SEED CRYOSTORAGE ENHANCES SUBSEQUENT PLANT PRODUCTIVITY IN THE FORAGE SPECIES *Teramnus labialis* (L.F.) SPRENG.

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Abstract

BACKGROUND: *Teramnus labialis* is an herbaceous legume that serves as a source of carbohydrates and proteins for animals and humans, and is valued for its nitrogen contribution to soil. The benefits of this species are, however, limited by low seed availability, small seed size and low *in situ* seed germination levels, due to physical dormancy. Cryostorage has been shown to be beneficial for both seed storage and breaking physical dormancy in seeds of various species. However, its potential effects on subsequent seedling emergence, plant growth and seed production need to be studied before large-scale implementation for *T. labialis.* **OBJECTIVE:** To record agricultural traits of *T. labialis* after seed exposure to liquid nitrogen. **MATERIALS AND METHODS:** Seeds were maintained at 5°C (control) or stored in LN before sowing. Seedling emergence percentage and traits related to plant growth and seed production were evaluated for 6 months. **RESULTS:** Except for seed weight, all traits differed significantly between seedlings generated from cryostored and control seeds. Except for pod number, seedling emergence and plant growth traits were enhanced by cryostorage to a greater extent than seed production traits. Cryostorage resulted in cracks and breaks in the seed coat which were absent in control seeds (scanning electron microscopy), and in breaking physical dormancy may have facilitated more rapid seedling emergence than for control seeds. **CONCLUSION:** Seed cryostorage enhances subsequent plant productivity in terms of growth and to a lesser extent seed production in *Teramnus labialis*, validating its use for commercial growth of this species.

Keywords: animal feed; crops; cryopreservation; legumes; nitrogen fixation; seed dormancy

INTRODUCTION

Legumes play an important role in agriculture, not only as a source of carbohydrates and proteins for animals and humans but also as nitrogen fixers in different ecosystems (17). In the tropical Latin America, there is a rich diversity of legumes, e.g. the genera *Neonotonia*, *Teramnus*, *Stylosanthes*, *Centrosema* and *Macroptilium* (18). *Teramnus labialis* (Fabaceae), which is a herbaceous legume used to treat rheumatism, tuberculosis and psychiatric disorders (3), is gaining popularity as a cover crop in diverse agricultural systems (35), including fruit orchards (24).

The use and management of *Teramnus labialis* is, however, far from being maximized

owing largely to low seed production, small seed size and low germination percentages, due to physical dormancy (22). Physical dormancy in legumes is the result of the presence of one or more water-impermeable palisade cell layer(s) in the seed coat (6). Under natural conditions, the seed coat becomes permeable by the stressing action of environmental factors, but this can take several weeks, to months, which delays the germination and establishment of the species (43).

Researchers and farmers have developed a series of techniques to make dormant seeds permeable, including mechanical scarification, and treatments with sulfuric acid, enzymes, organic solvents, high atmospheric pressures, hot water, dry storage and low temperatures (6). Interestingly, physical dormancy in several species has been broken by cryo-treatment, i.e. exposure to cryogenic (~160-196°C), temperatures [e.g. *Trifolium repens*, *Lotus corniculatus* (20); *Medicago sativa* (1)]. Moreover, exposure of plant materials to liquid nitrogen (LN, -196°C) has been described as a suitable technology to conserve genetic resources of several species: *Solanum tuberosum* (46), *Colocasia esculenta* var. *esculenta* (42), *Malus domestica* (30), *Chrysanthemum morifolium* (47), *Lilium* sp (8), *Dioscorea alata* (5), *Dianthus caryophyllus* (9), *Cucumis sativus* (2), *Rubus ulmifolius* (34) and *Helianthus tuberosus* L. (51).

The potential effects of LN exposure and associated dormancy breaking on subsequent *ex vitro* seedling establishment and plant growth in such studies are rarely reported but reports suggest that exposure to LN can alter germination and early seedling growth in crop species such as maize (4). At the time of this study there were no published reports on the effects of cryostorage on *ex vitro* seedling emergence and plant growth for the leguminous forage species *T. labialis*. This hinders the large-scale implementation of cryo-treatment to break seed dormancy and seed cryostorage in this species.

