PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF PLANTS REGENERATED FROM NON-CRYOPRESERVED AND CRYOPRESERVED WILD *SOLANUM LYCOPERSICUM* MILL. SEEDS

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Abstract

BACKGROUND: Before cryopreservation is routinely used, its effect on the trueness-to-type of the regenerated plant material needs to be evaluated. **OBJECTIVE**: In this work, we studied the effect of seed cryopreservation on the phenotypic and molecular characteristics of wild *Solanum lycopersicum* Mill. plants. **METHODS**: Thirty-five morphological traits of plants regenerated from cryopreserved seeds were compared to those measured on plants regenerated from non-cryopreserved seeds. **RESULT**: No statistically significant differences were observed between cryopreserved and non-cryopreserved samples, either in the first or in the second generation post-liquid nitrogen exposure. However, at the molecular level, the genetic analyses performed on the second generation plants germinated from control and cryopreserved seeds using 14 nuclear Simple Sequences Repeats (SSR) markers uncovered some changes in microsatellite length between control and cryopreserved samples. These results confirm at the botanical phenotype level the effectiveness of seed cryostorage for conservation and regeneration of true-to-type *S. lycopersicum* plants. **CONCLUSION**: Further experiments are required to clarify potential phenotypic effects of the changes observed in the DNA.

Keywords: tomato; cryostorage; phenotypic variation, microsatellites

INTRODUCTION

The word tomato refers both to the plant *Solanum lycopersicum* Mill. and to the edible, typically red, fruit that it bears. Originating in South America, the tomato has spread around the world, the fruit is consumed in diverse ways, including raw, as an ingredient in many dishes and sauces, and in drinks. The vegetable is rich in lycopene, which may have beneficial health effects. The plants typically grow to 1-3 m in height and have a weak stem that often sprawls over the ground and vines over other plants. It is a perennial in its native habitat, although often grown outdoors in temperate climates as an annual (1, 25). Tomato is the world's most important vegetable crop in economic terms. Wild relatives of tomato have great value as genetic resources (24, 32). Consequently, the conservation of wild *Solanum* species, both *ex situ* in germplasm banks and *in situ* in the form of natural populations in order to preserve the genes in their place of origin, is a high priority.

At present, the Polytechnic Agriculture and Cattle Husbandry University of Manabi (ESPAM), Ecuador is conducting a research project, which aims at collecting, characterizing and conserving the wild tomato (*S. lycopersicum* Mill.) genetic diversity occurring in Bolivar Canton (North-central section of the Manabi Province, 537.8 km^2). These populations represent important genetic resources and most of all are potentially very useful in future breeding programs. The objectives of the research project include the characterization of the *in situ* environmental conditions where seeds are collected; *ex situ* phenotypic description and molecular marker analysis of regenerated plants; and establishment of a wild tomato cryobank.

With the unprecedented loss of valuable plant germplasm occurring globally, which also concerns wild *S. lycopersicum* Mill., it becomes increasingly important to conserve seeds *ex situ* in genebanks (29). Seed storage is certainly the most effective and efficient method for *ex situ* conservation of plant genetic resources (19). Recommended optimal seed storage conditions include conservation in hermetically sealed containers at 3-7% moisture content (fresh weight basis) depending on the species and at -18°C or below (10); such conditions guarantee the retention of high levels of viability over extended time spans, possibly centuries (27).

The possibility that much longer periods of seed storage are attainable at ultra-low temperature using cryopreservation has been previously highlighted (27). Under cryopreservation (liquid nitrogen [LN], -196°C), the plant material can be stored without alteration or modification for very long time periods. Moreover, cultures are stored in a small volume, protected from contamination, requiring therefore limited maintenance (7).

