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*Original paper*

# *Histology of maize seeds and young germinating embryos after liquid nitrogen exposure*

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## Abstract

Maize represents a staple food crop and is the second most important agricultural commodity globally. Considering the important role of maize for food security, the long-term conservation of valuable germplasm is critical to ensure that high levels of genetic diversity are available for breeding superior cultivars to face future challenges. Cryopreservation is regarded as the most appropriate tool for long-term germplasm preservation and has been investigated in different crops. This short communication adds to the existing knowledge on maize cryopreservation by describing histological changes observed in maize seeds and young germinating embryos after liquid nitrogen (LN) exposure. Plants were examined immediately after recovery from LN (day zero) and following 3 days of germination. At day 3, seeds exposed to LN showed lower germination rates than non-cryostored seeds, i.e., 60.7% *vs*. 83.3%. Histological evaluation at day 3 revealed that the thickness of the conical endosperm and the scutellum did not show any statistically significant differences between control and cryopreserved seeds. In contrast, for the other histological evaluations made, mostly regarding the thickness of mesocarp, mealy endosperm, plumule, radicle and the epidermis, significant differences were observed between control and cryostored seeds with the former consistently displaying higher average values than the latter.

**Keywords** Conservation of plant genetic resources, cryopreservation, cryostorage, seed anatomical characterisation, *Zea mays* L.

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# Introduction

The challenge of producing sufficient food for an ever-increasing global population is exacerbated by changing climate patterns, which require the development of superior cultivars, in conjunction with improved management practices, to mitigate the adverse environmental effects. The goal of developing new, improved cultivars is reliant on a diverse gene pool to allow for the selection of cultivars with the desired traits, appropriate for the changing agricultural landscape. This is particularly relevant for staple food crops, on which a significant percentage of the population relies as a substantial part of their diet. In this context, maize (*Zea mays* L.) represents the second most important agricultural crop worldwide, after rice (CIMMYT, 2016]). Clearly, there is an urgent need to focus efforts globally to preserve valuable genotypes in repositories, thus avoiding germplasm losses (HALMAGYI and DELIU, 2006; PAUNESCU, 2009; BERJAK et al, 2013; OZUDOGRU et al, 2013; GONZALEZ-ARNAO el at, 2014; MAQSOOD et al, 2015; MIRA et al, 2015). Cryopreservation in liquid nitrogen provides an option for the long-term storage of plant germplasm. Indeed, protocols have been developed for cryopreservation of a range of species (TEHRIM and SAJID, 2011; BERJAK and PAMMENTER, 2014; GONZÁLEZ-ARNAO et al, 2014; COSTE et al, 2014; FORTE-GIL et al, 2017; LAMBARDi et al, 2018; VILLALOBOS-OLIVERA et al, 2019b). Considering the various stresses imposed on germplasm during the cryopreservation process and following retrieval from storage, it is crucial to ensure that the phenotypic and genetic integrity of stored germplasm is unchanged following re-growth. This includes many different aspects, from seed viability following cryogenic exposure and germination capacity, to biochemical, physiological and agronomic characteristics and molecular integrity for the production of true-to-type plants. The abovementioned parameters need to be evaluated for each crop to ensure that cryopreservation does not result in unintended adverse effects (ENGELMANN et al, 2012).