With the unprecedented loss of valuable plant germplasm occurring globally, it has become increasingly important to conserve plant germplasm of important forage legumes and crop species *ex situ* in seedbanks (19). Seed storage is certainly the most effective and efficient method for *ex situ* preservation of plant genetic resources (31). The recommended storage conditions for seeds are 3–7% (fresh weight basis) moisture content (depending on the species) and –18°C (39), or below. It is clear that future food and ecosystem security will depend greatly on seed cryopreservation (41). There are several publications which describe seed cryopreservation techniques (21, 28, 29). Given the above, an increasing number of studies have been designed to investigate the effects of seed cryostorage on germination and subsequent plant growth (4, 13, 14, 16, 38, 44, 49). The present study investigated the effects of seed cryostorage on subsequent *ex vitro* seedling emergence, plant growth and seed production in *T. labialis*, which is known to exhibit physical dormancy.

MATERIALS AND METHODS

Plant material and study site

Seeds were collected from 50 mature plants in Ciego de Avila, Cuba, and treated as suggested in the manual for seed management in germplasm banks (40). Seeds exhibited a moisture content (fresh mass basis) of 9.8% at harvest and were stored for 5 months in hermetically sealed glass containers at 5°C (6) until further use.

Seed treatments

Nine hundred and thirty-six true-to-type seeds were treated as follows: one half was maintained at 5°C (control) and the other half was immersed in LN. Seeds were exposed to LN within cryo-vials (156 seeds/vial; cooling rate: c. -200°C/min). After 24 h of cryostorage, the cryo-vials were retrieved from cryostorage and placed at room temperature to reach ambient (11).

Seed sowing and ex vitro *measurements*

Seeds were sown in at a site representative of typical fersialitic soil farmland in Ciego de Avila, Cuba where *T. labialis* is commonly found/planted. Control and cryostored seeds were sown in three 4 × 3 m plots each (three seeds in each of four rows of 13 places per plot; distance: 0.30 m x 0.7 m) that were randomly allocated. Plots were irrigated via sprinklers every 7 d until fruit formation. Border plants were not evaluated to exclude potential edge effects. The planting procedures were based on Mazorra-Calero et al. (35).

The following traits were measured according to Machado et al. (33) between 28 and

180 days after sowing (DAS): seedling emergence (%, 28 DAS), plant height (cm, 28 DAS), leaf number (28 DAS), leaf coverage (m^2; 30, 60, 90, 120, 150 and 180 DAS), inflorescence number per m^2 (104 DAS), total pod number per m^2 (139 DAS), pod length (cm; 139 DAS), seeds per pod (139 DAS), and weight of 1000 seeds (g, at harvest 180 DAS).

Scanning electron microscopy (SEM) of seed coats

After retrieval from cryostorage, as described above, seeds were processed for SEM using conventional processing methods, which included mounting seeds onto metal stubs using carbon tape and sputter coating with gold using a Quorum Q150R ES sputter coater. Seeds stored at 5°C were processed in a similar manner. The seed coats of 20 cryostored and control seeds were then assessed for cracks/breaks using a Zeiss LEO 1450 SEM and photomicrographs were captured.

Data analysis

All data were statistically analyzed using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY). Data were tested for normality using a Kolmogorov – Smirnov test and means were compared using t-tests ($p \leqslant 0.05$). The overall coefficients of variation (OCV) were calculated as follows: (standard deviation/average) * 100. In this formula, we considered the average values of the two treatments compared to calculate the standard deviation and average. For this comparison, the higher the difference between the two treatments compared, the higher is the OCV (32). OCVs were classified as low = 1.0 to 22.5%, medium = 22.5 to 43.9% and high = 43.9 to 65.4%.

RESULTS

Assessment of seed coat using SEM

The seed coats of control seeds were largely intact and devoid of breaks (i.e. parts of the coat that were missing) but a small proportion (20%) of these seeds exhibited superficial cracks in the seed coat (Fig. 1A). In contrast, a large proportion (80%) of the cryostored seeds exhibited multiple cracks and