An earlier prediction carried out by Pritchard (26) suggested that seed longevity at LN temperature could be about 175 times longer than at conventional seed bank temperature. More recently, cryogenic half-lives up to ca. 3,400 years were predicted for lettuce seeds, based on experiments running for longer than 10 years (34). Comparable estimates of lettuce seed longevity at -18°C, based on the seed viability equation developed by Walters *et al*. (34), were approx. 46-70 years, i.e. 74 times less than at LN temperature. Therefore, as an extra insurance policy for conservation, the use of cryopreservation should be considered for all orthodox seeds, and one sub-sample of any accession systematically stored in LN, in addition to the samples stored under classical gene bank conditions (9, 18).

The physiological state of germplasm before it is cryobanked has important implications for its long-term stability and viability (2). Moreover, physical and biochemical parameters of the plant material have an effect on its storability over long periods (28). Thermal-stress induced fractures of biological materials may cause serious damage to stored samples: fractures typically occur in large organs such as whole seeds and are less common in cell

suspensions and meristems. Most reports of physical cracking are with animal organs (liver slices, veins, and arteries) rather than plant specimens.

Our group has already published some results on the effects of seed cryopreservation on morphological, agricultural, biochemical and physiological indicators of regenerated plants. Cejas *et al.* (5) did not observe any phenotypic changes during the early germination stages (0-14 days) of cryopreserved *Phaseolus vulgaris* seeds. However, several significant effects of seed cryopreservation were recorded at the biochemical level. No statistically significant phenotypic differences were observed for the parameters measured on first or second generation plants (4). The genetic analyses performed on the second generation plants using six nuclear Simple Sequences Repeats (SSR) markers revealed no changes in microsatellite length between control and cryopreserved samples, implying that there was no effect of seed LN exposure on genome integrity.

In case of wild *S. lycopersicum* Mill. seeds, Zevallos *et al.* (35) showed that LN exposure increased the percentage of seed germination at 5 days but at 7 days, the conversion into plantlets and the plant fresh mass were not statistically different between non-cryopreserved and cryopreserved samples. Several significant effects of cryopreservation were recorded at the biochemical level at 7 days of germination under controlled conditions. After LN storage for different time periods (0 to 28 days), control and cryopreserved seeds displayed about 60% germination without statistically significant differences. No phenotypic changes were observed visually in seedlings recovered from the different treatments 7 days after onset of germination (36). However, some biochemical changes were observed between control and cryopreserved samples for 7, 14 and 21 days LN storage.

In this work, we studied the effect of seed cryopreservation on the development of wild *S. lycopersicum* Mill. plants. The main morphological traits of first and second generation plants grown from cryopreserved seeds were compared to those measured on plants grown from non-cryopreserved seeds. Molecular studies were also performed on second generation plants with a set of 14 nuclear Simple Sequence Repeats (SSR). As far as we know, this is the first report on the morphological and molecular characterization of wild *S. lycopersicum* Mill. plants originating from cryopreserved seeds.

MATERIALS AND METHODS

Accession 56 was collected at 0° 55□ 32.7□ South and 80° 02□ 47.5□ West at 95 m above sea level in the Zapote region in Ecuador. The population consisted of a few healthy plants. Flower anatomy seemed to confirm autogamy, which is typical in this plant species. Soil slope varied between 16 and 30%, with average drainage, 65.8 ppm of soluble salt contents and pH 7.0. The soil was black with some isolated stones. After collecting seeds from a single plant in the wild, seeds were sown and grown until harvest in a field experimental station (semi-controlled environment) to increase seed availability.

Seeds were stored for 4 months at 4°C in the dark, in hermetically closed containers. Seeds with 12% moisture content [based on fresh weight (16)] were used. One half of the seeds were placed in cryovials (volume: 2 ml; 50 seeds per cryovial) and immersed in liquid nitrogen (LN) for 2 weeks. 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 (31). From each treatment, seeds were randomly selected to perform the following steps of experimentation.

Morphological analyses of first and second generation plants grown in the field (90 plants per treatment) were performed using the experimental design described in Fig. 1. Seeds were germinated in a seed bed and transplanted 15 days after germination.