Considering the global importance of maize as a crop, some studies have been conducted on the effects of cryopreservation on maize seeds following retrieval from liquid nitrogen (ARGUEDAS et al, 2018a; PEREIRA et al, 2019; ARGUEDAS et al, 2018b). The parameters evaluated ranged from immediate germination of seeds following cryoexposure (0, 7 and 21 days post-retrieval) to longer-term phenotypic evaluation (following 3-4 months of growth in the field) of cryostored seeds, e.g. leaf dimensions, plant height, internode characteristics, number of ears, kernel characteristics and 100 seed weight (ARGUEDAS et al, 2018b). These authors reported no notable differences in the measured parameters between cryopreserved and non-stored seeds. In contrast, significant differences were noted in the biochemical attributes of plants following 7 and 14 days of growth post cryogenic storage (ARGUEDAS et al, 2018a). The biochemical indicators that were the most strongly affected were the levels of carotenoids, malondialdehyde and other aldehydes. Interestingly, in this study it was also reported that cryopreserved seeds showed a lag effect in terms of their development when compared with control plants. There are indications that the abovementioned biochemical and developmental changes observed in cryopreserved seeds might be transient as the field performance of fully mature maize derived from cryostored seeds was similar to that of control plants developed from non-cryostored seeds. Furthermore, Pereira et al. (2019) highlighted that the changes observed in the shikimate pathway of seedlings regenerated from cryostorage were predominantly in the maternal (cotyledonary) tissue rather than in the vegetative tissues and that these changes were likely to revert to normal following shedding of the cotyledons.

This short communication describes histological changes in maize seeds and young germinating embryos following exposure to liquid nitrogen (LN). Observations were made immediately after recovery from LN (day zero) and following 3 days of germination (day 3).

# Materials and Methods

### *Seed processing and treatment*

Maize seeds (cv. Tuzón) were harvested from mature, field-grown plants, and were air dried at room temperature, from an initial water content of 15% to 6% moisture content. Following drying, seeds were stored in hermetically sealed containers at reduced temperature (4°C), in the dark, for four months. The seed drying and storage conditions followed the recommendations from the International Seed Testing Association (ISTA 2005). After four months of storage, one half of the seeds were exposed to LN and the remaining seeds were kept under the same storage conditions and served as the control. For exposure to LN, seeds were placed directly in 2 ml cryovials (five seeds per cryovial), which were immersed into LN for 24 h. Retrieval of seeds from LN followed the procedure described by Stanwood and Bass (1981). To assess survival following exposure to the cryogen, seed germination was determined by placing the seeds (cryostored and control) onto filter paper in Petri dishes (10 cm diameter), moistened with 15 ml of distilled water. There were five replicates per treatment and ten seeds per Petri dish. The Petri dishes were incubated in the dark at 27 ± 1°C for 3 d.

### *Evaluations performed at day 0 and day 3*

Non-cryostored (NC) and cryostored (C) seeds were examined for germination and histological measurements at day 0 and at day 3. For the histological analysis, seeds were first fixed in a mixture of formaldehyde: ethanol: acetic acid (40:70:10, in %). Samples were then thoroughly washed with water for 5 min (PACHECO et al, 2016). For sectioning, free-hand transversal sections

were made (15-25 µm width), which were briefly stained with safranin (30 s), followed by toluidine blue (60 s). Histological determinations were performed using an inverted microscope [NIB-100; camera HDCE-50B (E); China]. The histological evaluations were conducted using nine seeds (three seeds/replicate) that were randomly selected from each treatment. The following parameters were measured: thickness of the testa, epidermis, mesocarp, pericarp, seed coat, conical endosperm, mealy endosperm, endosperm, scutellum, plumule and radicle.

### *Data analysis*

All data were statistically analysed using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY). For comparisons involving two treatments, i.e., non-cryopreserved and cryopreserved seeds, t-tests ($p = 0.05$) were performed. The results presented represent means with standard deviations. As an additional comparison, the overall coefficient of variation (OCV) was calculated. This was done by using the equation: OCV = (standard deviation/average) * 100.

For interpretation of OCV values, a high OCV indicates a substantial difference between the two groups of seeds, i.e. cryopreserved and non-cryopreserved seeds (LORENZO et al, 2015). To categorise OCVs, the following classification was established: a ¨Low¨ OCV ranges from 1.90 to 3.40%, ¨Medium¨ OCV from 3.40 to 4.89% and ¨High¨ OCV from 4.89 to 6.38%. The OCV for germination at day 3 was not classified as ¨High¨ because this indicator was beyond the range of the other indicators.

