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SHORT COMMUNICATION

Maize seed cryo-storage modifies chlorophyll, carotenoid, protein, aldehyde and phenolics levels during early stages of germination

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Abstract

We recorded the crypreservation effects (direct immersion) on various parameters of early germination stages of maize seeds (0, 7 and 14 days). Percentages of germination; fresh mass of different seedling parts; levels of chlorophyll pigments (a, b); carotenoids; malondialdehyde; other aldehydes; phenolics (cell wall-linked, free) and proteins were determined. Various statistically significant effects of seed exposure to liquid nitrogen (LN) were recorded. Maize seeds did not seem to be affected by LN exposure either visually or regarding fresh weight or germination rate. However, delayed growth was observed in seedlings recovered from cryopreserved seeds. This trend indicated an increase in the effect of seed cryopreservation on growing plants. The most significant effects of LN exposure were recorded in the combined fresh weight of stems and leaves at day 7 of germination and in fresh weights of roots, stems and leaves at day 14. At the biochemical level, numerous indicators varied following LN exposure, but the most significant effects were recorded in carotenoids, malondialdehyde and other aldehyde contents. LN exposure modified 50.0% of indicators in cotyledons, 48.1% in stems and leaves, 38.8% in roots and 11.1% in seeds. LN storage modified 11.1% of the variables measured at day 0 of germination, 37.0% at day 7, and 52.7% at day 14. Field performance of cryostored seed-derived plants should be evaluated to measure the durability of the changes observed.

Keywords Biochemical changes · Cryopreservation · Germplasm preservation · Liquid nitrogen · *Zea mays* L

Introduction

With the climate change occurring globally, it becomes increasingly important to preserve plant germplasm in genebanks (Berjak and Pammenter 2014; Gonzalez-Arnao et al. 2014; Mira et al. 2015). Conservation of seeds is one of the most proficient method for *ex situ* preservation of plant genetic resources, specially through cryopreservation (Pérez-Rodríguez et al. 2017).

Maize is among crops which seeds are cryo-banked. It represents the second most important agricultural plant worldwide, following rice (CIMMYT 2016). Our laboratory in Cuba is breeding maize to increase tolerance to abiotic stresses (PI 67). Therefore, we are also interested in using LN for storage of elite seeds.

We studied some effects of LN exposure of maize seeds on early stages of germination post LN storage (0, 7 and 14 days). Levels of chlorophylls (a, b), carotenoids, proteins, malondialdehyde, other aldehydes, free and cell wall-linked phenolics were determined. These compounds are related to relevant biological pathways, such as stress response and photosynthesis (Salisbury and Ross 1992; Nguyen et al. 2009; Hatfield and Prueger 2015; Pérez-Rodríguez et al. 2017). Moreover, such a study could provide a broad picture of the possible effects of cryopreservation on early stages of germination. To our knowledge, such data on maize seeds has not been informed to date.

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Materials and methods

After harvesting, maize seeds (cv. Tuzón) were air dried at room temperature from 15 to 6% moisture content and then stored for 4 months at 4 °C in the dark in hermetically closed containers. Seeds with 6% moisture content based on fresh weight (ISTA 2005) were used in the experiments shown here. One-half of the seeds was placed in cryo-vials (volume: 2 ml; five seeds per cryo-vial) and directly plunged in LN for 24 h. The other half remained in the same conditions as described above (control treatment). Recovery of seeds from LN was performed according to Stanwood and Bass (1981). From each treatment, 90 seeds were randomly selected to perform the following steps of experimentation.

Three series of experiments were carried out: (1) analyses of seeds immediately after cryopreservation: day 0 of germination (three replicates of 10 seeds per treatment), (2) analyses at day 7 after onset of germination (three replicates of 10 seeds per treatment), and (3) analyses at day 14 after onset of germination (three replicates of 10 seeds per treatment). To perform series 2, seeds were placed on filter paper in Petri dishes (Ø: 100 mm) with 15 ml distilled water for 7 days (dark, 27 ± 1 °C). To perform series 3, seeds were placed in pots (Ø: 75 mm) containing a mixture of ferralytic-red soil and filter-cake-sugarcane ashes (1:1, v:v; 27 ± 1 °C; 16/8 h photoperiod; PPFD: 80 µmol m^{-2} s^{-1}, solar radiation).

