehydration

Treatment 7: Direct immersion in LN

Treatment 8: Desiccation and immersion in LN

Treatment 9 (only for embryonic axes): Droplet-vitrification and immersion in LN

For each treatment, 8 replicates of 10 seeds or embryonic axes were made.

Germination Test and Seedling Growth

Seeds germination assays were performed by placing eight replicates of 10 seeds in 9-cm Petri dishes on top of two sheets of filter paper with 3.5 ml distilled water in the dark at 25ºC. The criterion for germination was radicle protrusion, and it was quantified every two days for up to 10 days. Results are expressed as the germination percentages (%) and as the number of days required to reach 50% of final germination (T50). The length of the radicle(mm) and the length of shoots (mm) was also determined.

The embryonic axes were *in vitro* cultured in sterile conditions with MS medium (Murashige & Skoog, 1962) supplemented with 0.3 M sucrose. The germination temperature was 25 ° C with a 16:8 h light/dark photoperiod with illumination from cool white fluorescent light (40 µmol m^{-2} s^{-}

[1]). Eight replicates of 10 embryonic axes per culture vessel were studied, every two days until 10 days, and the percentage of germinated embryos, the T50, the length of the radicles (mm) and the shoots (mm) were measured.

Seeds desiccation

For the desiccation of seeds, they were placed in petri dishes (9 cm in diameter) containing a layer of dehydrated silica gel covered by a filter paper disc on which the seeds were placed. Ten seeds and about 5 g of silica gel were placed in each petri dish, which, once sealed, were maintained for 72 hours at 25 ° C.

For moisture content determination of seeds and embryonic axes of each treatment a device Messtechnik Schaller GF-12 for measuring humidity was used.

Criopreservation

Control and desiccated seeds were introduced into cryovials and submerged directly into liquid nitrogen. The cryovials were removed from the LN after 1 h and rapidly warmed in a water bath at 40°C, and placed under the germination conditions indicated above.

Droplet-vitrification and cryopreservation of embryonic axes.

After dissecting the embryonic axes under sterile conditions, were placed for 24 hours on solid MS base culture media supplemented with 20 g/l sucrose. They were then placed in petri dishes containing the loading solution (0.4 M sucrose + 2 M glycerol), at room temperature and for 30 minutes. Then, embryonic axes were treated with 2 ml of PVS2 vitrification solution (Sakai et al., 1990) containing 30% glycerol + 15% ethylene glycol + 15% DMSO in culture medium with 0.4 M sucrose, at room temperature for 30 minutes. On sheets of aluminum foil, of approximately 20

x 7 mm, 15 µl drops of the PVS2 were placed and, then, the embryonic axes were introduced inside the drops. The sheets of aluminum foil were rapidly immersed, with the samples, directly into the liquid nitrogen. For thawing, the sheets were taken out of liquid nitrogen and dipped directly into liquid MS culture medium supplemented with 1.2 M sucrose at room temperature for 15 minutes. The washed embryonic axes were surface dried with filter paper and then transferred to the germination culture medium and maintained in continuous darkness.

Electrolyte Leakage

Electrolyte leakage was determined by placing four replicates of 5 seeds/embryonic axes (FW were recorded) into 50 ml of bi-distilled water at 25ºC and measuring the conductivity of the medium with a conductivity-meter (Accumt AB 30 Basis, Fisher Scientific) after 24 h. Results are expressed as µS g^{-1} FW. The seeds were then boiled for one hour in a water bath, then the conductivity was measured at room temperature. The percentage of electrolyte loss was calculated with the formula:

Electrolyte leakage (%) = (Initial conductivity - H_2O conductivity) X 100 / (Final conductivity - H_2O conductivity)

Data Analysis

Data were treated by analysis of variance (ANOVA), which was performed using Statgraphics Centurion XV statistical software (Stat Point Technologies, Warrenton,Virginia, USA) and InfoStat version 2008 (Di Rienzo et al., 2008). The LSD Fisher test was also used for comparing means ($\alpha = 0.05$). In the tables, different letters after the values within the same column express significant difference. The values

of germination percentages were arc-sine transformed.

RESULTS:

In common beans, both in seeds and embryonic axes, the moisture content never exceeded 8.1% and the drying treatments employed lowered the water content, up to 7.6% (Tables 1 and 2). In groundnuts (Tables 3 and 4), the initial moisture percentages were even lower, not exceeding 5%. After the desiccation treatments, it decreased to a maximum of 4.2%.