Neither fertilizers nor pesticides were applied. The morphological characterization was performed at 120 days of plant growth using a series of morphological traits and characterization descriptors defined by the International Plant Genetic Resources Institute [IPGRI (15)] (Table 1). Border plants, which had more space to grow, were not considered. The Statistical Package for Social Sciences (Version 17.0 for Windows, SPSS Inc.) was used to perform t-tests and compare results of the two studied treatments: non-cryopreserved and cryopreserved seeds (p≤0.05).

To evaluate the genetic stability of the second generation plants germinated from control and cryopreserved samples, five seeds were randomly selected on different plants from each experimental treatment. Seeds were placed on three layers of Whatman No. 1 filter paper in disposable plastic Petri dishes (Ø: 9 cm). The filter papers were imbibed with 4 ml distilled water and seeds were germinated under constant dark conditions at 25°C. As seeds were never in contact with culture media enriched with molecules known to promote somaclonal variation, we can exclude any effects related to the culture procedure. DNA was extracted for analyses. Fully

Figure 1. Experimental design carried out on ferralytic-red soil. Dots symbolize tomato plants. Furrow irrigation was done three times: at transplanting the tomato seedlings from the seed bed, and at 7 and 21 days after transplantation. Plants were illuminated at 458 µmol m^{-2} s^{-1} with sunlight. Border plants, which had more space to grow, were not evaluated in the experiment.
A: Thirty plants from non-cryostored seeds. **B:** Thirty plants from cryostored seeds.

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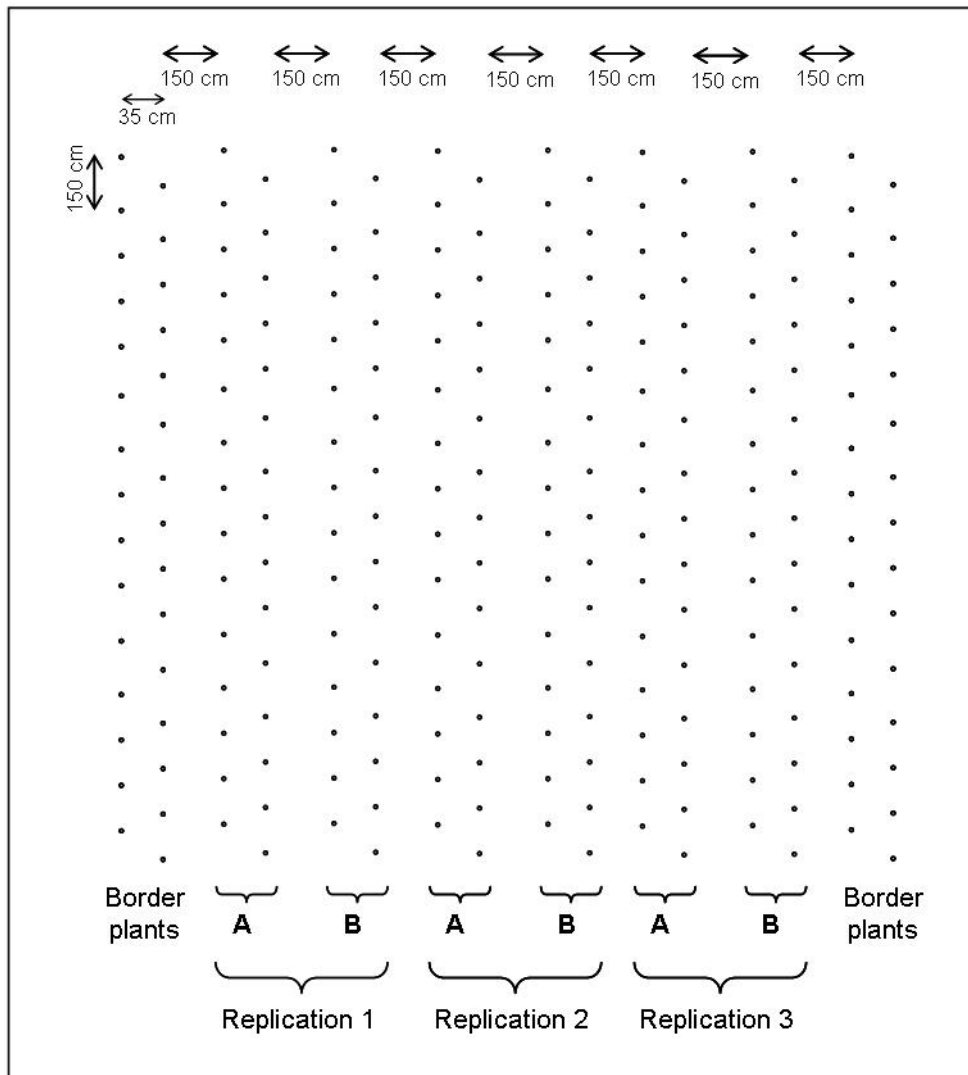
developed young leaves were collected at the same date from 10 plants: five cryopreserved (C) and five non-cryopreserved (NC). From each plant, DNA was isolated using the DNeasy Plant mini kit (Qiagen, Valencia, USA) following the manufacturer's instructions; DNA quality and integrity were checked by gel electrophoresis and spectrophotometric assay.