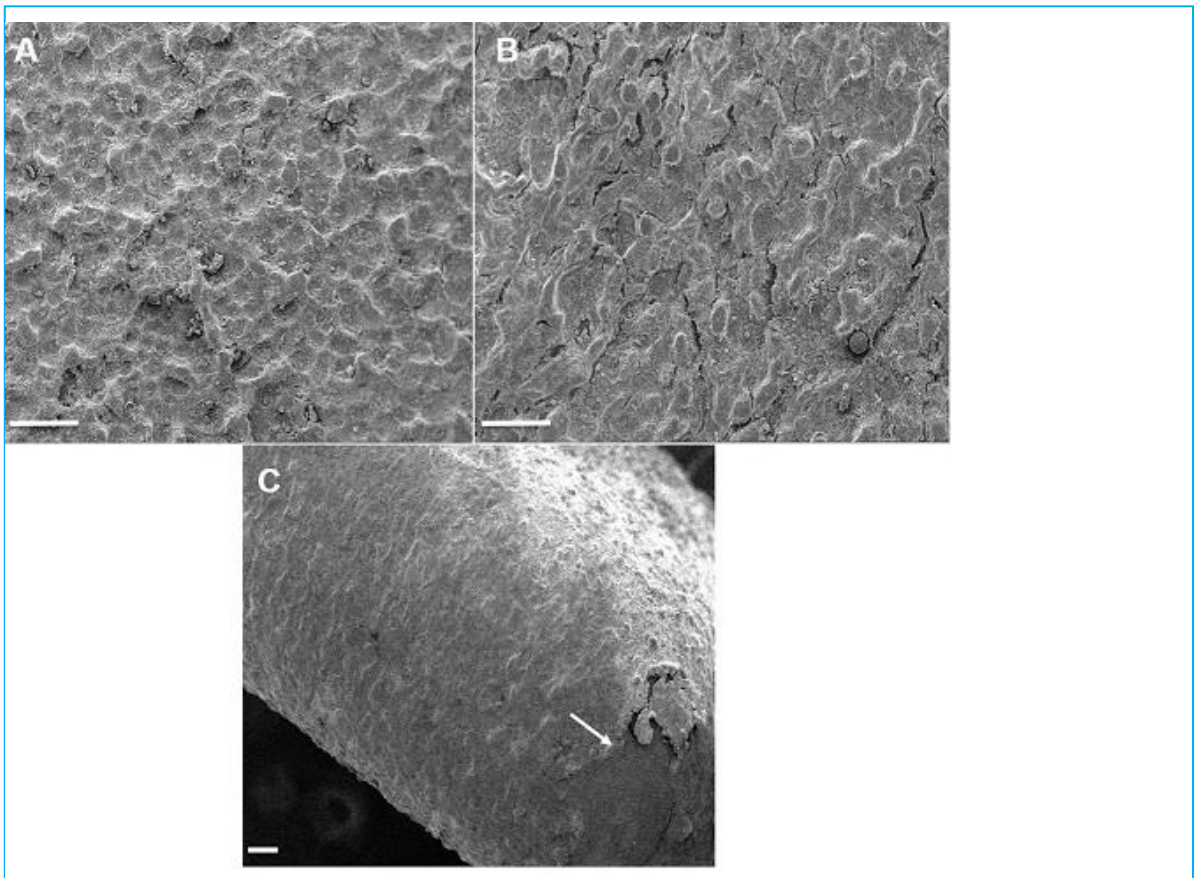


Fig. 1: Scanning electron micrographs showing intact coat of control seed (A) and multiple cracks (B) and break (C, arrow) in coat of seed recovered from cryostorage (Bar=100 um).