When the effect of different treatments on the germination of *P. vulgaris* seeds was analyzed (Table 1), it was observed that, at 10 day of germination assay, the percentages of germination of all seeds, from all treatments, was greater than 80% and no significant differences from control were detected.

Regarding cryopreservation of *P. vulgaris* seeds, it was found that it is not necessary to desiccate the seeds before immersion in liquid nitrogen. Both non-dried and dried seeds survive cryopreservation and reach germination percentages not significantly different from control (Table 1).

The effect of the different treatments on the in vitro germination of isolated embryonic axes of bean is shown in Table 2. It can be observed that the percentages of germination reached are, in general, higher than those of the complete seeds, a trend that can be observed already two days after germination begins. Aging treatments not negatively affect the final percentage of germination.

The cryopreservation of the embryonic axes of *P. vulgaris* was successful in two of the three treatments tested, and even the cryopreserved embryonic axes began to germinate before the controls and those undergoing aging treatments (Table 2). Common bean embryonic axes cryopreserved with the droplet vitrification method not survive LN treatment.

When the same studies were carried out with complete seeds of *A. hypogaea* (Table 3), it was also possible to verify that the aging treatments not negatively affect the final percentage of germination, and in 2, 4, 5 and 6 treatments values higher than the control were obtained.

It is noteworthy that not-desiccated groundnut seeds not survive immersion in liquid nitrogen (Table 3) and were not able to germinate. On the other hand, when they were dried before the immersion in LN they showed germination rates similar to those of the control, and even lower values of T50, and the rest of treatments.

The effect of the different treatments on the in vitro germination of isolated groundnut embryonic axes is shown in Table 4. It can be observed that, as in the case of the groundnut embryonic axes, the percentages of germination reached are higher to those of the complete seeds, although they initiate later the germination. The aging treatments evidences that in the cases where the embryonic axes were not rehydrated after the periods at 45 ° C, they did not survive. On the contrary, those that were rehydrated gave rise to germination values not significantly different from control.

Cryopreservation of groundnut embryonic axes was successful with non-crioprotected and silica gel desiccated embryos (Table 4). It should be noted that the embryonic axes that had been subjected to the cryoprotective method of droplet vitrification, survive cryopreservation, but, instead of germinating, they gave rise to dedifferentiated tissues (callus) (Fig. 1).

When the growth of shoots and radicles of seedlings from the germination of bean seeds from the different treatments was quantified at ten days of initiates the tests of germination (Table 1), it was possible to observe that all the treatments of aging (except treatment 3) caused that the shoots had longer lengths than those obtained from control seeds. This is not the case with the shoots form seed that had been cryopreserved without desiccation, which showed values similar to control. In

contrast, the dried and cryopreserved seeds gave rise to seedlings with stems with an average length much higher than the control. The radicles had, in all the cases (except treatment 2), sizes remarkably higher than the control (Table 1).

The same studies developed with isolated embryonic axes of bean (Table 2) gave results similar to those obtained from seeds. The major difference was that the shoots from embryonic axes germinated in vitro were clearly longer. The radicles were always of smaller length than those formed in seedlings, but larger or equal than those of their control.

Shoots from groundnut seeds subjected to artificial aging treatments and from seeds dried before cryopreservation in LN (Table 3) showed significantly longer lengths than control. The radicles of the seedlings from seeds that survive the previous treatment, were also of greater size (except in treatment 3) than those of the control. With respect to shoots from in vitro germinated embryonic axes of groundnut (Table 4), all those surviving previous treatments gave rise to shoots of smaller sizes than the control (except in treatment 3) and radicles smaller than the control in treatments 3, 7 and 8 but significantly longer in 1 and 3 treatments. Radicles of plantlets form embryonic axes were shorter than those of seedlings from seeds. It should be remembered that the embryonic axes cryopreserved by the drop-vitrification method, resulted in growth of callus and not of differentiated organs (Fig 1).

Electrolyte leakage (Figs. 2 and 3) was studied in treatments C, 3, 7 and 8, applied to the four types of materials BS, GS, BEA and GEA, and treatment 9 applied to BEA and GEA.

It should be noted that, under study conditions, the electrolyte leakage was always greater in the embryonic axes than in the whole seeds, regardless of the treatment to which they were subjected.

If we consider media conductivity values in $mScm^{-1}g^{-1}$ (Fig. 2), in *P. vulgaris* there are no significant differences between control and treated seeds, both with temperature and with LN. The embryonic axes that have been cryopreserved have lower values than their control and those that come from the treatment of aging. In *A. hypogaea*, control seeds have lower electrolyte leakage than those pretreated. In the case of the embryonic axes, greater electrolyte leakage was detected in those submitted to artificial aging and smaller in the cryopreserved without previous treatment, with respect to its control and to the other treatments.

