Agricultural Science
Volume 4, No. 3 (2016), 6-12
ISSN 2291-4471 E-ISSN 2291-448X
Published by Science and Education Centre of North America

Short-Term Liquid Nitrogen Storage of Maize, Common Bean and Soybean Seeds Modifies Their Biochemical Composition

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Received: April 25, 2016 Accepted: June 24, 2016 Online Published: August 12, 2016

DOI: 10.12735/as.v4n3p06 URL: http://dx.doi.org/10.12735/as.v4n3p06

Abstract

We studied the effects of liquid nitrogen storage of maize, common bean and soybean seeds on their germination, electrolyte leakage, levels of chlorophylls, phenolics, aldehydes, proteins and peroxidase activity. After storage for 28 days, seeds were retrieved from liquid nitrogen, some were set to germinate and others were analyzed biochemically. No phenotypic modifications were observed visually 5 days after beginning of germination, although percentage of seed germination was reduced by LN in maize and soybean. Moreover, numerous significant effects of seed cryopreservation were recorded at the biochemical status. In maize seeds, the most important and statistically significant modifications were observed in the increased levels of chlorophyll b and total chlorophyll pigments and in the decreased contents of free phenolics after 28 days of exposure to LN, compared to the control treatment. In common bean, relevant changes were observed in the increased electrolyte leakage and in the reduced levels of chlorophyll pigments (*b*, total) and free phenolics. In soybean, modifications were observed in the increased levels of chlorophyll pigments (*a*, *b*, total), malondialdehyde and electrolyte leakage, and in the decreased peroxidase activity. We have shown for the first time that immersion of maize, common bean and soybean seeds in liquid nitrogen modified the levels of different biochemicals.

Keywords: *Zea mays*; *Phaseolus vulgaris*; *Glycine max*; cryostorage; phenotypic variation

Abbreviation: Liquid nitrogen (LN)

How to cite this paper: Arguedas, M., Pérez, A., Abdelnour, A., Hernández, M., Engelmann, F., Martínez, M. E., Yabor, L., & Lorenzo, J. C. (2016). Short-term liquid nitrogen storage of maize, common bean and soybean seeds modifies their biochemical composition. *Agricultural Science*, *4*(3), 6-12. http://dx.doi.org/10.12735/as.v4n3p06

1. Introduction

Seed conservation is the most useful and proficient technique for *ex situ* maintenance of plant genetic resources (Linington & Pritchard, 2001). A great amount of crop species have seeds i.e. that can be dried out to small wet contents and can thus be stored at low temperature for extensive periods (Roberts, 1973). In cryogenic temperatures e.g. at liquid nitrogen temperature (LN, -196°C), the plant material can be stored without modification for extended time periods. Furthermore, samples are stored in a small volume, protected from contamination, and require very limited maintenance (Engelmann, 2011).

An examination of plant species researched shows substantial attention in the cryopreservation of socio-economically key species. For orthodox seeds the most of these species are effortlessly conserved at cryogenic temperatures and there are many papers which illustrate seed cryopreservation procedures (Pritchard, 1995; Pritchard & Dickie, 2003; Walters, 2004; Walters, Wheeler & Stanwood, 2004; Pritchard, 2007; Pritchard & Nadarajan, 2008; Salinas-Flores, Adams, Wharton, Downes & Lim, 2008; Berjak *et al*., 2011; Engelmann, 2011; Forni *et al*., 2010). Contrastingly, just a few researches have been developed on the understanding of the biochemical special effects of LN exposure and of the physiological modifications taking place in seeds after LN storage (Uragami, Lucas, Ralambosoa, Renard & Dereuddre, 1993; Lakhanpaul, Babrekar & Chandel, 1996; Dussert *et al*., 2003; Harding, 2004; Varghese & Naithani, 2008; Cejas *et al*., 2012; Engelmann & Ramanatha, 2013; Zevallos *et al*., 2013a; Zevallos *et al*., 2013b). From the genebank and agronomic perspectives, the consequence of LN exposure on seed viability, germination, biochemistry and physiology should be evaluated for each plant material before using cryopreservation for long-term conservation.