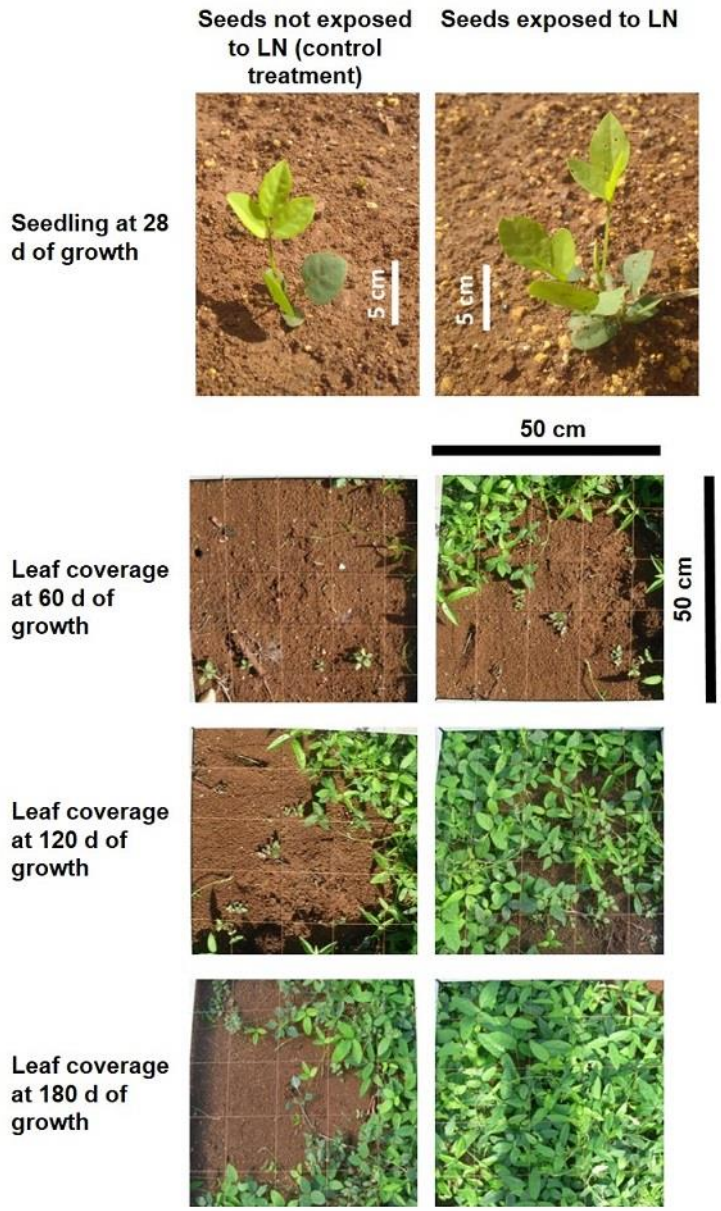
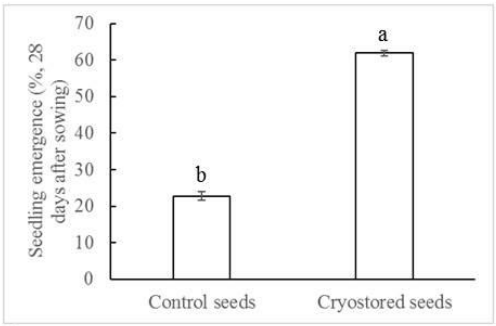


Fig. 2: Field growth of *Teramnus labialis* (L.f.) Spreng seeds after exposure to LN.

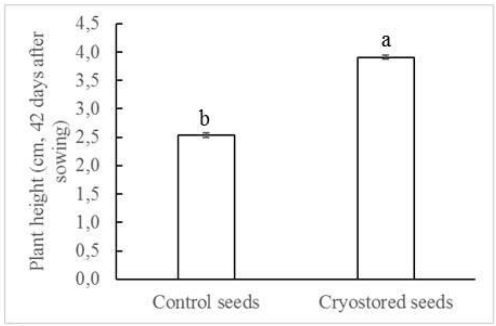
breaks in the seed coat (Fig. 1B, C). These cracks did not appear to extend into the cotyledonary tissues but were in some cases deep.

Seedling emergence and plant growth related traits

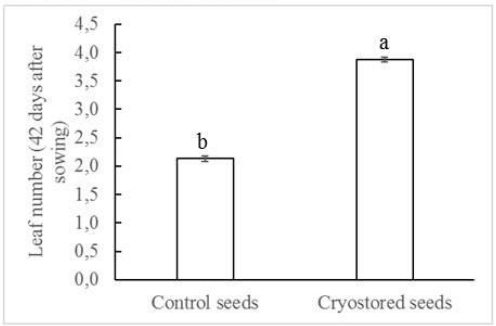
The cryostorage enhanced seedling emergence and all growth related traits significantly relative to the control (Fig. 2). The effect on seedling emergence was particularly marked (high OCV = 65.4%), while plant height and leaf number were enhanced to a relatively lower extent (medium OCVs of 30.1 and 41%, respectively) (Fig. 3A, B, C). Leaf coverage was assessed at 20 DAS intervals for 6 months and while plants produced by cryostored seeds exhibited higher leaf coverage than control plants throughout the experiment, these differences were greater during the first half of the growth period (OCVs of 44.8-48.8% for 30-90 DAS versus 21.2-40.3% for 120-180 DAS) (Fig. 3D).