When the percentage of electrolyte leakage is considered (Fig. 3), greater percentages were observed in the isolated embryonic axes than in their respective seeds especially in common bean. It is also verified that the percentages of electrolyte leakage were higher in groundnut seeds than in beans. In bean seeds, the aging treatment causes a clear increase in the percentage of electrolyte leakage, which is not the case in the isolated embryonic axes. In these, cryopreservation without pretreatment, causes a lower percentage of electrolyte leakage. In groundnut seeds, it is again the aging treatment that causes higher values followed by desiccation-cryopreservation. In groundnut embryonic axes, the treatment of droplet-vitrification seems to cause the highest percentage of electrolyte leakage and cryopreservation without cryoprotective treatment, the lowest.

DISCUSSION:

The deterioration and loss of seed viability is a common process even when are stored under, theoretically, optimal conditions (Khan et al, 2013). The vigor of seeds gradually diminishes with the storage period, due to the aging, which leads to a slower and less uniform germination. Aging of seeds affects orthodox and recalcitrant species (Murthy et al., 2003).

Studies to investigate metabolic deterioration during aging of seeds are usually developed under artificial aging treatments (McDonald, 1999), with conditions of high temperature and humidity. Seeds subjected to these storage conditions usually lose their viability (Khan et al, 2013). Preliminary studies on the effect of artificial aging on seeds suggested a decrease in vigor of seedlings with the passage of the aging period (Rodo and Marcos-Filho, 2003, Ghani and Golpayegani, 2011, Khan et al. In the present study, aging treatments not negatively affect the germination or vigor of the bean and groundnut seedlings from the germination of seeds or isolated embryonic axes. Only the embryonic axes of the groundnut lost its viability after artificial aging treatments that not apply rehydration treatments before inoculation in the culture medium.

Previous studies on cryopreservation of *P. vulgaris* seeds (Cejas et al, 2012) indicate that the roots suffered the greatest damage due to cryopreservation and during the thawing stage they describe the rupture of the testa and the detachment of the cotyledons. In our study, we not observed any of these negative effects. In contrast, the radicles from seedling from cryopreserved seeds were longer than the controls. In the case of seedlings from cryopreserved embryonic axes, radicles were of shorter length than when they came from seeds, but no smaller than controls. This fact could be explained because they developed through the solid culture medium.

The results reported in our study, are not in coincidence with a growing number of reports in several species which indicate that embryonic axes recovering from cryopreservation often fail to develop normally (e.g. Berjak et al., 2011; Normah et al., 2011; Wesley-Smith et al., 2014). Sershen et al. (2016), consider that dehydration does not necessarily affect the viability of the explants and that they are the processes of cooling and subsequent thawing, which leads to losses of viability (Sershen et al., 2012). This may explain why the recovery of seedlings after cryopreservation of embryos and embryonic axes that survive without anomalies to partial

dehydration is very low, between 0-30% (Ballesteros et al., 2014) and / or with a high incidence of abnormal seedlings, i.e. no roots, no stems or only callus (Sershen et al., 2007). In our studies, moisture contents were low even in control seeds and embryonic axes before each treatment (7.9-8.1% in beans and 5% in peanuts). After the different treatments, the moisture content decreased slightly (7.6-7.9% in beans and 4.2-4.6 in peanuts). These low moisture contents may explain why such high germination rates and excellent vigor of the seedlings are obtained.

The increase in electrolyte leakage (increase in conductivity in leachates) has been correlated with the decrease of germination and vigor of seedlings. Tajbakhsh, (2000) mentions that high electrolyte leakage is related to the aging and the deterioration of the seeds. These electrolytes are mainly potassium, phosphates, sugars and amino acids and are lost as result of damage to the cell membrane.

We observed greater percentages of electrolyte leakage in the isolated embryonic axes than in their respective seeds. It is also verified that the percentages of electrolyte leakage were higher in groundnut seeds than in beans. In bean seeds, the aging treatment causes a significant increase in the percentage of electrolyte leakage, which is not the case in the isolated embryonic axes. In these, cryopreservation without pretreatment, causes a significantly lower percentage of electrolyte leakage. In groundnut seeds, it is again the aging treatment that causes higher values followed by desiccation-cryopreservation. In groundnut embryonic axes, the treatment of droplet-vitrification seems to cause the highest percentage of electrolyte leakage and cryopreservation without cryoprotective treatment, the lowest.