## Results

The first measured indicator of survival was seed germination at day 0. As expected, there was no germination immediately after recovery from LN (day 0). However, by the third day after retrieval, seeds that were not cryopreserved (control) displayed significantly higher levels of germination, 83.3%, than those exposed to the cryogen, with 60.7% germination rate (Figure 1A). All the histological evaluations made at day 0 indicated that there were no significant differences in the measured parameters between seeds that had been cryostored and non-cryostored, control seeds (Figures 1, 2).

Three days after regeneration, the only histological parameters that did not show significant differences between cryopreserved and control seedlings were conical endosperm (Fig. 2A) and scutellum (Fig. 2D) thickness. For all other measured parameters, the obtained values were higher in control than in cryopreserved seedlings. Considering the calculated OCVs values, ¨Low¨ OCVs were obtained for the thickness of the testa (Fig. 1B), pericarp (Fig. 1E), seed coat (Fig. 1F) and endosperm (Fig. 2C). ¨Medium¨ OCVs were observed for the thickness of the mesocarp (Fig. 1D), mealy endosperm (Fig. 2B), plumule (Fig. 2E) and radicle (Fig. 2F). The only parameter that resulted in a ¨High¨ OCV was the epidermis thickness (Fig. 1C).

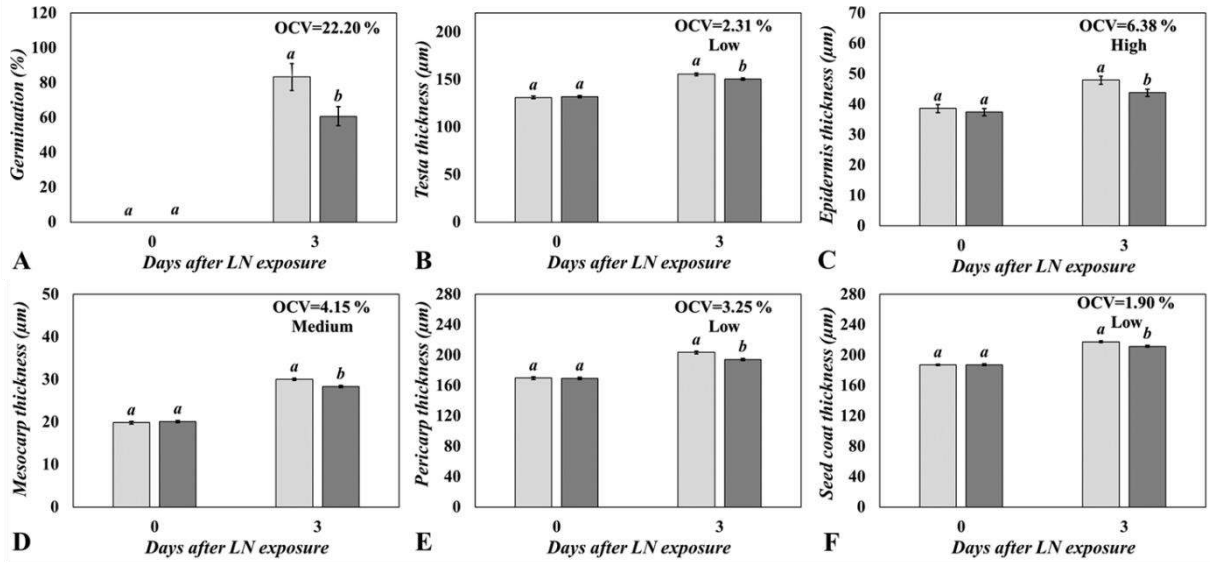