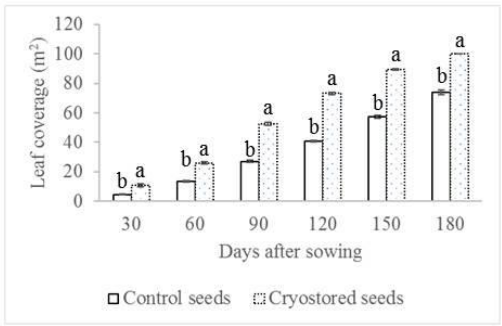
A OCV = 65.4% (*High*)



B OCV = 30.1% (*Medium*)



C OCV = 41.1% (*Medium*)



D OCV at 30 DAS = 58.8% (*High*); at 60 DAS = 44.8% (*High*); at 90 DAS = 45.7% (*High*); at 120 DAS = 40.3% (*Medium*); at 150 DAS = 30.8% (*Medium*); and at 180 DAS = 21.2% (*Medium*).

Fig. 3: Effects of seed cryostorage on *Teramnus labialis* seedling emergence (A), plant height (B), leaf number (C) and leaf coverage (D). Days after sowing (DAS) at which they were measured are given. Results with the same *letter* are not statistically different (t-test, $p>0.05$). Vertical bars represent means ± SE. Overall coefficient of variation (OCV) = (Standard deviation/Average)*100. To calculate this coefficient, the two average values (treatments) were considered. The higher the difference between results, the higher the overall coefficient of variation: *Low* = 1.0 to 22.5%, *Medium* = 22.5 to 43.9% and *High* = 43.9 to 65.4.

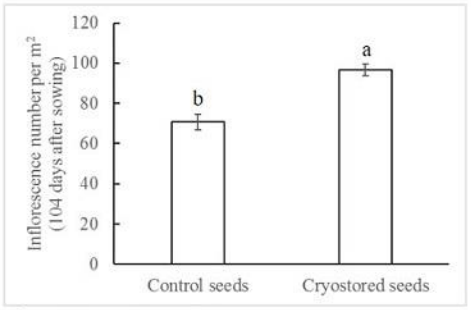
Seed production related traits

Except for seed weight, which was statistically comparable between plants generated from cryostored seeds and the control, all traits related to seed production were significantly higher in plants generated following cryostorage compared to control seeds (Fig. 4). Of the traits that were enhanced, pod number/m^2 was increased the most relative to the control (OCV = 54.7%), while OCVs for the remaining traits (number of inflorescences/m^2, number of seeds/pod, and pod length) were low (2.5-22.0%).

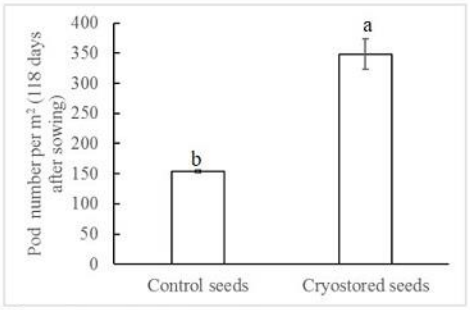
DISCUSSION

The physiological state of germplasm before it is cryobanked and during storage has important implications for its long-term stability and viability (7). Thermal-stress induced fractures of seeds during and after exposure to LN for example (45), may influence their ability to germinate. Fractures typically occur in large samples such as whole seeds and are less common in cell suspensions and meristems. Despite this, seed cryostorage has been reported to have no negative effects on subsequent germination and seedling morphology in *Phaseolus vulgaris* (15) and even enhance germination in *Solanum lycopersicum* (50).

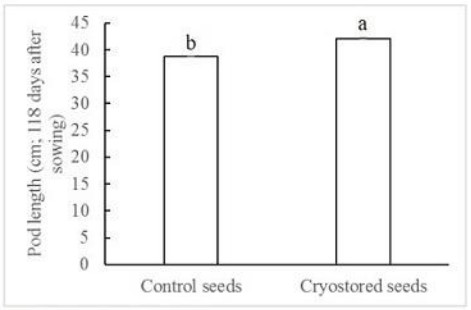
Similarly, our results showed that cryostorage induced cracks and breaks in the coat of *T. labialis* seeds, which may explain the enhanced seedling emergence percentage in these seeds relative to the control. Given that this species exhibits physical dormancy (22), we believe that this cryostorage-induced enhancement in seedling emergence may have



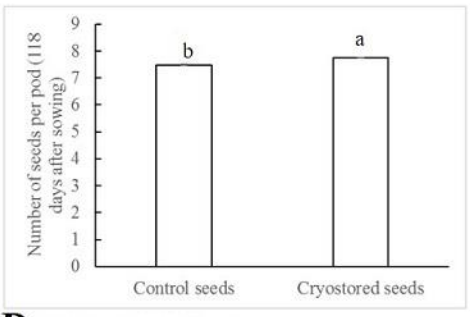
A OCV = 22.0% (*Low*)