The testa provides a physical and chemical barrier to protect seed to resist environmental attacks (Arc et al., 2011). In legume species, a variable effect of fractures and microfractures of seed coat has been found (Cardoso et al., 2000). The presence of the intact coat would avoid leakage of

283 solutes from the seed (Arc et al., 2011). Magnitskiy and Plaza (2007) state that solute leakage is
284 directly related to the thickness and integrity of the seed coat, which is consistent with the greater
285 losses shown by groundnut seeds compared to those of beans, and by the embryonic axes with
286 respect to the seeds in both species, in the present study.

287 In our study, we could see that there was no direct relationship between loss of electrolytes and
288 the germination capacity of seeds and embryonic axes. Our results coincide with the opinion of
289 Sershen et al. (2016), who consider that electrolyte leakage does not necessarily predict loss of
290 viability. It should also be noted that the conclusions obtained for some species using electrolyte
291 leakage data may be erroneous if not interpreted in terms of morphology and anatomy of the
292 explants (Varghese & Naithani 2002).

293 In a previous work on cryopreservation of groudnut embryonic axes, Abdulmalik et al. (2014)
294 describe that the excised embryonic axes were dehydrated in laminar flow chamber, for different
295 periods of time, before being introduced into LN, where they were held for 1 h. The samples were
296 then thawed in a water bath at 40 °C for 2 min, then the explants were cultured for recovery in an
297 MS medium supplemented with 15 mg / l BAP. The highest survival (96.67% - 100%) and shoot
298 formation (91.67% - 96.67%) were obtained with an average moisture content of 17%, obtained
299 after 4-5 hours of drying. They found that no-desiccated embryonic axes not survive the storage
300 in liquid nitrogen. Similarly, the embryonic axes dried for 1 hour not survive cryogenic treatment
301 either (Abdulmalik et al, 2014). This result corroborates those of Gagliardi (2002), who observed
302 that the embryonic axes of non-dehydrated *Arachis* species did not survive when they were
303 cryopreserved. Runthala et al. (1993) reported a protocol for the conservation of embryonic axes
304 using glycerol and programmed freezing, obtaining variable survival levels according to genotype
305 (40-90%).

Our results indicate that no pretreatment is required to the seeds or embryonic axes of beans to achieve high percentages of germination and normal seedlings. In the case of groundnut seeds, it is necessary a previous desiccation with silica gel to obtain results comparable to non-cryopreserved seeds. In the case of isolated embryonic axes, both the non-desiccated and the silica gel desiccated, perfectly tolerate cryopreservation. In contrast, the droplet-vitrification treatment used in our study, caused the embryonic axes to survive but form callus instead of seedlings.

The fact that the use of silica gel guarantees a more accurate and reproducible dehydration of the embryos, compared to dehydration in laminar flow chamber, has been pointed out by other authors (Sisunandar et al., 2010). The use of silica gel allows a better standardization of the method than when laminar flow chambers are used, where air flows of different intensity may be present (N'Nan et al, 2012).

Abdulmalik et al. (2014) indicate that it is necessary to optimize the exposure time to PVS2 to obtain a high percentage of shoot formation after groundnut embryonic axes vitrification. In their study, they were able to obtain survival and shoot formation from dehydrated cryopreserved embryonic axes with PVS2 for 2 h. Other authors also observed very high survival rates in cryopreserved peanut embryonic axes after 2 h vitrification in PVS2 (Gaglardi et al., 2002; Ozudogru et al., 2009). In our study, the treatment of vitrification with the PVS2 solution was not only advantageous to simpler methods, but to induce callus formation in the explants during the recovery phase.

The methodology reported here is simple, efficient and cost effective and can therefore be applied on a routine basis for long term storage and management of seed germplasm of *P. vulgaris* and *A. hypogaea* in cryogenic genebanks.

CONCLUSIONS:

- Aging and cryopreservation treatments in bean seeds not affect the germination percentage.
- Germination percentage was higher when isolated bean embryonic axes were studied. In groundnut seeds, aging treatments and cryopreservation positively affected germination and vigor of seedlings.
- Undried groundnut seeds not survive cryopreservation, while the dried ones showed a germination behavior superior to the control.
- Embryonic axes tolerate immersion in liquid nitrogen but when droplet-vitrification was used, formed callus instead of seedlings.
- Electrolyte leakage was always higher in embryonic axes than in seeds, both in beans and groundnut, and not to have a significant effect, under the conditions studied, on germination and vigor of seedlings.
- The present study evidences the possibility of cryopreserving, by simple procedures, germplasm of beans and groundnut, and to verify the aging tolerance of the seeds and embryonic axes of these important species.

