



DESICCATION METHOD AND CRYOCONSERVATION AFFECTS AMINO ACIDS COMPOSITION OF EMBRYONIC AXES OF PHASEOLUS VULGARIS L AND ARACHIS HYPOGAEA L.

M. Tacán., C. Tapia and C. Pérez*

Abstract

The effect of seeds desiccation and cryopreservation on amino acids content in embryonic axes of two Leguminosae of great economic importance, *Phaseolus vulgaris* L and *Arachis hypogaea* L, was studied. Proline content in bean embryonic axes was higher, at 24 h of germination, in the control than in those from desiccated and cryopreserved seeds. There were, later, a proline decrease in the control and embryonic axes from desiccated seeds while increased in axes from cryopreserved seeds. In groundnut, at 24 h, in the control and in embryonic axes from cryopreserved seeds, proline content was lower than in those obtained from desiccated seeds. Absolute glutamate content in bean embryonic axes, was much higher when seeds were dried at 45 °C. In groundnut, the control showed the highest values with a clear increase in the content of glutamate, in all cases, at 72 h. The relative content of 16 amino acids, showed, in bean, a generalized decrease compared to control at 24 h, except asparagine that increased when the seeds were dried at 45°C. In groundnut, the results were more heterogeneous. The results suggest that some amino acids may have a role in adaptation to stressful conditions, especially in the sub-orthodox species.

Key Words: Leguminosae, *Phaseolus vulgaris*, *Arachis hypogaea*, desiccation, cryopreservation, proline, glutamate, amino acids relative contents.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume in human food. Approximately 12 million metric tons are produced annually. Groundnut (*Arachis hypogaea* L.) is also an important crop throughout the world. It is grown in more than 100 countries, with a global production of 43.9 million tonnes [13].

P. vulgaris seeds have an orthodox storage behavior [42]. In addition, they are tolerant to desiccation and immersion in LN [6, 7, 36]. Groundnut seeds, however, cannot tolerate standard storage conditions of seed banks for long periods as true seeds true seeds. As a consequence, there is consensus that groundnut seeds should be considered sub-orthodox [40, 15]. The viability of several peanut seed lines has been studied [1], and 75% of them lost viability after two years of storage.

There are several mechanisms that plants use to adapt to changes in temperature. Thus, they can synthesize heat and cold shock proteins [21, 17, 32] and amino acids [9, 33, 27]. There is an extensive literature [41, 24, 23], on the accumulation, induced by low temperatures, of metabolites, particularly low molecular weight carbohydrates and amino acids, such as proline and glutamine [39, 11]. These compounds have effective protective roles in the freezing tolerance in plants and have, therefore, been frequently used as cryoprotective agents in the cryopreservation protocols of various types of explants [5]. However, few studies [12, 28, 16, 37] have been published on the changes occurring in the rest of the amino acids in relation to stressful situations.

Proline content undergoes significant fluctuations, in plant tissues, in response to different types of environmental stress [3], and accumulation increases the tolerance of plants to such stressful situations [2, 18] although it is not well known which is the mode of action. Water stress conditions also tend to increase the accumulation of other low molecular weight compounds [3]. The present study aims to contribute to a better understanding of the role of amino acids in relation to the long - term conservation of germplasm, such as seed desiccation and cryopreservation tolerance, of two important legumes, with orthodox (common bean) and sub - orthodox (groundnut) storage behavior by comparing the results.

MATERIAL AND METHODS

Plant material

Seeds of *P. vulgaris* cv Canela and *A. hypogaea* cv Valencia were provided by local commercial suppliers. Seeds were randomly selected from seed lots stored at 5°C until utilization. Seeds 5 min in 70% alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 to 3 drops of tween 20, rinsed thrice with sterile distilled water and immersed in 5% NaOCl plus 2 to 3 drops of tween 20 for 10 min with occasional stirring and rinsed twice with sterile distilled water. Thereafter, seeds were soaked in sterile distilled water for 3 h.

Treatments

Bean seeds (BS) and groundnut seeds (GS) were subjected to the following treatments, prior to germination and embryonic axes aseptically excision from surface sterilized seeds:

Control (C): Seeds without pretreatment.

Treatment 1: Seeds maintained in an oven at 42 ° C for 72 h.

Treatment 2: Seeds maintained on silica gel for 72 h.