Seed germination was evaluated at 14 days. At 0, 7 and 14 days, the fresh mass of the different seedling organs was recorded. Contents of malondialdehyde and other aldehydes (Heath and Packer 1968); chlorophylls (a, b) (Porra 2002); carotenoids (Lichtenthaler 1987) and phenolics (cell wall-linked, free) (Gurr et al. 1992) were determined. Total protein content was recorded according to Bradford (1976). Each biochemical determination was performed using three independent samples (100 mg each). The whole experiment was repeated three times.

SPSS 8.0 (SPSS Inc.) was used to perform *t* tests and compare results of the two treatments studied: non-cryopreserved and cryopreserved seeds ($p \leq 0.05$). Then the overall coefficients of variation (OCV) were calculated as follows: (Standard deviation/Average) × 100. In this formula, to calculate the standard deviation and average of the two treatments, we considered the arithmetic average values of non-cryopreserved and cryopreserved seeds. Therefore, the higher the difference between the two materials compared, the higher the OCV (Lorenzo et al. 2015). OCVs were categorized "Low", "Medium" or "High" as described in tables and figures.

Results and discussion

Maize seeds were not affected by exposure to LN either visually (Fig. 1a) or regarding fresh weight or germination (Fig. 2a, b). However, seedling growth was significantly delayed by cryoexposure (Figs. 1b, c, 2c, d). This growth delay was not observed in our previous experiments with common bean (Cejas et al. 2012) or tomato (Zevallos et al. 2013a), although (Mikuła et al. 2010) reported a one-month delay in cryopreserved *Asplenium cuneifolium* Viv. gametophyte regeneration, and a complete regrowth inhibition of LN-exposed explants was recorded with *Ajania pacifica* (Nakai) Bremer et Humphries (Kulus and Abratowska 2017). In maize, the most significant effects of LN were recorded in the combined fresh weight of stems and leaves at day 7 of germination (Fig. 2c); and in fresh weights of roots, stems and leaves at day 14 (Fig. 2d).

There are several publications which describe cryopreservation techniques (Engelmann 2000, 2004, 2010; Engelmann and Takagi 2000; Salinas-Flores et al. 2008; Berjak et al. 2010; Forni et al. 2010; Gonzalez-Arnao et al. 2014; Kulus and Zalewska 2014; Kalaiselvi et al. 2017; Matsumoto 2017). An increasing number of studies has been carried out to know the effects of LN exposure and the changes occurring after storage of seeds (Uragami et al. 1993; Lakhanpaul et al. 1996; Harding et al. 2000; Cejas et al. 2012, 2013, 2015, 2016; Zevallos et al. 2013a, b, 2014; Arguedas et al. 2016; Song et al. 2016; Coelho et al. 2017; Pérez-Rodríguez et al. 2017).

Our study on maize seeds provided evidence that at the biochemical level, numerous indicators varied due to LN exposure (Tables 1, 2, 3). Based on "High" OCVs, the most significant effects of LN were recorded in contents of carotenoids, malondialdehyde and other aldehydes. At day 7 of germination, LN increased carotenoid content by 1.8 times from 6.93 to 12.53 µg g^{-1} cotyledon fresh weight, but decreased other aldehydes in roots from 0.098 to 0.055 nmol g^{-1} fresh weight (Table 2). At day 14, LN increased malondialdehyde content in stems by 2.2 times from 0.005 to 0.012 nmol g^{-1} fresh weight, but decreased carotenoid concentration in cotyledons from 27.80 to 15.98 µg g^{-1} fresh weight (Table 3).