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Table 1(on next page)

Table 1

Table 1.- Percentages of germination (mean value + standard error) at 2,4,6,8 and 10 days of initiation of germinatión tests and T50 in *P. vulgaris* seeds (BS), submitted to the following pre-treatments: (C) Untreated Control; (1) 45 ° C for 72 hours and rehydration 24 h in sterile water; (2) 45 ° C for 96 hours and rehydration 24 h in sterile water; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (4) 45 ° C for 72 hours without rehydration; (5) 45 ° C for 96 hours without rehydration; (6) 45 ° C for 120 hours without rehydration; (7) Direct immersion in LN and (8) Desiccation and immersion in LN. Shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In each column, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the LSD Fisher test.

PLANT MATERIAL	Water content (%)	Germination (%) day 2 Mean±SE	Germination (%) day 4 Mean±SE	Germination (%) day 6 Mean±SE	Germination (%) day 8 Mean±SE	Germination (%) day 10 Mean±SE	T 50 (days) Mean±SE	Shoot length (mm) day 10 Mean±SE	Root length (mm) day 10Mean±SE
BSC	8.1	26±15.2a	47.1±11.1ab	67.5±23.1cd	78.8±27bc	85±20ab	4	7.9±2.9ef	63.1±17.6de
BS1	7.9	0	40±19.3bc	90±9.3ab	92.5±7.1ab	92.5±7.1ab	5	12.8±4.8bc	94.8±26.4a
BS2	7.9	10±0.1a	20±12.3c	77.5±14.9bc	81.3±12.5abc	83.8±14.1ab	6	11.1±5.8cd	54.6±28.1e
BS3	8.0	0	63.8±15.1a	93.8±7.4a	93.8±7.4a	93.8±7.4a	4	9.8±5.6de	72.2±28.5bc
BS4	7.7	11.3±0.2a	51.3±22.3ab	87.5±8.9ab	87.5±8.9abc	87.5±8.9ab	5	13.5±5.6b	93.4±26.1a
BS5	7.6	0	37.5±16.7bc	81.3±15.5abc	83.8±13abc	83.8±13ab	5	17.1±12.1a	89.2±26.8a
BS6	7.7	10±0.1a	50±20.7ab	92.5±7.1a	92.5±7.1ab	92.5±7.1ab	5	12.9±7.1bc	69.9±27.9cd
BS7	8.1	12.5±0.4a	22.5±10.4c	55±9.3d	76.3±11.9c	80±10.7b	6	7.2±7f	72.2±33.1bc
BS8	7.8	11.3±0.2a	61.2±20.3a	87.5±10.4ab	90±7.6abc	90±7.6ab	4	16.7±7.3a	79.7±19.6b

Table 2(on next page)

Table 2

Table 2.- Percentages of germination (mean value + standard error) at 2,4,6,8 and 10 days of initiation of germinatión tests and T50 in *P. vulgaris* embryonic axes (BEA), submitted to the following pre-treatments: (C) Untreated Control; (1) 45 ° C for 72 hours and rehydration 24 h in sterile water; (2) 45 ° C for 96 hours and rehydration 24 h in sterile water; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (4) 45 ° C for 72 hours without rehydration; (5) 45 ° C for 96 hours without rehydration; (6) 45 ° C for 120 hours without rehydration; (7) Direct immersion in LN; (8) Desiccation and immersion in LN and (9) Droplet-vitrification and immersion in LN. Shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In each row, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the least significant difference test.CF= callus formation.