SSR analysis was performed using fourteen primers (Table 2): LE 20592, LE21085, LE EF1Aa and LELE25 from the 44 sets described by Smulders *et al.* (30); LE at002, LE aat002, LE aat007, LE caa001, LE ct001, LE ctt001, LE ga003, LE ta003, LE tat015 and LE tat002 from the 129 sets described by He et al. (14). Optimization of annealing temperatures and $MgCl_2$ concentration for each primer pair was accomplished by gradient PCR.

PCR reaction in 10 µl final volume consisted of 1×PCR reaction buffer, 0.2 µM of each dNTP, 2 mM $MgCl_2$, 0.3 µM of each primer, 1 U of Taq DNA polymerase Recombinant (Life Technologies, Carlsbad,

Table 1. Botanical characterization of *Solanum lycopersicum* Mill. plants from non-cryopreserved and cryopreserved seeds during *in vivo* growth.

	Plants from non-cryostored seeds	Plants from cryostored seeds	OCV (%)[**]
Length of the longest stem (cm)[*]	280.3 a	271.8 a	2.17
Petal length (mm)[*]	11.1 a	11.0 a	0.63
Sepal length (mm)[*]	5.1 a	5.1 a	0.00
Number of fruits per inflorescence[*]	6.9 a	7.3 a	3.98
Fruit mass (g)[*]	1.5 a	1.5 a	0.00
Fruit length (cm)[*]	1.3 a	1.3 a	0.00
Fruit width (cm)[*]	1.5 a	1.5 a	0.00
Pedicel length (mm)[*]	8.1 a	8.1 a	0.00
Pericarp thickness (mm)[*]	1.3 a	1.3 a	0.00
Fruit core diameter (cm)[*]	0.4 a	0.4 a	0.00
Number of loculus per fruit[*]	2.1 a	2.2 a	3.28
Fruit brix (%)[*]	8.1 a	7.9 a	1.76
Fruit pH[*]	3.8 a	3.9 a	1.83
Type of growth[***]	Semi-determined	Semi-determined	
Stem pubescence density[***]	Intermediate	Intermediate	
Stem internodes length[***]	Short	Short	
Foliage density[***]	Intermediate	Intermediate	
Leaf position[***]	Horizontal	Horizontal	
Leaf type[***]	*Peruvianum*	*Peruvianum*	
Grade of leaf dissection[***]	Intermediate	Intermediate	
Anthocyanin-like colour of leaf veins[***]	Normal	Normal	
Corolla color[***]	Yellow	Yellow	
Type of corolla[***]	Open	Open	
Style position[***]	Inserted	Inserted	
External color of immature fruits[***]	Light green with white veins	Light green with white veins	
Fruit pubescence[***]	Limited	Limited	
Predominant fruit shape[***]	Slightly flattened	Slightly flattened	
Uniformity of fruit size[***]	Intermediate	Intermediate	
External colour of fruits[***]	Red	Red	
Intensity of external fruit colour[***]	High	High	
Facility to separate the fruit from the pedicel[***]	Intermediate	Intermediate	
Shape of the fruit shoulder[***]	Flattened	Flattened	
Facility to peal the fruit[***]	Intermediate	Intermediate	
Pericarp colour[***]	Dark red	Dark red	
Consistence of the fruit at 10 days of harvest[***]	Good	Good	

[*] 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.
[***] According to IPGRI (15).