**Figure 1.** Histology of maize seeds and young germinating embryos, at day 0 and day 3 after exposure to liquid nitrogen (LN) for 24 h; light bars: control seeds, dark bars: cryostored seeds. Bars represent germination percentages (**A**) and the thickness (in µm) of the seed testa (**B**), epidermis (**C**), mesocarp (**D**), pericarp (**E**), or seed coat (**F**). In each day after LN exposure (0 or 3 d), results with the same *letter* are not statistically different (t-test, $p < 0.05$). Vertical bars represent SE. OCV were only calculated when statistically significant differences were observed. A High OCV is indicative of a large difference between the two groups compared. Classification of OCVs: ¨Low¨ from 1.90 to 3.40%, ¨Medium¨ from 3.40 to 4.89%, and ¨High¨ from 4.89 to 6.38%. OCV of germination at day 3 (Fig. 1A) was not classified as ¨High¨ because this indicator was beyond the range of the other indicators.

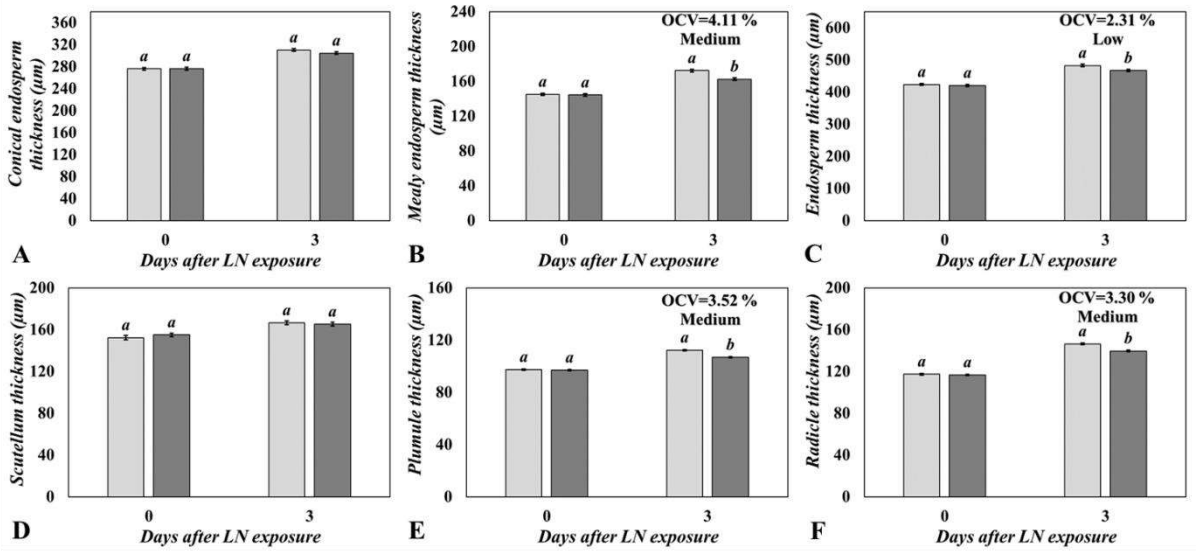


**Figure 2.** Histology of maize seeds and young germinating embryos, at day 0 and day 3 after exposure to liquid nitrogen (LN) for 24 h; light bars: control seeds, dark bars: cryostored seeds. Bars represent the thickness (in µm) of the seed conical endosperm (**A**), mealy endosperm (**B**), endosperm (**C**), scutellum (**D**), plumule (**E**) or radicle (**F**). In each day after LN exposure (0 or 3 d), results with the same *letter* are not statistically different (t-test, $p < 0.05$). Vertical bars represent SE. OCV were only calculated when statistically significant differences were observed. A High OCV is indicative of a large difference between the two groups compared. Classification of OCVs: ¨Low¨ from 1.90 to 3.40%, ¨Medium¨ from 3.40 to 4.89%, and ¨High¨ from 4.89 to 6.38%.