PLANT MATERIAL	Water content (%)	Germination (%) day 2 Mean±SE	Germination (%) day 4 Mean±SE	Germination (%) day 6 Mean±SE	Germination (%) day 8 Mean±SE	Germination (%) day 10 Mean±SE	T 50 (days) Mean±SE	Shoot length (mm) day 10 Mean±SE	Root length (mm) day 10 Mean±SE
BEAC	8.1	40±7.6d	75±7.6c	90±9.3a	92.5±7a	92.5±7a	3	31.8±14.4de	23.9±12.7d
BEA1	7.9	40±0.1d	82.5±10.3bc	90±7.6a	90±7.6a	90±7.6a	3	34.7±15.1cd	27.3±17bc
BEA2	7.9	70±11.9c	87.5±8.8ab	90±9.2a	90±9.2a	90±9.2a	2	38.9±16.7b	30.5±12.8ab
BEA3	8.0	72.5±7bc	87.5±7ab	95±7.6a	97.5±4.7a	97.5±4.7a	2	46.4±14.2a	31.9±12.7a
BEA4	7.7	66.3±11.8c	75±10.7c	92.5±10.3a	92.5±10.3a	92.5±10.3a	2	31.5±5de	30.8±3.9a
BEA5	7.6	63.8±17.6c	77.5±7c	92.5±8.8a	92.5±8.8a	92.5±8.8a	2	26.6±9.5f	24.4±7.4cd
BEA6	7.6	67.5±13.8c	87.5±10.3ab	92.5±7a	92.5±7a	92.5±7a	2	29.1±6.8ef	23.1±3.5d
BEA7	8.1	92.5±7.1a	95±7.6a	95±7.6a	95±7.6a	95±7.6a	2	36.8±2.7bc	22.8±3.5d
BEA8	7.8	85±5.3ab	87.5±4.6ab	95±5.3a	97.5±4.6a	97.5±4.6a	2	25.7±3.6f	22.6±2.1d
BEA9	CF	CF	CF	CF	CF	CF	CF	CF	CF

Table 3(on next page)

Table 3

Table 3.- Table 1.- Percentages of germination (mean value + standard error) at 2,4,6,8 and 10 days of initiation of germinatión tests and T50 in *A. hypogaea* seeds (GS), submitted to the following pre-treatments: (C) Untreated Control; (1) 45 ° C for 72 hours and rehydration 24 h in sterile water; (2) 45 ° C for 96 hours and rehydration 24 h in sterile water; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (4) 45 ° C for 72 hours without rehydration; (5) 45 ° C for 96 hours without rehydration; (6) 45 ° C for 120 hours without rehydration; (7) Direct immersion in LN and (8) Desiccation and immersion in LN. Shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In each column, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the LSD Fisher test

PLANT MATERIAL	Water content (%)	Germination (%) day 2 Mean±SE	Germination (%) day 4 Mean±SE	Germination (%) day 6 Mean±SE	Germination (%) day 8 Mean±SE	Germination (%) day 10 Mean±SE	T 50 (days) Mean±SE	Shoot length (mm) day 10 Mean±SE	Root length (mm) day 10Mean±SE
GSC	5.0	15±5.4b	40±28.8d	53.8±21.3b	65±16.9b	65±16.9b	5	7,6±4,8d	13,1± 9,2f
GS1	4.6	0	40±11.9d	72.5±18.3ab	77.5±14.9ab	77.5±14.9ab	3	13,2± 9,9c	19,6±16,5de
GS2	4.5	25±13.8ab	60±16.9cd	77.5±12.8a	85±5.3a	85±5.3a	6	17,5±5,6ab	29,4±17,9ab
GS3	4.3	35±9.2a	70±9.2abc	73.8±5.1a	75±5.3ab	75±5.3ab	4	15,9±11,4bc	16,8±10,6ef
GS4	4.2	30±11.6ab	71.3±18.8abc	82.5±10.3a	82.5±10.3a	82.5±10.3a	3	15,3± 7,6bc	23,8±11,6cd
GS5	4.3	35±18.5a	81.3±15.5a	82.5±12.8a	82.5±12.8a	82.5±12.8a	4	20,1±13,3 a	32,5±14a
GS6	4.4	23.8±11.9ab	78.8±12.4ab	82.5±11.6a	82.5±11.6a	82.5±11.6a	4	15,7± 4,1bc	27,7± 3,7bc
GS7	5.0	0	0	0	0	0	0	0	0
GS8	5.0	35±12.9a	65±10.7bc	80±10.7a	80±10.7ab	80±10.7ab	3	14,9± 3,6bc	24,4± 5,4c

Table 4(on next page)

Table 4

Table 4.- Percentages of germination (mean value + standard error) at 2,4,6,8 and 10 days of initiation of germinatión tests and T50 in P. vulgaris embryonic axes (BEA), submitted to the following pre-treatments: (C) Untreated Control; (1) 45 ° C for 72 hours and rehydration 24 h in sterile water; (2) 45 ° C for 96 hours and rehydration 24 h in sterile water; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (4) 45 ° C for 72 hours without rehydration; (5) 45 ° C for 96 hours without rehydration; (6) 45 ° C for 120 hours without rehydration; (7) Direct immersion in LN; (8) Desiccation and immersion in LN and (9) Droplet-vitrification and immersion in LN. Shoots and roots length (mean value + standard error) in 10-day seedlings is also shown. In each row, values followed by the same letter are not significantly different at p≤ 0,05 as determined by the least significant difference test.CF= callus formation