USA) and 30 ng of genomic DNA. Amplifications were performed in a 96-well Mastercycler® ep system (Eppendorf AG, Hamburg, Germany) under the following conditions: 2 min at 94°C, followed by 35 cycles at 94°C for 45 s, 45-57°C (see Table 2) for 45 s, 72°C for 1 min and a final extension at 72°C for 5 min. Amplification products were firstly separated on 2% ultra-pure agarose gel to check PCR products.

Capillary electrophoresis fluorescence-based SSR analyses were conducted on an ABI PRISM® 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Forward primers were labeled with either FAM (blue), HEX (green) and GenScanTM 500 ROX TM standard (red-labeled) was used as internal size standard (Applied Biosystems), according to manufacturer's instructions; co-loading was then performed when size or colour did not overlap. Sequencing raw data were analyzed with Peak Scanner software (Applied Biosystems, version 1.0) to estimate the variant size.

RESULTS

No statistically significant phenotypic differences were observed for the characters studied between plants originating from non-cryopreserved and from cryopreserved seeds (Fig. 2, Table 1). The phenotypic evaluation of second generation plants did not reveal any statistically significant differences between both treatments (t-test, $p > 0.05$, data not shown but similar to Fig. 2 and Table 1).

Table 2. Description of the 14 SSR markers used to assess the genome integrity of *Solanum lycopersicum* Mill. plants from non-cryopreserved and cryopreserved seeds. For each marker, the respective name, forward (Fw) and reverse (Rv) primer sequences, repeat motifs, expected size, and annealing temperature (Ta) are reported.

SSR name	Primer sequences (5' to 3')	Repeat motif	Size (bp)	Anneal. T°C
LE 20592	Fw_CTGTTTACTTCAAGAAGGCTG Rv_ACTTTAACTTTATTATTGCCACG	$(tat)_{15-1}(tgt)_4$	166	50
LE21085	Fw_CATTTTATCATTTATTTGTGTCTTG Rv_ACAAAAAAAGGTGACGATACA	$(ta)_2(tat)_{9-1}$	104	45
LE at002	Fw_ACTGCATTTCAGGTACATACTCTC Rv_ATAAACTCGTAGACCATACCCTC	$(at)_9$	203	56
LE aat002	Fw_GCGAAGAAGATGAGTCTAGAGCATAG Rv_CTCTCTCCCATGAGTTCTCCTCTTC	$(aat)_{12}$	106	54
LE aat007	Fw_CAACAGCATAGTGGAGGAGG Rv_TACATTTCTCTCTCTCCCATGAG	$(aat)_{12}$	100	50
LE caa001	Fw_AGAAGGCGTGAGAGGCAAC Rv_CTTAGCACTTGATGTTGATTGG	$(caa)_7$	105	50
LE ct001	Fw_TCCAATTTCAGTAAGGACCCCTC Rv_CCGAAAACCTTTGCTACAGAGTAGA	$(ct)_{12}$	111	54
LE ctt001	Fw_CCTCTCTTCACCTCTTTACAATTTCC Rv_CACTGGTCATTAAGTCTACAGCC	$(ctt)_9$	101	57
LE EF1Aa	Fw_AAATAATTAGCTTGCCAATTG Rv_CTGAAAGCAGCAACAGTATTT	$(tat)_8(ata)_9$	131	49
LE ga003	Fw_TTCGGTTTATTCTGCCAACC Rv_GCCTGTAGGATTTTCGCCTA	$(ga)_{20}$	241	50
LELE25	Fw_TTCTTCCGTATGAGTGAGT Rv_CTCTATTACTTATTATTATCG	$(ta)_{13-1}$	225	51
LE ta003	Fw_GCTCTGTCCTTACAAATGATACCTCC Rv_CAATGCTGGGACAGAAGATTTAATG	$(ta)_9$	111	52
LE ta015	Fw_ATATGCATGGACAAATCTTGAGGG Rv_CTCGCGCATCAAATTAATGTATCAG	$(ta)_{15}$	107	50
LE tat002	Fw_ACGCTTGGCTGCCTCGGA Rv_AACTTTATTATTGCCACGTAGTCATGA	$(tat)_{12}$	196	52