According to "Medium" OCVs, at day 0 of germination, exposure to LN increased seed chlorophyll a (Table 1). At day 7, LN increased chlorophyll a and b concentration in roots, and malondialdehyde content in stems and leaves but contrastingly, the following indicators were decreased: chlorophyll a and other aldehydes in cotyledons and free phenolics in roots (Table 2). At day 14, cryoexposure increased chlorophyll a, b and malondialdehyde contents in cotyledons; carotenoids in roots; free proteins, other aldehydes and cell wall-linked phenolics in stems; and cell

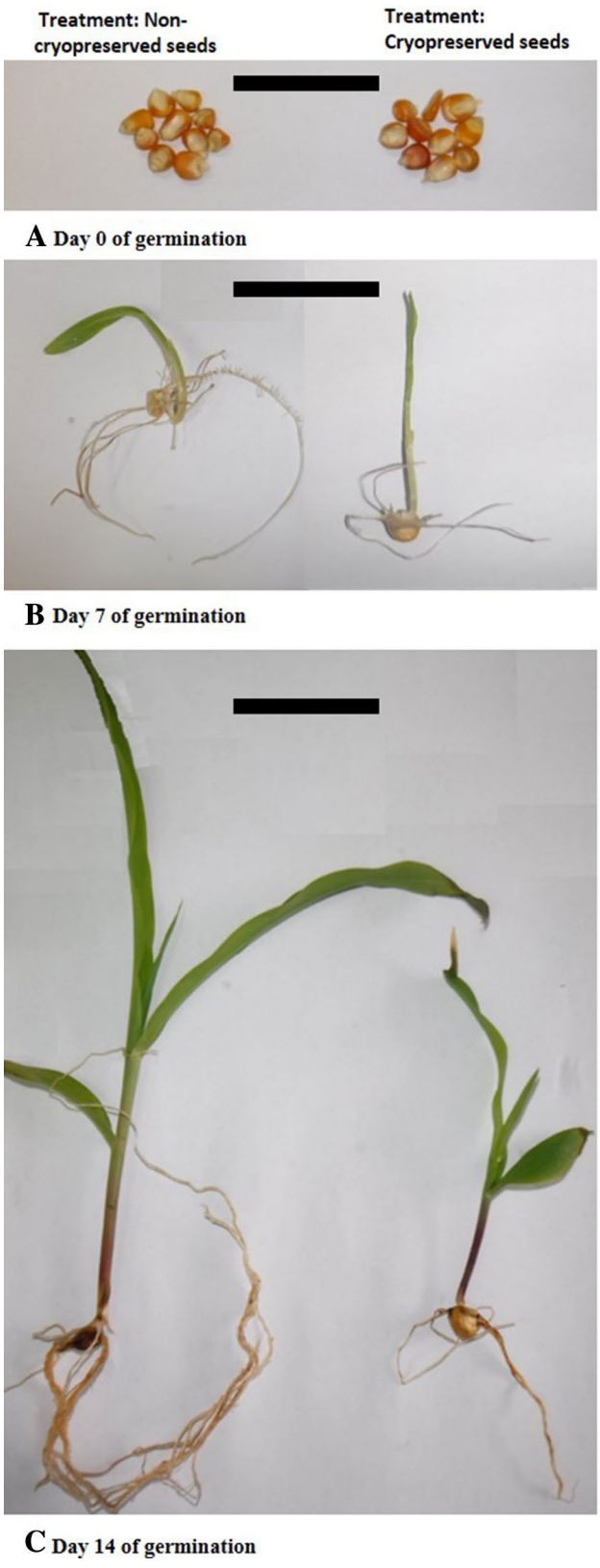

Fig. 1 Effect of cryopreservation of maize seeds on early stages of germination (0, 7 and 14 days). Bars represent 5 cm

wall-linked phenolics in leaves (Table 3). Contrastingly, LN decreased cell wall-linked phenolics in cotyledons; chlorophyll a in roots; chlorophyll b and carotenoids in stems; free phenolics and aldehydes in leaves (Table 3). Other biochemical indicators showed "Low" OCVs indicating low effects of cryopreservation (Tables 1, 2, 3).

Regarding biochemical changes observed by seed exposure to LN, Cejas et al. (2012) studied the effects of cryopreservation on *Phaseolus vulgaris* L.. LN decreased phenolics contents in roots at day 7 after onset of germination. In wild *Solanum lycopersicum* Mill. seeds, Zevallos et al. (2013a) informed highly significant LN effects in leaves: increased peroxidase activity and cell wall-linked phenolics. Decreased contents of chlorophylls and cell wall-linked phenolics were also significant in roots.