Treatment 3: Seeds maintained on silica gel for 72 hours, cryopreserved in LN 24 h and thawed at room temperature for 1 h.

For each treatment, 2 replicates of 10 seeds were made.

Germination Test

Germination assays were done by placing the seeds in 9 cm Petri dishes on two sheets of filter paper (moistened with 3.5 ml of distilled water) at 25 °C, in the dark. The criterion for germination was the emergence of the radicle and was quantified every two days until day 10. The results are expressed as percentages of germination (%) and represent the mean of two replicates of 10 seeds per treatment.

Silica gel desiccation

For the silica gel 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 h at 25 °C.

Criopreservation

Silica gel desiccated seeds were introduced into cryovials and submerged into liquid nitrogen (LN). The cryovials were removed from the LN after 24 h and thawed at room temperature for 1 h and placed under the germination conditions indicated above.

Amino acids determination

Reagents and solvents: Most of the standard compounds used for this study were obtained from Sigma (St. Louis, USA). N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Supelco (Bellefonte, USA). The two stable isotope reference compounds [2H3]-proline, and [13C5]-glutamic acid were purchased from Campro (Veenendaal, The Netherlands).

Plant extraction: A total of 100 mg of frozen plant material was transferred into 2 mL screw-cap tubes filled with ceramic beads (MAGNALyser Green Beads), 1 ml of a chloroform:methanol:H₂O (20:60:20) mixture including the internal standards ([2H3]-proline, [13C5]-glutamic acid) was added, and the plant material crushed in a MAGNALyser Instrument (Roche, Mannheim, Germany) at 6,500 rpm for 1 min. The final concentration of each reference compound in the solvent mixture was 15 ng mL⁻¹. Next, the samples were centrifuged (10 min, 14,000 rpm), and 200 µL aliquots of the supernatant transferred to fresh tubes. Samples were taken to dryness in a speed-vac concentrator for subsequent derivatization. Derivatization: Prior to GC-MS analysis, samples were derivatized as given in the following: All acidic protons were derivatized by the addition of 30 µL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA+1% TMCS) for 1 h at room temperature (22 °C). All samples were analyzed in randomized order and the first sample was analyzed by GC-MS 30 min after the silylation reagents were added.

GC-MS: One µL of the derivatized sample was injected splitless by an CombiPal autosampler (CTC, Zwingen, Switzerland) into a BRUKER Daltonics 451 gas chromatograph equipped with a 30 m x 0.25 mm i.d. fused silica capillary column with a chemically bonded 0.25 µm DB 5-MS stationary phase (BRUKER Daltonics). The injector

temperature is set to 270 °C. After 1 min, the split is opened (1:100). During the first 1.10 min a pressure pulse (30 psi) supports sample application onto the column. The gas flow rate through the column is adjusted to 1 mL min⁻¹, the column temperature is held at 70 °C for 2 min, then increased by 20 °C min⁻¹ to 325 °C, and held there for 5 min. The column effluent is introduced into the ion source of a Scion-TQ triple quadrupole mass spectrometer, GC-QQQ-MS (BRUKER Daltonics). The transfer line and the ion source temperatures are maintained at 250 °C. Ions are generated by a 70 eV electron beam at an ionization current of 80 µA, and 30 spectra s⁻¹ are recorded in the mass range 50 to 600 m/z. The acceleration voltage was turned on after a solvent delay of 390 s. All data were processed by MS Workstation 8 (rev2) software (Bruker Daltonics, Bremen, Germany). Automatic peak detection and mass spectrum deconvolution was performed with a peak width set to 1.0 s. Peak areas were calculated using selected quantification masses for each metabolite and internal standard. Mass spectra of all detected compounds were compared with spectra in the NIST 11 mass spectral library and an in-house reference database.

RESULTS

Bean and groundnut seeds tolerated the desiccation and cryopreservation treatments, showing at 72 h of the germination assay, in all cases, a germination percentage higher than 10% (Fig. 1). In bean seeds, desiccation treatment with silica gel, seem to slightly delay the onset of germination. Desiccated cryopreserved seeds showed germination rates similar to control, at 24 h and 72 h, but the seeds of cryopreserved common beans showed at 72 h, significantly higher percentages of germination than control. As for groundnut seeds (Fig. 1), the behavior was different, so that the desiccation treatment with silica gel not delayed the onset of germination and even, at 72 h, the germination percentage was higher than in control seeds. On the other hand, high temperature desiccation delayed the onset of germination. Cryopreservation had a germination promoting effect in both species, which was most evident at 72 hours after initiation of germination tests.