Figure 2. Effect of cryopreservation of wild *Solanum lycopersicum* seeds on *ex vitro* plant growth. **A:** Typical middle-aged leaves at 120 days of plant growth. **B:** Typical flowers after anthesis at 120 days of plant growth. **C:** Typical ripen fruits at 120 days of plant growth.
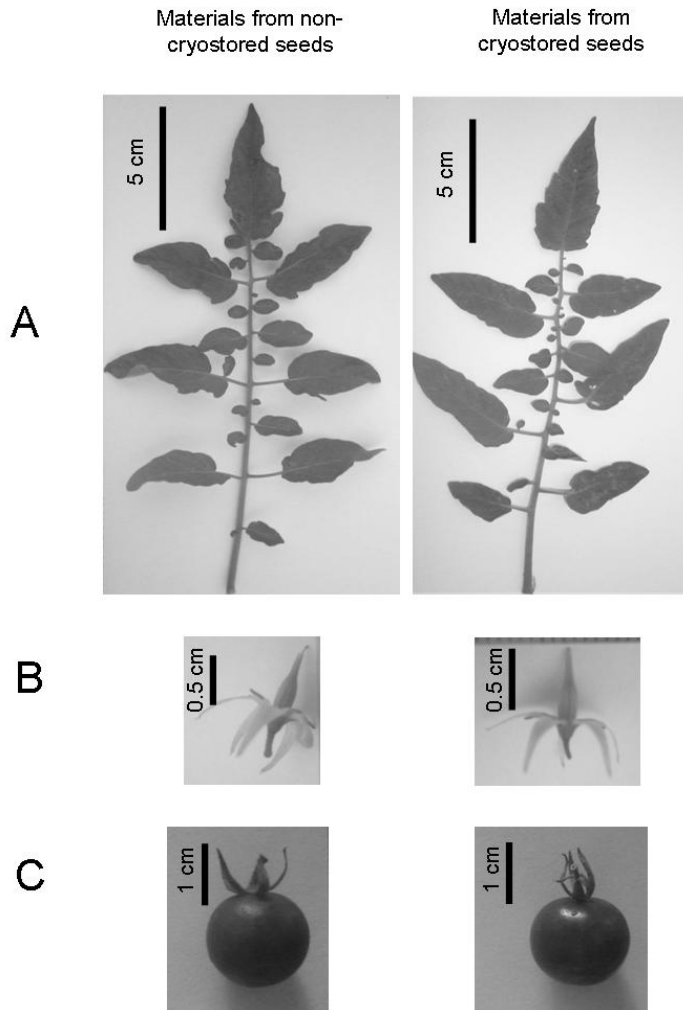
Fourteen SSR loci were selected for their high polymorphism and their small size PCR products (Table 2) and used for genotyping the 10 second generation selected plants. Twelve loci were monomorphic and 18 alleles were detected in total (Table 3). Two SSR loci (LE at002 and LE EF1Aa) showed differences in microsatellite length in one and two cryopreserved plants, respectively. As for LE at002 locus, all samples were homozygous with microsatellite length of 207-207, while the C4 sample was homozygous with a 201-201 SSR size. The same plant was found to be different, together with C6 plant, having a 156-156 locus profile differing from the 204-206 heterozygous profile of the other eight plants. These results indicate that cryopreservation caused some changes in DNA sequence that may potentially affect phenotypic traits not evaluated in the present study (Table 1) or that accession 56 used in this study was not totally homogeneous.