In a preceding research (Zevallos *et al*., 2013b), we evaluated the result of short-term LN conservation of *Solanum lycopersicum* Mill. seeds on their germination and phenolics contents. Following storage in LN for diverse time periods (0 to 28 days), seeds were recovered from LN and placed to germinate. Equally control and cryopreserved seeds showed about 60% germination without statistically significant differences. No phenotypic modifications were recorded visually in seedlings obtained from different treatments 7 days after start of germination. After 7 days, contents of phenolics (free, cell-wall linked and total) were determined in roots, stems and leaves of seedlings. When seeds were deepened in LN for 7, 14 and 21 days, the contents of cell wall-linked, free and total phenolics decreased considerably in roots and stems, compared to non-cryopreserved controls. However, their level in general augmented when seeds were deepened in LN for 28 days. In leaves, an analogous model to that recorded in roots and stems was noted with the free phenolics level but cell wall-linked phenolics were higher in leaves of seedling derived from seeds immersed in LN for 7 or 14 days. We concluded that immersion of tomato seeds in LN for diverse periods of time (0-28 days) changed the contents of phenolics, demonstrating that some modifications could have happened beyond the physiological state at which seeds were located in cryostorage. Additional experiments including longer periods of LN conservation are necessary to elucidate the mechanisms underlying the modifications observed by Zevallos *et al*. (2013b).

To increase our understanding of the consequences of LN storage length on biochemical modifications, we conducted an experiment with seeds of three dissimilar plant species, maize, common bean and soybean, on which a group of biochemical parameters were measured. We studied the results of short-term LN storage (28 days) of seeds on their germination, electrolyte leakage, contents of chlorophylls (*a*, *b*, total), phenolics (free, cell wall-linked, total), malondialdehyde, other aldehydes, proteins and peroxidase activity. We selected several biochemical compounds connected to a wide variety of significant biochemical and physiological pathways linked to storage compounds, membrane and cell wall injure, and ROS production depending of physiological processes such as germination, plant reaction to stress and photosynthesis (Gross, Hultenby, Mengarelli, Camner & Jarstrand, 2000; Moller, 2001; Porra,

2002; Hörtensteiner, 2006; Palma *et al*., 2006; Hörtensteiner & Kräutler, 2011; Xu, Lu, Tong & Song, 2011). After storage in LN for 28 days, seeds were retrieved from LN, 30% were placed to germinate and 70% were processed biochemically.

Instinctively, it is predictable that cryopreserved cells should not modify further than the physiological status at which they were placed in cryostorage. It is generally recognized that cryostorage guarantees stabilization of cells, based on the idea that germplasm remains viable, does not experience long-term modifications and can be recovered healthy and unchanged after conservation (Benson, 2008). The present experiment was conducted to examine this theory. As far as we know, such results on maize, common bean and soybean seeds have not been reported previously.

2. Materials and Methods

Seeds were located in cryo-vials, deepened in LN for 28 days and then recovered (Stanwood & Bass, 1981). Control seeds were placed to germinate or analyzed directly, without LN exposure. To assess germination, seeds (three replicates of 10 seeds per treatment) were located on filter paper and partially immersed with 25 ml distilled water for 5 days in Petri dishes (Ø: 150 mm). For biochemical evaluations, three independent samples of 25 seeds each were ground in LN to generate seed powder. Powder samples (1000 mg each) were used to carry out every biochemical determinations. Contents of chlorophylls (Porra, 2002), phenolics (Friend, 1992), malondialdehyde, other aldehydes (Heath & Packer, 1968), proteins (Bradford, 1976) and peroxidase activity (Hammerschmidt, Nuckles & Kuc, 1982) were determined. The electrolyte efflux examination was used for electrolyte escape determination (Martínez-Montero *et al*., 2002) from unbroken seeds (3 replicates of 15 seeds each per treatment). Seeds were brought back to ambient temperature before the germination and electrolyte leakage evaluations. SPSS was used to make t-tests ($p \leq 0.05$). Normality (Kolmogorov-Smirnov) and homogeneity of variances (Levene) were confirmed. The overall coefficients of variation (OCVs) were calculated too. For the statistical analysis only, percentages of germination were transformed according to $y' = 2*\arcsin(y/100)^{0.5}$.

3. Results and Discussion

No phenotypic modifications were observed visually 5 days after beginning of germination, although percentage of seed germination was reduced by LN in maize and soybean (Table 1). Moreover, numerous significant effects of seed cryopreservation were recorded at the biochemical status. In maize seeds, the most important and statistically significant modifications were observed in the increased levels of chlorophyll b and total chlorophyll pigments and in the decreased contents of free phenolics, with ¨medium¨ OCVs, after 28 days of exposure to LN.

In common bean, changes with ¨high¨ and ¨medium¨ OCVs were observed in the increased electrolyte leakage and in the reduced levels of chlorophyll pigments (*b*, total) and free phenolics (Table 1). In soybean, modifications were observed in the increased levels of chlorophyll pigments (*a*, *b*, total), malondialdehyde and electrolyte leakage, and in the decreased peroxidase activity (Table 1).