PLANT MATERIAL	Water content (%)	Germination (%) day 2 Mean±SE	Germination (%) day 4 Mean±SE	Germination (%) day 6 Mean±SE	Germination (%) day 8 Mean±SE	Germination (%) day 10 Mean±SE	T 50 (days) Mean±SE	Shoot length (mm) day 10 Mean±SE	Root length (mm) day 10Mean±SE
GEAC	5.0	0	77.5±17.5ab	90±9.2ab	90±9.2ab	90±9.2ab	4	16,7± 3,9a	9,4± 2,2c
GEA1	4.6	0	85±9.2a	92.5±7ab	92.5±7ab	92.5±7ab	4	14,7± 3,8c	11,8± 1,8b
GEA2	4.5	0	87.5±4.6a	88.8±3.5b	88.8±3.5b	88.8±3.5b	4	15,4± 2,6bc	15± 2,4a
GEA3	4.3	0	85±11.9a	92.5±7.1ab	92.5±7.1ab	92.5±9.1ab	4	17,6±10,2a	8,3± 3,5d
GEA4	4.2	0	0	0	0	0	0	0	0
GEA5	4.4	0	0	0	0	0	0	0	0
GEA6	4.2	0	0	0	0	0	0	0	0
GEA7	5.0	0	80±9.2ab	95±5.3a	95±5.3a	95±5.3a	6	15,3± 2,4bc	7,6± 4d
GEA8	5.0	15±7	62±21.2b	92±7.1ab	95±5.3a	95±5.3a	4	13,9± 3,4c	7,5± 3,4d
GEA9	0	0	0	CF	CF	CF	CF	CF	CF

Figure 1(on next page)

Fig.- 1

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Fig.1. Groundnut embryonic axes development 10 days after cryopreservation in LN whit silicagel desiccation method (Left) and droplet vitrification method (Right). The photographs are originals of the authors.

Figure 2(on next page)

Fig. 2

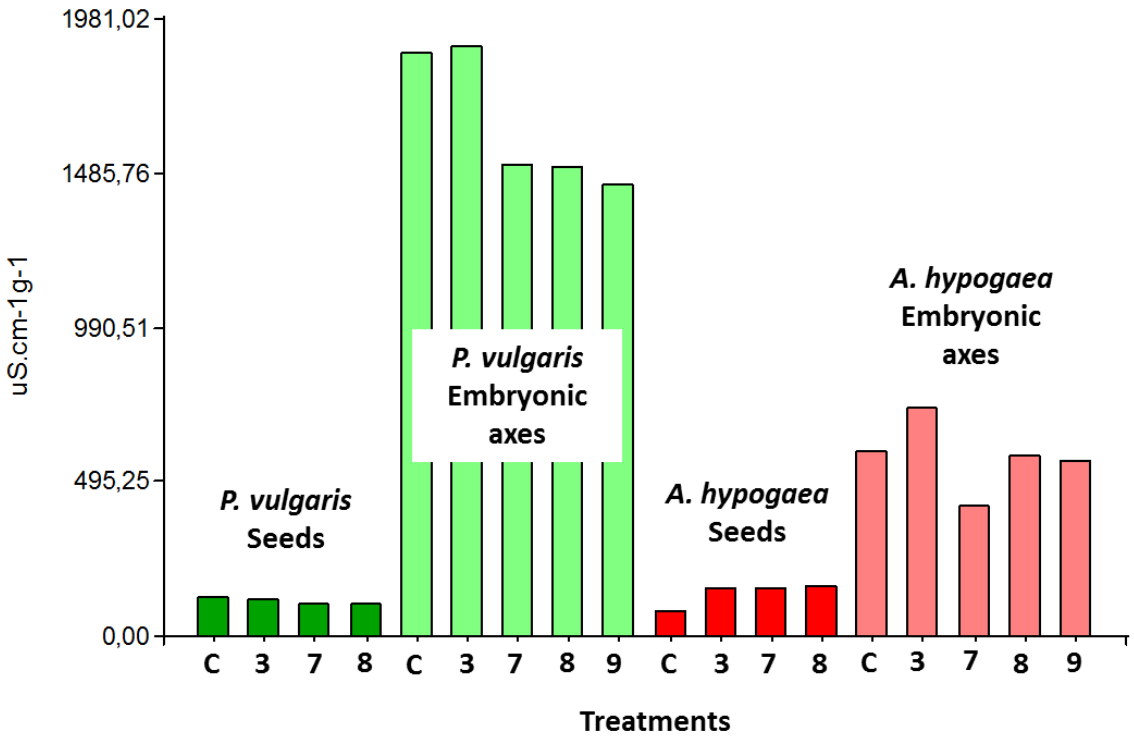

Fig.2.- Electrolyte leakage determined by placing four replicates of 5 seeds/embryonic axes (FW were recorded) into 50 ml of bi-distilled water at 25ºC and measuring the conductivity of the medium after 24 h. Results are expressed as μS g-1 FW and represent the means of four measurements. (C) Untreated Control; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (7) Direct immersion in LN; (8) Desiccation and immersion in LN and (9) Droplet-vitrification and immersion in LN.