Biochemical changes in seeds exposed to LN seem to be species-dependent. In maize, stress conditions could modify the growth pattern of the plant, altering the functioning of the most efficient routes of production of metabolic energy, as in other crops under environmental conditions (Tadeo and Gómez-Cadenas 2008). According to Singh et al. (2014) maize has a higher antioxidant capacity compared to other grains like wheat, oat, and rice. In our present experiment with maize seeds, the most significant effects of LN were recorded in modifications of carotenoid, malondialdehyde and other aldehyde contents following different patterns (Mikuła et al. 2010; Cejas et al. 2012; Zevallos et al. 2013a).

Extreme temperature stress has different effects: reduced rate of growth, inhibition of photosynthesis and respiration, activation of senescence and abscission, since most stress conditions influence the vegetative growth of aerial part of the plant when presented under environmental conditions (Qin et al. 2007; Anjum et al. 2011). In addition, Pérez-Rodríguez et al. (2017) associated these physiological indicators to the oxidative state. Some studies have shown that low temperature is also responsible for the production of reactive oxygen species (ROS) in plant cell and they are involved at all stages of the seed life, including embryogenesis through germination (Berjak et al. 2011; Weitbrecht et al. 2011; Leymarie et al. 2012). Martínez-Montero et al. (2002) found that the level of malondialdehydes and others aldehydes in the microsomal fraction was higher in cryopreserved sugarcane callus compared to unfrozen controls, during the first three days following LN exposure.

Regardless of which tissue is considered, temperature slightly affects the pigment content (Haldimann 1996). Maize contains high levels of carotenoid pigments and phenolics, which have antioxidant and bioactive capacities (Singh et al. 2014). Accumulation of ROS can result in seed death (Fernández-Marín et al. 2014) and a rapid reaction with other molecules may cause lipid peroxidation. Lipophilic antioxidants such as carotenoids protect cells from lipid peroxidation. Higher plants have developed efficient