In Table 1, the results of the analysis of the relative content in amino acids, respect the controls, in embryonic axes extracted from the seeds (24 h) or young seedlings (72 h), both control seeds and those subjected to the treatments, at 24 and 72 h of initiating germination conditions, are shown. The amino acid profile in the embryonic axes of *P. vulgaris* was very similar for 13 of the 16 amino acids analyzed (Table 1). Only glutamate, asparagine and tryptophan showed values higher than the control in any of the treatments. The rest (alanine, leucine, isoleucine, serine, proline, threonine, methionine, cysteine, phenylalanine, lysine, glutamine, histidine and tyrosine), were found in concentrations much lower than control in embryonic axes from all treatments. Even the embryonic axes from non-treated seeds, at 72 h showed a drastic reduction in the contents of the 13 amino acids mentioned. L-glutamate content was very similar in almost all treatments, at 24 and 72 h, and with values slightly lower or higher than control. Only the embryonic axes from seeds dried with silica gel showed contents, at 24 h, more than four times the control. Regarding asparagine, two results are worthy of note: the large increase, at 24 h, in axes from

Table 1 Relative content of amino acids (relative to controls at 24 h) in embryonic axes of *P. vulgaris* and *A. hypogaea* at 24 and 72 hours after initiation of germination conditions. Control: Seeds without pretreatment; T1: Seeds maintained in an oven at 42 ° C for 72 hours; T2: Seeds maintained on silica gel for 72 hours; T3: Seeds maintained on silica gel for 72 hours, cryopreserved in LN 24 hour and thawed at room temperature for 1 hour. For each treatment, 2 replicates of 10 seeds were made.

Amino acid	<i>P. vulgaris</i>								<i>A. hypogaea</i>							
	Control		T1		T2		T3		Control		T1		T2		T3	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
L-Alanine	1	0.49	0.14	0.13	0.09	0.02	0.65	0.06	1	0.32	11.74	1.59	30.51	1.12	3.22	6.15
L-Leucine	1	0.06	0.06	0.06	0	0.03	0.15	0.03	1	0.1	5.41	6.51	1.79	0.07	1.18	4.1
L-Isoleucine	1	0.13	0.07	0.06	0.01	0	0.16	0.05	1	1.5	81.42	157.3	62.51	13.67	4.09	6.06
L-Serine	1	0.05	0.07	0.06	0.01	0.15	0.61	0.14	1	1.43	8.64	7.06	43.37	3.32	1.24	1.42
L-Proline	1	0.49	0.44	0.23	0.09	0.05	0.16	0.54	1	6.14	15.87	35.54	23.28	7.19	1.03	4.15
L-Threonine	1	0.11	0.05	0.09	0.04	0.13	0.13	0.13	1	1.1	4.79	8.92	10.15	0.75	0.91	1.18
L-Methionine	1	0.05	0.03	0.05	0	0.04	0.06	0.13	1	1.97	3.5	2.45	13.06	1	0.53	0.38
L-Cysteine	1	0.11	0.06	0.1	0.02	0.06	0.07	0.09	1	0.29	2.66	8.59	7.08	0.20	0.96	1.3
L-Phenylalanine	1	0.09	0.07	0.05	0.08	0.08	0.21	0.06	1	1.15	5.16	8.54	16.59	0.31	2.3	5.15
L-Glutamate	1	1.12	0.97	1.54	4.36	0.96	1.93	1.19	1	1.32	0.45	0.71	0.4	0.77	0.06	0.25
L-Asparagine	1	1	5.61	0.03	0.17	0.05	0.69	5.7	1	0.45	125.03	58.97	69.94	1.78	21.89	2.42
L-Lysine	1	0.27	0.8	0.03	0	0	1.53	0.72	1	0	8.54	3.53	5.26	0	1.22	1.04
L-Glutamine	1	0.01	0.07	0.01	0.14	0.08	0.06	0.01	1	16.8	8.77	11.5	16.02	2.9	0.66	3.7
L-Histidine	1	0	0.08	0.06	0.22	0.11	0.22	0.04	1	1.66	27.15	4.14	52.61	2.38	1.58	2.98
L-Tyrosine	1	0.03	0.04	0.02	0.44	0.1	0.58	0.12	1	0.55	3.28	1.14	20.37	1.09	0.22	0.85
L-Tryptophane	1	0.87	1.83	0.33	0.06	1.18	0.86	0.45	1	0.82	5.16	12.55	39.45	3.34	1.15	2.68