Figure 3(on next page)

Fig. 3

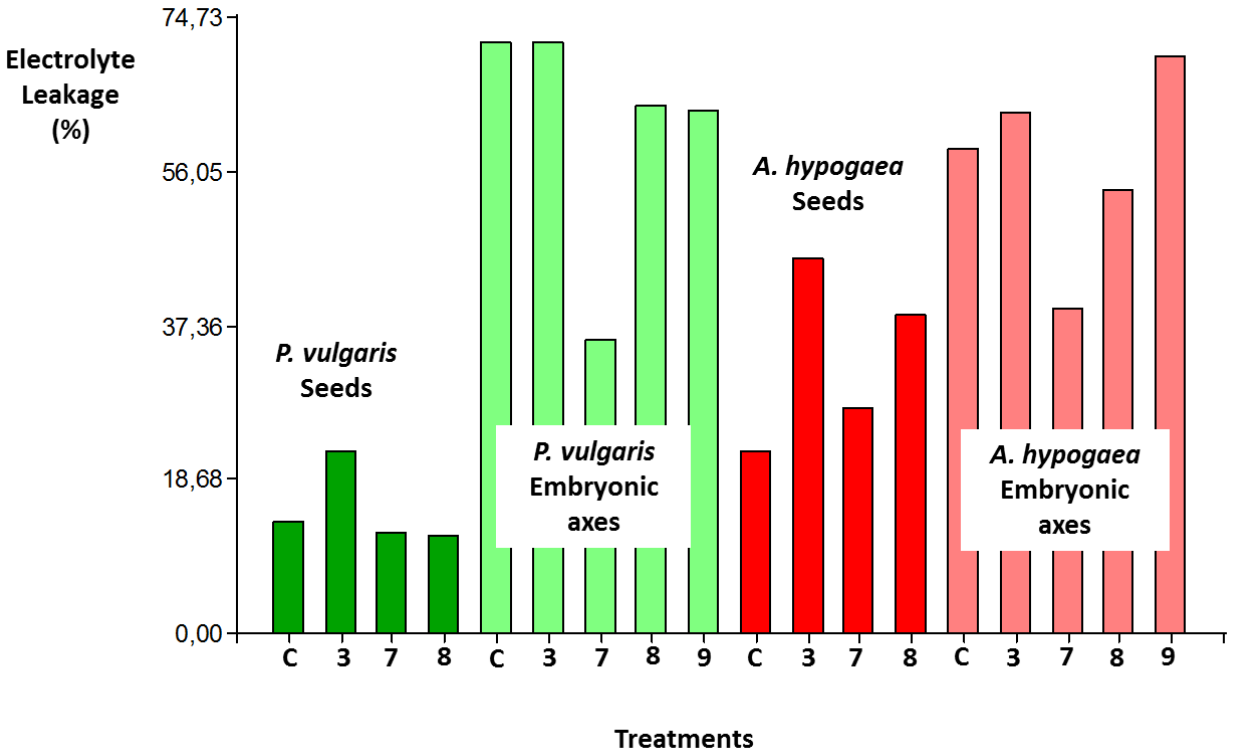

Fig. 3.- Electrolyte leakage determined by placing four replicates of 5 seeds/embryonic axes (FW were recorded) into 50 ml of bi-distilled water at 25ºC and measuring the conductivity of the medium. Results are expressed as µS g-1 FW represent the means of four measurements. The seeds were then boiled for one hour in a water bath, then the conductivity was measured at room temperature. The percentage of electrolyte loss was calculated: Electrolyte leakage (%) = (Initial conductivity - H2O conductivity) X 100 / (Final conductivity - H2O conductivity). (C) Untreated Control; (3) 45 ° C for 120 hours and rehydration 24 h in sterile water; (7) Direct immersion in LN; (8) Desiccation and immersion in LN and (9) Droplet-vitrification and immersion in LN.