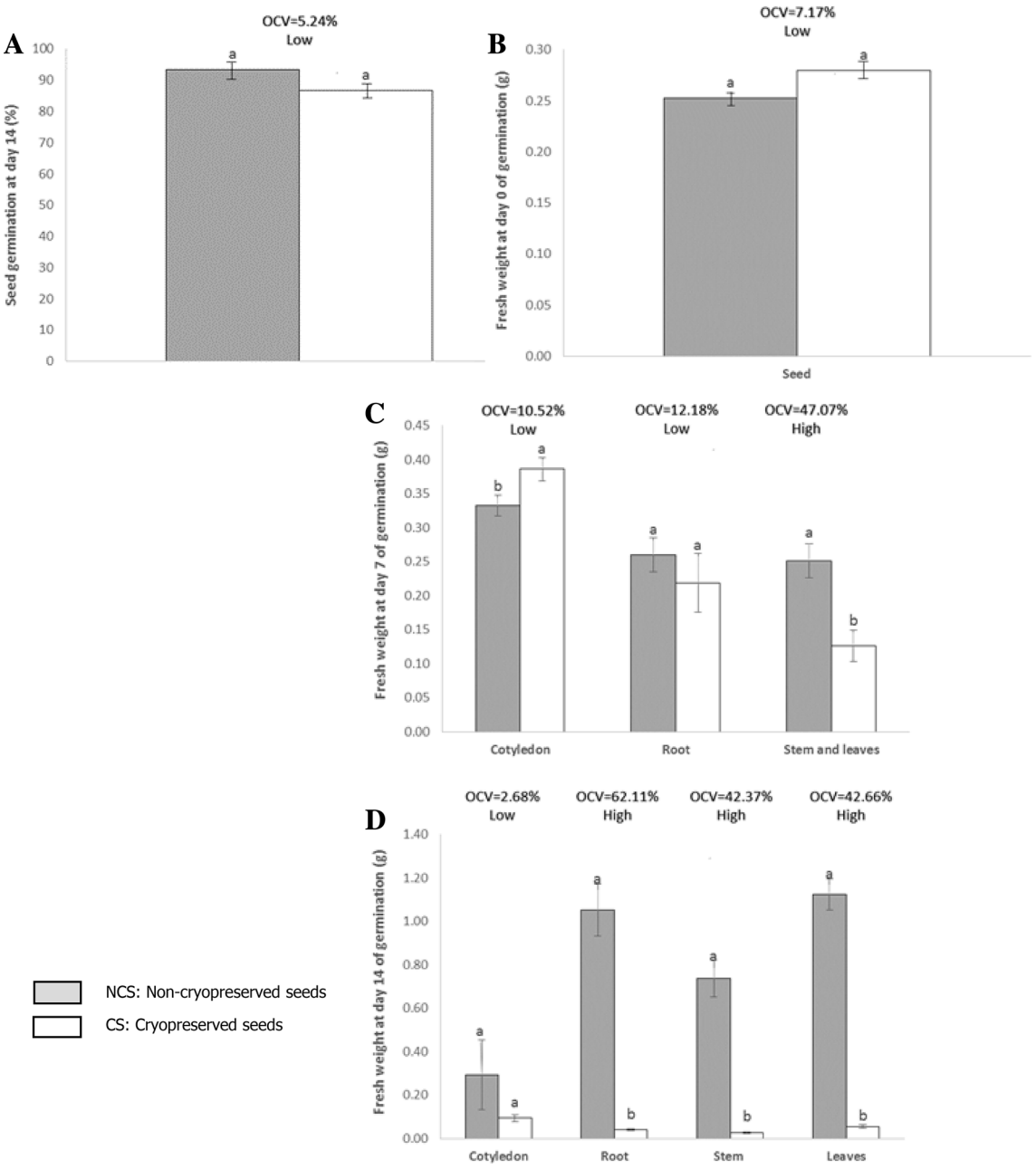

Fig. 2 Effect of cryopreservation of maize seeds on germination and fresh weight. In each plant part, results with the same letter are not statistically different (*t* test, $p > 0.05$). Vertical bars represent SE. OCV (Overall coefficient of variation) = (Standard deviation/Average) × 100 To calculate this coefficient, average values of non-cryopreserved and cryopreserved seeds were considered. The higher difference between the two materials compared, the higher the overall coefficient of variation. Classification of OCVs: "Low" from 2.68 to 22.49%, "Medium" from 22.49 to 42.30% and "High" from 42.30 to 62.11%. "Medium" OCVs were not observed

antioxidant systems to neutralize ROS produced in response to stresses. Under unfavourable conditions, plants show superoxide dismutase activity and high levels of carotenes (Sytykiewicz 2014).

Table 4 summarizes the impact of LN exposure on the germination pattern of maize seeds. In cotyledons, 50.0% (9/18) of indicators were increased or decreased by cryoexposure; 48.1% (13/27) in stems and leaves; 38.8% (7/18) in roots and 11.1% (1/9) in seeds. Approximately 11.1% (1/9) of the variables measured were modified by LN exposure at day 0 of germination; 37.0% (10/27) at day 7; and 52.7% (19/36) at day 14. This trend indicated an increase in the effect of seed cryostorage on growing plants which was not recorded in previous researches with common bean

Table 1 Effects of cryopreservation of maize seeds at 0 days of germination

	NCS	CS	OCV (%)**
Chlorophyll a (μg g^{-1} fresh weight)*	18.62 ± 2.35^{b}	26.54 ± 7.62^{a}	24.83 (Medium)
Chlorophyll b (μg g^{-1} fresh weight)*	28.01 ± 4.02^{a}	35.71 ± 6.21^{a}	17.12 (Low)
Carotenoids (μg g^{-1} fresh weight)*	18.22 ± 2.59^{a}	16.92 ± 1.61^{a}	5.26 (Low)
Free proteins (mg g^{-1} fresh weight)*	0.154 ± 0.007^{a}	0.151 ± 0.008^{a}	1.09 (Low)
Malondialdehyde (nmol g^{-1} fresh weight)*	0.009 ± 0.001^{a}	0.008 ± 0.001^{a}	7.00 (Low)
Other aldehydes (nmol g^{-1} fresh weight)*	0.123 ± 0.005^{a}	0.120 ± 0.002^{a}	1.40 (Low)
Free phenolics (mg g^{-1} fresh weight)*	1.89 ± 0.15^{b}	1.83 ± 0.06^{a}	2.38 (Low)
Cell wall-linked phenolics (mg g^{-1} fresh weight)*	26.92 ± 1.02^{a}	28.40 ± 1.48^{a}	3.77 (Low)