It is considered that metabolic action and cell division stop at the temperature of LN or its vapor (Benson, 2008). Nevertheless, experiments conducted by Walters *et al.* (2004) cautioned that, even though cryogenic conservation is supposed to supply long life, the result of timescales on viability has not been evaluated. Walters (2004) studied the biophysical immovability of dehydrated seeds using the Kauzmann temperature, i.e. the position as which the molecular constancy of glasses is almost nil, as a pointer of molecular mobility. Walters (2004) concluded that molecular mobility

Table 1. Biochemical changes produced in maize, common bean and soybean seeds after exposure to LN

	Maize			Common bean			Soybean		
Seed exposure to LN (days)	0	28	OCV (%) **	0	28	OCV (%)**	0	28	OCV (%)**
Percentage of germination at 5 days after exposure to LN *	100.00 a	80.00 b	15.71	93.33 a	86.67 a	5.23	93.33 a	73.33 b	16.97
Electrolyte leakage (%) *	14.96 b	23.44 a	31.23	0.49 b	29.15 a	136.74	0.65 b	4.37 a	104.80
Chlorophyll *a* concentration (µg g^{-1} fresh weight) *	2.50 b	3.73 a	27.92	5.81 a	3.31 b	38.76	1.60 b	8.04 a	94.48
Chlorophyll *b* concentration (µg g^{-1} fresh weight) *	4.21 b	10.53 a	60.63	4.70 a	1.69 b	66.61	5.69 b	12.45 a	52.70
Total chlorophyll concentration (µg g^{-1} fresh weight) *	6.71 b	14.26 a	50.91	10.51 a	5.01 b	50.11	7.29 b	20.49 a	67.20
Content of free phenolics (µg g^{-1} fresh weight)	522.46 a	268.90 b	45.31	326.71 a	88.38 b	81.19	793.52 a	534.11 b	27.63
Content of cell wall-linked phenolics (µg g^{-1} fresh weight) *	5865.48 a	6226.56 a	4.22	7243.31 b	9473.48 a	18.86	9189.32 b	11382.64 a	15.08
Total content of phenolics (µg g^{-1} fresh weight) *	6387.94 a	6495.46 a	1.18	7570.02 b	9561.87 a	16.44	9982.84 b	11916.74 a	12.49
Malondialdehyde content (µmol g^{-1} fresh weight) *	12.61 b	16.87 a	20.43	185.73 a	119.26 b	30.82	58.22 b	136.01 a	56.64
Other aldehyde content (µmol g^{-1} fresh weight) *	1.52 a	1.50 a	0.93	5.40 a	4.15 b	18.51	3.95 a	3.93 a	0.36
Total protein content (mg g^{-1} fresh weight) *	11.89 b	15.91 a	20.45	29.45 a	30.89 a	3.37	37.65 a	36.34 a	2.50
Peroxidase activity (U mg^{-1} fresh weight) *	9.67 b	13.37 a	22.71	52.91 b	67.91 a	17.55	7.63 a	3.46 b	53.18
Peroxidase specific activity (U mg^{-1} of protein) *	0.87 a	0.85 a	1.64	1.80 b	2.20 a	14.14	0.20 a	0.09 b	53.64

* In each crop, results with the same *letter* are not statistically different (t-test, $p>0.05$). For the statistical analysis only, percentages of germination were transformed according to $y'=2*\arcsin(y/100)^{0.5}$

** Overall coefficient of variation = (Standard deviation/Average)* 100. To calculate this coefficient, average values were considered. The higher difference between the two times compared, the higher the overall coefficient of variation.

Classification of the OCV: From 0.35 to 45.82% was regarded as ¨low¨, from 45.82 to 91.28% was regarded as ¨medium¨ and from 91.28 to 136.74% was regarded as ¨high¨.

could slowly take place still at cryogenic temperatures. Buitink, Leprince, Hemminga and Hoekstra (2000) used electron paramagnetic resonance spectroscopy to measure the rotational movement of a spin probe in the cytoplasm of seed, pollen and various plant species as a function of water level and temperature. Their results recommended that unfavorable changes were linked to molecular mobility in the cytoplasm.

Even though the time scales evaluated by Buitink *et al*. (2000) and Walters (2004) are different from that used in our experiment (years in their case, days in ours), their results can be used to elucidate some of our results. The molecular mobility happening in the cytoplasm for the duration of cryostorage might, for example, provoke oxidative stress throughout the development of free radicals, which may injure the cell membrane organization and create microfractures on it. These modifications in the cell partition may combine some biochemicals that are separated under normal situations and then provoke alterations in diverse metabolic pathways.

In this work, we have shown for the first time that immersion of maize, common bean and soybean seeds in LN modified the levels of different biochemicals, demonstrating that some changes can have taken place beyond the point at which seeds were placed in cryostorage.

Acknowledgements

This research was supported by the Bioplant Centre. We are grateful to Julia Martínez and Alitza Iglesias for their assistance.

The authors declare that they have no conflict of interest.

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