Table 2 Absolute contents of amino acids (ng/g FW) in embryonic axes of *P. vulgaris* and *A. hypogaea* at 24 and 72 hours after initiation of germination conditions. Control: Seeds without pretreatment; T1: Seeds maintained in an oven at 42 ° C for 72 hours; T2: Seeds maintained on silica gel for 72 hours; T3: Seeds maintained on silica gel for 72 hours, cryopreserved in LN 24 hour and thawed at room temperature for 1 hour. For each treatment, 2 replicates of 10 seeds were made.

Amino acid	<i>P. vulgaris</i>								<i>A. hypogaea</i>							
	Control		T1		T2		T3		Control		T1		T2		T3	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
L-Proline	271.63	132.97	119.53	62.76	23.94	13.93	43.32	147.52	2.61	16.01	41.43	92.75	60.77	18.77	2.69	10.82
L-Glutamate	365.2	408.8	355.1	562.2	1588.5	351.5	705.1	435.0	878.7	1162.5	394.2	621.0	351.0	679.5	51.8	224.8

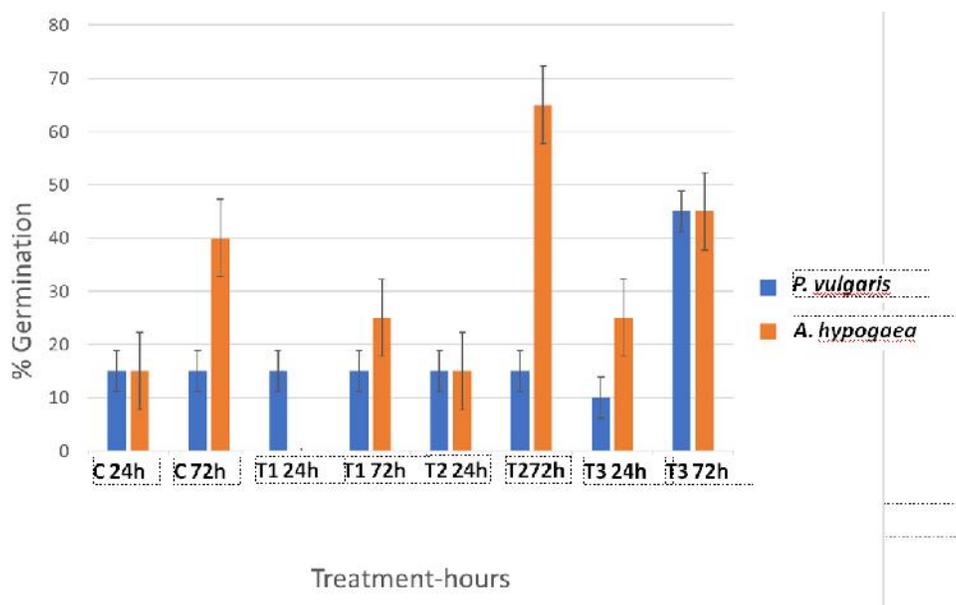


Figure 1. Percentage of germination (+SE) of *P. vulgaris* and *A. hypogaea* seeds at 24 and 72 hours after initiation of germination conditions. Control: Seeds without pretreatment; T1: Seeds maintained in an oven at 42 ° C for 72 hours; T2: Seeds maintained on silica gel for 72 hours; T3: Seeds maintained on silica gel for 72 hours, cryopreserved in LN 24 hour and thawed at room temperature for 1 hour. Seeds germination assays were performed by placing two replicates of 10 seeds in 9-cm Petri dishes on top of two sheets of filter paper (previously moistened with 3.5 ml distilled water, which was periodically added) in the dark at 25°C.