NCS non-cryopreserved seeds, *CS* cryopreserved seeds. Average ± SE

*Results with the same *letter* are not statistically different (*t* test, $p > 0.05$)

**(Overall coefficient of variation) = (Standard deviation/Average) × 100. To calculate this coefficient, average values of non-cryopreserved and cryopreserved seeds were considered. The higher difference between the two materials compared, the higher the overall coefficient of variation. Classification of OCVs: "Low" from 0.02 to 18.23%, "Medium" from 18.23 to 36.43% and "High" from 36.43 to 54.63%

Table 2 Effects of cryopreservation of maize seeds at 7 days after initiation of germination

	Cotyledons			Roots			Stem and leaves		
	NCS	CS	OCV (%)**	NCS	CS	OCV (%)**	NCS	CS	OCV (%)**
Chlorophyll a (μg g^{-1} fresh weight)*	11.82 ± 0.99^{a}	9.11 ± 0.98^{b}	18.25 (Medium)	5.08 ± 1.38^{b}	8.56 ± 1.86^{a}	36.16 (Medium)	54.12 ± 5.10^{a}	63.44 ± 1.38^{a}	11.21 (Low)
Chlorophyll b (μg g^{-1} fresh weight)*	18.96 ± 1.85^{a}	14.81 ± 2.40^{a}	17.36 (Medium)	9.01 ± 2.20^{b}	14.22 ± 3.22^{a}	31.74 (Medium)	37.98 ± 4.71^{a}	35.49 ± 0.65^{a}	4.79 (Low)
Carotenoids (μg g^{-1} fresh weight)*	6.93 ± 0.24^{b}	12.53 ± 1.00^{a}	40.69 (High)	8.13 ± 0.11^{a}	7.97 ± 0.23^{a}	1.45 (Low)	38.46 ± 1.98^{a}	34.63 ± 1.49^{a}	7.41 (Low)
Free proteins (mg g^{-1} fresh weight)*	0.096 ± 0.001^{a}	0.090 ± 0.007^{a}	4.65 (Low)	0.125 ± 0.003^{a}	0.113 ± 0.003^{a}	6.80 (Low)	0.098 ± 0.003^{a}	0.101 ± 0.006^{a}	2.34 (Low)
Malondialdehyde (nmol g^{-1} fresh weight)*	0.027 ± 0.001^{a}	0.023 ± 0.001^{a}	10.60 (Low)	0.011 ± 0.000^{a}	0.009 ± 0.000^{a}	12.45 (Low)	0.011 ± 0.001^{b}	0.014 ± 0.001^{a}	18.53 (Medium)
Other aldehydes (nmol g^{-1} fresh weight)*	0.156 ± 0.002^{a}	0.119 ± 0.009^{b}	18.85 (Medium)	0.098 ± 0.001^{a}	0.055 ± 0.002^{b}	39.69 (High)	0.071 ± 0.002^{a}	0.068 ± 0.001^{a}	3.46 (Low)
Free phenolics (mg g^{-1} fresh weight)*	3.09 ± 0.26^{a}	2.48 ± 0.24^{a}	15.48 (Low)	3.54 ± 0.35^{a}	2.51 ± 0.15^{b}	23.93 (Medium)	3.65 ± 0.082^{a}	3.44 ± 0.34^{a}	4.31 (Low)
Cell wall-linked phenolics (mg g^{-1} fresh weight)*	18.41 ± 0.59^{a}	19.49 ± 0.61^{a}	4.04 (Low)	8.16 ± 0.26^{a}	6.78 ± 0.27^{a}	13.05 (Low)	4.47 ± 0.25^{a}	4.60 ± 0.11^{a}	2.03 (Low)