## Discussion

To use cryopreservation as a viable tool for the long-term preservation of germplasm, it is necessary to assess the effects of exposure to cryogenic temperatures on subsequent seed development. From the studies performed to date, it is commonly suggested that the response of seeds to LN can be classified into three categories: ***i)*** non-reactive seeds, where exposure to LN does not affect germination, e.g. in common bean (CEJAS et al, 2012); ***ii)*** seeds which display a positive response, where immersion in LN seemingly results in the promotion of germination, e.g. in *Teramnus labialis* (ACOSTA et al, 2020a; ACOSTA et al, 2020b), *Neonotonia wightii* (ACOSTA et al, 2020c) or *Solanum lycopersicum* (ZEVALLOS et al, 2013); and ***iii)*** seeds which display a negative response, where exposure to the cryogen causes delayed germination, e.g. in sorghum (VILLALOBOS-OLIVERA et al, 2019a) or maize (ARGUEDAS et al, 2018a). Considering the above classification, the results of the present study confirm that maize seeds fall within the third category, where germination is delayed following immersion in liquid nitrogen.

Exposure of seeds to sub-zero temperatures, followed by warming after retrieval from storage, imposes several stresses on seed tissues. The ability of seeds to limit and/or overcome the damage caused by cryopreservation will influence subsequent germination and seedling survival. One of the primary factors affecting maize seeds exposed to LN is likely to be the mechanical damage of external tissues (WALTERS et al, 2008; PÉREZ-RODRÍGUEZ et al, 2017; DUSSERT et al, 2006). The physical stress results in cellular rupture, leading to a loss of their protective function, causing internal structures within the seed to be exposed (BERJAK et al, 2013). Cryogenic temperatures are admittedly extreme, and it is not unexpected that some form of damage is incurred because of this treatment. Hence, when seeds are immersed in the cryogen, not only there is physical alteration of the external tissues, as discussed above, but also internal cellular damage, which can subsequently have an adverse effect on germination. These effects are usually exhibited during the initial stage of germination, i.e. from imbibition (ZARITZKY et al, 2015). Consequently, the affected tissues require a period of recovery to implement repair mechanisms and, therefore, germination might be delayed. This was observed in the current study, where cryopreserved seeds showed delayed onset of germination. However, as reported by others, this developmental lag has been shown to be transient in maize as mature plants grown from cryopreserved seeds did not display this trait (ARGUEDAS et al, 2018b).

An interesting observation made in some dormant seeds is that immersion in LN promotes germination rather than causing reduced or delayed germination. In the case of these seeds, immersion in the cryogen leads to the physical rupture of the testa (ACOSTA et al, 2020a), which characteristically has a thicker cell wall and higher lignin content than in non-dormant seeds (BASKIN et al, 2004; RODRIGUES-JUNIOR et al, 2014; CHOUDHARY et al, 2014; GALLO et al, 2019). Once this protective testa cracks due to immersion in LN, the physical barrier to germination is removed, allowing for imbibition and subsequent germination. This was demonstrated by Acosta et al. (2018b), who reported how exposure of *T. labialis* seeds to LN facilitated the breakdown of physical dormancy and resulted in increased seed germination.

Examination of the OCVs allowed for estimation of the extent of damage to cellular structures following cryopreservation. These results suggested that the primary site affected in maize seeds after exposure to sub-zero temperatures was the epidermis (as indicated by ¨High¨ OCVs, Fig. 1C). The epidermis constitutes the external seed tissue, and its damage is likely to cause the loss of its protective function. Immersion in LN also affected the mesocarp, mealy endosperm, plumule and radicle but to a lesser extent, as indicated by the ¨Medium¨ OCVs calculated for these structures.

## Conclusions

The results of the present study indicate that maize seeds are, to some extent, negatively affected by the cryopreservation process. However, in this case it is important to emphasise that the damage experienced by the seeds at very low temperatures, exemplified by the delayed onset of germination and alterations observed at the cellular level, appears to be only transient. As previously shown by Arguedas et al. (2018b), the developing maize plant is able to overcome the changes induced by cryostorage as it grows, without showing long-term effects.

## Authors' Contributions

All authors contributed equally to this article.

## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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