seeds subjected to 42 ° C (which then decreased to very low values at 72 h) and the increase, at 72 h on the axes of cryopreserved seeds. The tryptophan showed (24 h) slight increases with respect to the control after the two desiccation treatments. As shown in Table 1, the variations in the amino acid contents in the embryonic axes of *A. hypogaea*, was completely different to the case of bean. In groundnuts. Both desiccation methods (42 ° C for 72 h and silica gel) caused significant increases, at 24 h, in the relative content of all amino acids studied except glutamate. It is appreciated that at 72 h there is a marked decrease in all cases. In the embryonic axes from cryopreserved seeds, on the other hand, were detected significantly higher values than the control for L-alanine, L-Phenylalanine, L-isoleucine and L-asparagine. Note, also, the high increase of L-proline, L-isoleucine, L-alanine, L-asparagine and L-histidine attributable to desiccation treatments. The absolute contents of proline and glutamate in the control embryonic axes and those from all treatments were measured at 24 and 72 h (Table 2).

In *P. vulgaris*, the control embryonic axes showed at 24 h the highest proline content (271.63 ng / g FW). The content decreased, approximately, by half at 72 h. The embryonic axes of the seeds subjected to desiccation had smaller proline contents which also decreased at 72 h. In the case of the embryonic axes of cryopreserved seeds, the proline contents were lower than in the control, but a significant proline increase was detected at 72 h (147.52 ng / g FW) compared to 24 h (43.32 ng / g FW). In *A. hypogaea*, the content of proline in the embryonic axes showed, again, a very different behavior (Table 2). The content in proline was much higher in the desiccation treatments than in the control, being superior, except in the case of desiccation with silica gel, at 72 h than at 24 h. In the case of cryopreservation treatment, similar values to the control were detected. Comparing the two species (Table 2), the contents of proline were higher in common bean than in groundnut.

The absolute content of L-glutamate in the embryonic axes of bean showed in several of the treatments, important differences with respect to the control, 24 h (365.2 ng / g FW), as in the case of silica gel desiccation (1588.5 ng / g FW) or cryopreservation (705.1 ng / g FW). In general, except pretreatment at 42 ° C, absolute glutamate content fell at 72 h. In the case of groundnuts, the control has the highest values at 24 h (878.7 ng / g FW) and at 72 h (1162.5 ng / g FW). In all cases the glutamate content increased at 72 h.

DISCUSSION

Stress has been shown to induce proline accumulation in practically all living beings [41, 26]. In plants, a wide range of abiotic and biotic stress factors induce proline accumulation, constituting a very important mechanism of physiological adaptation to stress [34, 19, 35]. In particular, it has been shown that changes in intracellular proline content are related to protection against low temperature stress [18], and their accumulation in plants has a clear cryoprotective function [33, 14], although the precise mode of action of proline remains largely unknown [3]. Chang *et al.* [8] found that an acclimatization treatment at 10 ° C for 2-3 days, improved the tolerance of mungbean seedlings to cooling at 4 ° C. The concentration of free amino acids, including proline, increased in plants that had been preadapted to

cold, with respect to non-acclimated ones. With respect to cryopreservation, numerous studies have demonstrated the importance of proline for plant ultralow temperatures tolerance [20, 22, 4]. Our results showed that the proline content in isolated embryo axes of common bean descended after the seeds were subjected to desiccation stress treatments. In the case of cryopreservation treatment, there is a marked increase in developing embryos at 72 h after germination (See Table 2). Therefore, proline does not appear to increase in response to stress in bean. It is true that the control embryonic axes already have elevated proline content (271.63 ng / g FW), which may be sufficient to exert a protective effect against the stress treatments to which they were subjected. In the case of groundnuts, the results were as expected, according to the literature. Proline levels increased significantly after desiccation treatments (See Table 2), especially in the data taken at 24 h. They reached values of more than twenty times the control. These results seem to indicate that proline have, in this species, a role in protecting against this type of stress. Treatment with liquid nitrogen, on the other hand, did not produce, in groundnuts, proline increases respect to the control. It seems clear that if correlate proline contents with stress levels, they are lower in dried and cryopreserved embryos than in only dried ones.