NCS non-cryopreserved seeds, *CS* cryopreserved seeds. Average ± SE

*In each plant part, results with the same letter are not statistically different (*t* test, $p > 0.05$)

**Overall coefficient of variation = (Standard deviation/Average) × 100. To calculate this coefficient, average values of non-cryopreserved and cryopreserved seeds were considered. The higher difference between the two materials compared, the higher the overall coefficient of variation. Classification of OCVs: "Low" from 0.02 to 18.23%, "Medium" from 18.23 to 36.43% and "High" from 36.43 to 54.63%

Table 3 Effects of cryopreservation of maize seeds on seedlings growth at 14 days after initiation of germination

	Cotyledons			Roots			Stems			Leaves		
	NCS	CS	OCV (%)**	NCS	CS	OCV (%)**	NCS	CS	OCV (%)**	NCS	CS	OCV (%)**
Chlorophyll a (µg g^{-1} fresh weight)*	12.48 ± 0.96[b]	16.29 ± 0.32[a]	18.73 (Medium)	16.23 ± 2.55[a]	11.43 ± 2.65[b]	24.54 (Medium)	59.24 ± 6.25[a]	46.48 ± 4.38[a]	17.07 (Low)	151.63 ± 26.06[a]	151.68 ± 27.41[a]	0.02 (Low)
Chlorophyll b (µg g^{-1} fresh weight)*	19.94 ± 1.15[b]	28.04 ± 0.82[a]	23.89 (Medium)	26.68 ± 4.13[a]	24.39 ± 1.41[a]	6.34 (Low)	38.19 ± 1.68[a]	27.45 ± 2.67[b]	23.15 (Medium)	96.53 ± 5.84[a]	105.74 ± 4.56[a]	6.44 (Low)
Carotenoids (µg g^{-1} fresh weight)*	27.80 ± 6.31[a]	15.98 ± 1.17[b]	38.19 (High)	4.34 ± 0.90[b]	7.08 ± 0.94[a]	33.99 (Medium)	30.20 ± 1.19[a]	20.04 ± 2.43[b]	28.61 (Medium)	102.92 ± 1.29[a]	119.54 ± 11.76[a]	10.56 (Low)
Free proteins (mg g^{-1} fresh weight)*	0.098 ± 0.001 a	0.118 ± 0.002[a]	13.55 (Low)	0.103 ± 0.006[a]	0.099 ± 0.003[a]	2.76 (Low)	0.070 ± 0.003[b]	0.094 ± 0.005[a]	21.04 (Medium)	0.126 ± 0.010[a]	0.129 ± 0.006[a]	1.61 (Low)
Malondialdehyde (nmol g^{-1} fresh weight)*	0.025 ± 0.001[b]	0.040 ± 0.001[a]	34.02 (Medium)	0.009 ± 0.001[a]	0.010 ± 0.001[a]	9.23 (Low)	0.005 ± 0.000[b]	0.012 ± 0.001[a]	54.63 (High)	0.014 ± 0.000[a]	0.016 ± 0.001[a]	8.53 (Low)
Other aldehydes (nmol g^{-1} fresh weight)*	0.087 ± 0.011[a]	0.093 ± 0.005[a]	4.68 (Low)	0.045 ± 0.002[a]	0.048 ± 0.001[a]	5.25 (Low)	0.041 ± 0.001[b]	0.067 ± 0.010[a]	34.45 (Medium)	0.097 ± 0.005[a]	0.069 ± 0.005[b]	23.69 (Medium)
Free phenolics (mg g^{-1} fresh weight)*	2.55 ± 0.06[a]	2.43 ± 0.13a	3.48 (Low)	2.54 ± 0.13[a]	3.09 ± 0.09[a]	13.75 (Low)	2.88 ± 0.56[a]	2.95 ± 0.18[a]	1.65 (Low)	4.03 ± 0.67[a]	2.80 ± 0.18[b]	25.32 (Medium)
Cell wall-linked phenolics (mg g^{-1} fresh weight)**	20.15 ± 0.57a	14.55 ± 1.56[b]	22.80 (Medium)	5.20 ± 0.08[a]	5.95 ± 0.28[a]	9.49 (Low)	3.46 ± 0.19[b]	4.68 ± 0.35[a]	21.11 (Medium)	4.50 ± 0.26[b]	6.67 ± 0.34[a]	27.54 (Medium)