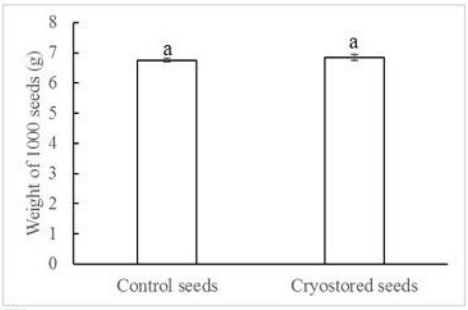
B OCV = 54.7% (*High*)



C OCV = 5.8% (*Low*)



D OCV = 2.5% (*Low*)



E OCV = 1.0% (*Low*)

Fig. 4: Effects of seed cryostorage on *Teramnus labialis* inflorescence number (A), pod number (B), pod length (C), number of seeds per pod (D) and weight of 1000 seeds (E). Days after sowing (DAS) at which they were measured are given. Results with the same *letter* are not statistically different (t-test, $p>0.05$). Vertical bars represent means ± SE. Overall coefficient of variation (OCV) = (Standard deviation/Average)*100. To calculate this coefficient, the two average values (treatments) were considered. The higher the difference between results, the higher the overall coefficient of variation: *Low* = 1.0 to 22.5%, *Medium* = 22.5 to 43.9% and *High* = 43.9 to 65.4.

been the consequence of increased water uptake facilitated by cracks in the seed coat. Abrasion of the seed coat can increase water uptake rates and hence, break dormancy in a number of legumes including members of the genus *Teramnus* (27). Mira et al. (36) observed high percentages of germination in *Medicago polymorpha* seeds following immersion in LN for 24 hours, while Bonilla et al. (10) also obtained 62% emergence increase under field conditions for LN-stored *Sapindus saponaria* seeds. In contrast, Cardoso et al. (11) showed LN treatment to have no significant effects on seed germination in *Lathyrus cicera*, *L. sativus*, *Lens culinaris*, *Lupinus albus*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia articulata*, *V. faba*, *V. monanthos* and *V. sativa*. These authors followed a protocol similar as the one described here. Their evidences showed that not all types of seeds respond equally when stored in LN. Their response depends on seed size, moisture content and the kind of dormancy present.

The seedling emergence phase is probably the most important phenological event that influences the success of a plantation (37). The emergence efficiency is related to the vigour, the growth rate and the establishment of the plantation (26). Our results show that cryostorage of *T. labialis* seeds also enhanced productivity in a number of traits including plant coverage, which is important given the use of this species as a cover crop in a number of

agricultural systems. It was also encouraging to note that the increased leaf coverage in plants derived from cryostored compared with control seeds was sustained throughout the growth period; and even more so during the early stages of the growth period, when this species has been reported to exhibit very slow growth rates (23). Low growth rate negatively affects the survival of seedlings when they share the same environment with other more invasive fast-growing plant species (12). The benefit of rapid germination seems to be associated with conferring a competitive advantage compared to other plants contending for the same resources (e.g. soil nutrients, light, water) (48). The significance of our results is strengthened by the fact that weed species such as *Digitaria decumbens*, *Amaranthus viridis* and *Paspalum notatum* were observed mainly in plots sown with control seeds (data not shown).

Few studies have been carried out on the biochemical and physiological effects of LN exposure/cryostorage on seeds (25). Studies by our group on the effects of cryostorage on the subsequent germination and growth of common bean, tomato, tobacco and maize seeds suggest that while cryostorage may induce some morphological, physiological and biochemical changes in seedling derived from cryostored seeds, these gradually disappeared with development (4, 15, 38, 49). However, information on the effects of seed cryostorage on traits related to reproduction and seed production in plants generated from cryostored seed is limited. It is therefore useful to note that except for seed weight, all traits related to seed production were significantly higher in plants generated from cryostored relative to control seeds. However, a comparison of OVCs across traits suggests that except for pod number/m^2, cryostorage enhanced growth related traits to a greater degree than traits related to seed production (e.g. number of inflorescences/m^2 and number of seeds/pod).

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Author contribution

YA, LH, CM, NQ, BEZ, IC, S, JCL, MEMM and DF designed the research; YA and LH conducted the experiment; YA, LH, CM, NQ, BEZ, IC, S, JCL, MEMM and DF analyzed the data and wrote the paper; and DF had primary responsibility for the final content. All authors have read and approved the final manuscript.

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