It should be noted that in the embryonic axes of control seeds and seeds treated with liquid nitrogen, proline contents were much lower (see table 2) in groundnut than in common bean. Respect to the absolute content of L-glutamate in the embryonic axes of bean, our results showed important differences in several of the treatments, as in the case of silica gel desiccation or cryopreservation, with respect to the control 24 h. In general, except pretreatment at 42 ° C, absolute glutamate content fell at 72 h. In the case of groundnuts, the control presented the highest values at 24 h and at 72 h. In all cases the glutamate content increased at 72 h. It should be noted, that both amino acids are metabolically related. Glutamate is a precursor of proline in a pathway regulated positively by different environmental factors, such as water stress, but is also a product of proline catabolism stimulated, among other factors, by rehydration [38]. Subbarayan *et al* [37] indicated that the total amount of soluble amino acids did not change during the cryopreservation of garlic meristems, however, they were slightly lower in the previous stage of dehydration. Alanine increased its concentration 2.5-fold during the dehydration step. During LN storage there were no significant changes in the concentration of alanine and glutamic acid, but after cryopreservation, glycine and serine increased.

In the present study, the relative amino acids content, in the embryonic axes of *P. vulgaris* was very similar for 13 of the 16 amino acids analyzed (Table 1). Only glutamate, asparagine and tryptophan showed values higher than the control after the treatments. The rest (alanine, leucine, isoleucine, serine, proline, threonine, methionine, cysteine, phenylalanine, lysine, glutamine, histidine and tyrosine), showed much lower concentrations than the control in embryonic axes from all treatments. Even the embryonic axes from non-treated seeds, at 72 h showed a drastic reduction in the contents of the 13 amino acids mentioned. Regarding asparagine, two results are worthy of note: the large increase, at 24 h, in axes from seeds subjected to 42 ° C (which then decreased to very low values at 72 h) and the increase, at 72 h on the axes of cryopreserved seeds. The

variations in the amino acid contents in the embryonic axes of *A. hypogaea*, was completely different to that indicated for the case of bean. Desiccation methods caused significant increases, at 24 h, in the relative content of all amino acids studied except glutamate. It is appreciated that at 72 h there is a marked decrease in all cases. In the embryonic axes from cryopreserved seeds, on the other hand, only were detected values higher than the control for control for L-alanine, L-Phenylalanine, L-isoleucine and L-asparagine and high contents of asparagine and histidine can be related to desiccation treatments.

Kaczmarczyk *et al* [25] showed the correlation between asparagine increase and freeze tolerance in potato, while glutamine concentration decreased in three accessions analyzed. Glutamine as well as asparagine belong to the class of nitrogen-containing amino acids, which are transported in plants from the source to sink tissues [29] and possibly glutamine was metabolized to synthesize other nitrogen-containing biomolecules [25]. Subbarayan *et al* [37] consider that alterations in the metabolism of alanine and glutamate observed in their study with cryopreserved garlic apices, were due to the anoxia conditions caused during cryoprotective treatments. They are supported by previous studies that found that alanine accumulated in plants of *Arabidopsis thaliana* [30] and *Hordeum vulgare* [31] under conditions of hypoxia. Similar results were reported by Limami *et al.* [28], who found that under hypoxic conditions alanine replaced asparagine as the predominant amino acid, while the concentration of glutamate decreased, which is consistent since it is precursor of alanine synthesis. In our study, the increase of alanine detected in *A. hypogaea* after stress, may also be associated with a decrease in glutamate content. Chawade *et al* [10] found that the concentration of glycine and serine increased in oats after cryopreservation and that these changes are similar to those occurring when plants are subjected to cold stress. It has also been noted that in *Lolium perenne*, under low temperature conditions, serine accumulates [12]. Serine is closely linked to the formation of glycine whose accumulation has been detected after storage in LN [37]. In the present study, serine increased, in the embryonic axes of the peanut, after all stress treatments, including storage in LN, the amino acid glycine was not quantified. Our results show the important differences, attributable to the genotype, regarding the content of proline and the rest of amino acids, in relation to stress conditions related to desiccation tolerance and cryopreservation. Perhaps these differences are involved in the orthodox behavior of *P. vulgaris* and the sub-orthodox behavior of *A. hypogaea* during storage under standard conditions of germplasm banks.