NCS non-cryopreserved seeds, *CS* cryopreserved seeds. Average ± SE

*In each plant part, results with the same *letter* are not statistically different (*t* test, $p > 0.05$)

*Overall coefficient of variation = (Standard deviation/Average) × 100. To calculate this coefficient, average values of non-cryopreserved and cryopreserved seeds were considered. The higher difference between the two materials compared, the higher the overall coefficient of variation. Classification of OCVs: "Low" from 0.02 to 18.23%, "Medium" from 18.23 to 36.43% and "High" from 36.43 to 54.63%

Table 4 Summary of the effects of cryopreservation of maize seeds on early stages of germination

Plant parts	Days after initiation of germination	Indicators			Percentage of indicators modified
		Not modified with respect to the control treatment (non-cryopreserved seeds)	Increased with respect to the control treatment (non-cryopreserved seeds)	Decreased with respect to the control treatment (non-cryopreserved seeds)	
Seeds	0	8 indicators: fresh mass, chlorophyll b, carotenoids, free proteins, malondialdehyde, other aldehydes, free phenolics, cell wall-linked phenolics	1 indicator: chlorophyll *a*	0 indicator	11.1% (1/9)
Cotyledons	7	5 indicators: chlorophyll b, free proteins, malondialdehyde, free phenolics, cell wall-linked phenolics	2 indicators : fresh mass, carotenoids	2 indicators: chlorophyll a, other aldehydes	44.4% (4/9)
	14	4 indicators: fresh mass, free proteins, other aldehydes, free phenolics	3 indicators: malondialdehyde, chlorophyll a, chlorophyll b	2 indicators : carotenoids, cell wall-linked phenolics	55.6% (5/9)
Roots	7	5 indicators: fresh mass, carotenoids, free proteins, malondialdehyde, cell wall-linked phenolics	2 indicators: chlorophyll a, chlorophyll b	2 indicators: other aldehydes, free phenolics	44.4% (4/9)
	14	6 indicators: chlorophyll b, free proteins, malondialdehyde, other aldehydes, free phenolics, cell wall-linked phenolics	1 indicator: carotenoids	2 indicators : fresh mass, chlorophyll a	33.3% (3/9)
Stems and leaves	7	7 indicators: chlorophyll a, chlorophyll b, carotenoids, free proteins, other aldehydes, free phenolics, cell wall-linked phenolics	1 indicator: malondialdehyde	1 indicator : fresh mass	22.2% (2/9)
Stems	14	2 indicators: chlorophyll a, free phenolics	4 indicators: free proteins, other aldehydes, malondialdehyde, cell wall-linked phenolics	3 indicators : fresh mass, chlorophyll b,carotenoids	77.8% (7/9)
Leaves	14	5 indicators: chlorophyll a, chlorophyll b, carotenoids, free proteins, malondialdehyde	1 indicator: cell wall-linked phenolics	3 indicator : fresh mass, other aldehydes, free phenolics	44.4% (4/9)

Classification supported by *t* tests (Fig. 2; Tables 1, 2, 3)

(Cejas et al. 2012) or tomato (Zevallos et al. 2013a). Field performance of cryostored seed-derived plants should be evaluated to measure the duration of the changes observed during early stages of maize germination.

Author contribution statement MA, DG, LH, FE, RG, IC, LY, MEMM and JCL designed the research; MA, DG and LH conducted the experiment; MA, DG and JCL analyzed data; MA, FE, RG, IC, LY, MEMM and JCL wrote the paper; JCL had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Human and animal rights This research did not involve experiments with human or animal participants.

Informed consent Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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