Acknowledgments

This work was sponsored by the PROMETEO Project of the Secretariat of Higher Education, Science, Technology and Innovation (SENESCYT) of the Republic of Ecuador "

References

1. Abdulmalik, M. M., Usman, I. S., Olarewaju, J. D., & Aba, D. A. (2014). Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by vitrification. *African Journal of Biotechnology*, 13(2).
2. Arora, S. and Saradhi, P. (1995), Light-induced enhancement in proline levels in *Vigna radiata* exposed to environmental stress. *Australian Journal of Plant Physiology*, 22, 383-386.
3. Bohnert, H.J.; Nelson, D.E.; Jensen, R.G. (1995) Adaptations to environmental stresses. *The Plant Cell*, Vol.7,1099-1111.
4. Burbulis, N.; Jonytiene, V.; Kupriene, R.; Blinstrubiene, A. (2011) Changes in proline and soluble sugars content during cold acclimation of winter rapeseed shoots in vitro. *Journal of Food Agriculture & Environment*, Vol.9,371-374.
5. Burritt, D.J. (2008) Efficient cryopreservation of adventitious shoots of *Begonia x erythrophylla* using encapsulation-dehydration requires pretreatment with both ABA and proline. *Plant Cell Tissue and Organ Culture*, Vol.95, 209-215.
6. Cejas, I., Vives, K., Laudat, T., González-Olmedo, J., Engelmann, F., Martínez-Montero, M. E., & Lorenzo, J. C. (2012). Effects of cryopreservation of *Phaseolus vulgaris* L. seeds on early stages of germination. *Plant Cell Reports*, 31(11), 2065-2073.
7. Cejas, R. Méndez, A. Villalobos, F. Palau, C. Aragón, F. Engelmann, D. Carputo, R. Aversano, M. Martínez and J. Lorenzo, (2013) "Phenotypic and Molecular Characterization of *Phaseolus vulgaris* Plants from Non-Cryopreserved and Cryopreserved Seeds," *American Journal of Plant Sciences*, Vol. 4 No. 4, pp. 844-849.
8. Chang, M.W, Chen, S.L., Lee, C.F. and Chen, Y.M. (2001), Cold-acclimation and root temperature protection from chilling injury in chilling-sensitive mungbean (*Vigna radiata* L.) seedlings. *Botanical Bulletin of Academia Sinica*, 42, 53-60.
9. Chapin, F. S. (1991). Integrated responses of plants to stress. *BioScience*, 41(1), 29-36.
10. Chawade, A., Lindén, P., Bräutigam, M., Jonsson, R., Jonsson, A., Moritz, T., & Olsson, O. (2012). Development of a model system to identify differences in spring and winter oat. *PLoS One*, 7(1), e29792.
11. Cook, D.; Fowler, S.; Fiehn, O.; (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.101,15243-15248. *Crop Science*, Vol.50,1037-1047.
12. Draper S. (1972) Amino acid changes associated with low temperature treatment of *Lolium perenne*. *Phytochemistry*, 11(2):639-41.
13. FAOSTAT (2014). <http://faostat.fao.org>.
14. Fretz, A.; Jahne, A. and Lorz, H. (1992), Cryopreservation of embryonic suspension cultures of barley (*Hordeum vulgare* L.). *Botanica Acta*, 105, 140-145.
15. Gagliardi RF, Pacheco GP, Coculilo SP, Vall JFM, Mansur E (2000). In vitro plant regeneration from seed explants of wild groundnut species (Genus *Arachis*, Section *Extranervosae*). *Biodiversity & Conservation*, 9:943-951.
16. Gill, S. S., Gill, R., & Anjum, N. A. (2014). Target osmoprotectants for abiotic stress tolerance in crop plants-glycine betaine and proline. *Plant adaptation to environmental change: Significance of amino acids and their derivatives*, 97-108.

17. Gimalov, F.P.; Chemeris, A.V. and Vakhitov, V.A. (1996), Synthesis of cold shock proteins in wheat tribe seedlings of the family Poaceae. *Russian Journal of Plant Physiology*, 43, 228-231.
18. Hare, P.D.; Cress, W.A. and Van Staden, J. (1998), Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment*, 21, 535-553.
19. Hare, P.D.; Cress, W.; van Staden, J. (1999) Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. *Journal of Experimental Botany*, Vol.50, 413-434.
20. Hoffman, L., DaCosta, M., Ebdon, J. S., & Watkins, E. (2010). Physiological changes during cold acclimation of perennial ryegrass accessions differing in freeze tolerance. *Crop Science*, 50(3), 1037-1047.
21. Howarth, C.J. and Skot, K.P. (1994), Detailed characterization of heat shock protein synthesis and induced thermotolerance in seedlings of *Sorghum bicolor* L. *Journal Experimental Botany*, 48, 1353-1363.
22. Javadian, N.; Karimzadeh, G.; Mahfoozi, S.; Ghanati, F. (2010) Cold-induced changes of enzymes, proline, carbohydrates, and chlorophyll in wheat. *Russian Journal of Plant Physiology*, Vol.57, 540-547.
23. Kaur, G., & Asthir, B. (2015). Proline: a key player in plant abiotic stress tolerance. *Biologia plantarum*, 59(4), 609-619.
24. Kavi Kishor, P. B. and Sreenivasulu, N. (2014), Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue?. *Plant Cell Environment*, 37: 300–311.
25. Kaczmarczyk, A., Shvachko, N., Lupysheva, Y., Hajirezaei, M. R., & Keller, E. J. (2008). Influence of alternating temperature preculture on cryopreservation results for potato shoot tips. *Plant Cell Reports*, 27(9), 1551.
26. Kostal, V.; Zahradnickova, H.; Simek, P. (2011) Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.108, 13041-13046.
27. Kuznetsov, V. V., & Shevyakova, N. I. (1997). Stress responses of tobacco cells to high temperature and salinity. Proline accumulation and phosphorylation of polypeptides. *Physiologia Plantarum*, 100(2), 320-326.
28. Limami AM, Glévarec G, Ricoult C, Cliquet JB, Planchet E. (2008) Concerted modulation of alanine and glutamate metabolism in young *Medicago truncatula* seedlings under hypoxic stress. *Journal Experimental Botany*, 59(9):2325-35.
29. Lohaus G, Buker M, Hussmann M, Soave C, Heldt HW (1998) Transport of amino acids with special emphasis on the synthesis and transport of asparagine in the Illinois low protein and Illinois high protein strains of maize. *Planta*, 205:181-188
30. Miyashita Y, Dolferus R, Ismond KP, Good AG. (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *The Plant Journal*, 49(6):1108-21.
31. Rolletschek, H., Melkus, G., Grafahrend-Belau, E., Fuchs, J., Heinzl, N., Schreiber, F., ... & Borisjuk, L. (2011). Combined noninvasive imaging and modeling approaches reveal metabolic compartmentation in the barley endosperm. *The Plant Cell*, 23(8), 3041-3054.
32. Sabehat, A.; Weiss, D. and Lurie, S. (1996), The correlation between heat-shock protein accumulation and persistence and chilling tolerance in tomato fruit. *Plant Physiology*, 110, 531-537.
33. Santarius, K. A. (1992), Freezing of isolated thylakoid membranes in complex media. VIII. Differential cryoprotection by sucrose, proline and glycerol. *Physiologia Plantarum*, 84, 87-93.
34. Saradhi, P.P.; Alia; Arora S; Prasad, KVS (1995) Proline accumulates in plants exposed to UV-radiation and protects them against UV induced peroxidation. *Biochemical and Biophysical Research Communications*, Vol.209, 1-5.
35. Siripornadulsil, S.; Traina, S.; Verma, D.P.S.; (2002) Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *The Plant Cell*, Vol.14, 2837-2847.
36. Stanwood PC (1985) Cryopreservation of seed germplasm for genetic preservation. In: Kartha KK (ed) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, pp 199-226.
37. Subbarayan, K., Rolletschek, H., Senula, A., Ulagappan, K., Hajirezaei, M. R., & Keller, E. J. (2015). Influence of oxygen deficiency and the role of specific amino acids in cryopreservation of garlic shoot tips. *BMC Biotechnology*, 15(1), 40.
38. Szabados, L., & Savoure, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science*, 15(2), 89-97.
39. Taji, T.; Ohsumi, C.; Iuchi, S.; Seki, M.; Kasuga, M.; Kobayashi, M.; Yamaguchi-Shinozaki, K.; Shinozaki, K. (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *The Plant Journal*, Vol. 29, 417-426.
40. Vazquez -Yanes C, Arechiga MR (1996). Exsitu conservation of tropical rain forest seed; problems and perspectives. *Interciencia*, 21:293-298.
41. Verbruggen, N.; Hermans, C. (2008) Proline accumulation in plants: a review. *Amino Acids*, Vol.35, 753-759.
42. Walters C, Wheeler L, Stanwood PC (2004) Longevity of cryogenically stored seeds. *Cryobiology*, 48:229-244.