DISCUSSION

Our data showed that first and second generation plants were true-to-type after LN exposure but that some structural changes at DNA level due to LN exposure affected the genome at the loci considered. Polymorphisms have been found in many plant species using a variety of molecular markers including SSR (3, 13).

Table 3 Detected SSR alleles (bp) obtained using 14 microsatellite markers on *S. lycopersicum* Mill. plants from cryopreserved (C) and non-cryopreserved (NC) seeds. In bold are reported the polymoprhic alleles between cryo- and non-cryopreserved plants.

Sample	LE 20592	LE 21085	LE at002	LE aat002	LE aat007	LE caa001	LE ct001
C1	171-171	109-109	207-207	104-104	99-99	100-100	98-98
C3	171-171	109-109	207-207	104-104	99-99	100-100	98-98
C4	171-171	109-109	**201-201**	104-104	99-99	100-100	98-98
C6	171-171	109-109	207-207	104-104	99-99	100-100	98-98
C7	171-171	109-109	207-207	104-104	99-99	100-100	98-98
NC1	171-171	109-109	207-207	104-104	99-99	100-100	98-98
NC2	171-171	109-109	207-207	104-104	99-99	100-100	98-98
NC3	171-171	109-109	207-207	104-104	99-99	100-100	98-98
NC5	171-171	109-109	207-207	104-104	99-99	100-100	98-98
NC6	171-171	109-109	207-207	104-104	99-99	100-100	98-98
Sample	LE ctt001	LE EF1Aa	LE ga003	LELE 25	LE ta003	LE ta015	LE tat002
C1	91-91	204-206	235-251	230-230	97-97	98-98	202-202
C3	91-91	204-206	235-251	230-230	97-97	98-98	202-202
C4	91-91	**156-156**	235-251	230-230	97-97	98-98	202-202
C6	91-91	**156-156**	235-251	230-230	97-97	98-98	202-202
C7	91-91	204-206	235-251	230-230	97-97	98-98	202-202
NC1	91-91	204-206	235-251	230-230	97-97	98-98	202-202
NC2	91-91	204-206	235-251	230-230	97-97	98-98	202-202
NC3	91-91	204-206	235-251	230-230	97-97	98-98	202-202
NC5	91-91	204-206	235-251	230-230	97-97	98-98	202-202
NC6	91-91	204-206	235-251	230-230	97-97	98-98	202-202

Cryopreservation imposes a series of stresses to the plant material, which can induce modifications in cryopreserved explants and regenerated plants (2). It is thus necessary to verify that genetic stability of cryopreserved material is not altered before routinely using this technique for long-term conservation of plant genetic resources (8).

There are very few reports of phenotypic variations occurring during *in vitro* culture of plant materials recovered from cryopreservation. One example concerns phenotypic alterations in *Chrysanthemum* flower colouring after regeneration of 106 cryopreserved apices (11). On the other hand, Medina *et al.* (22) found differences in strawberry fruit production among 50 plants derived from cryopreserved apices.

Recent studies comparing the vegetative and floral development in the field of plants originating from control and cryopreserved material performed with several species including oil palm [about 52 plants studied per clone (17)], potato [120 apices per variety per batch, (23)], and banana (6) did not reveal any differences in the studied characters. In our laboratory, we studied the field performance of sugarcane plants originating from control and cryopreserved embryogenic calluses, compared to plants produced using classical macropropagation [100 plants per treatment (20, 21)]. The results showed only transitory differences between plants originating from *in vitro* cultured materials, irrespective of their cryopreservation status and *in vivo* plants. Stems produced from *in vitro* cultured materials had a smaller diameter and a shorter height compared to those produced from macropropagated buds. These differences were not seen after 12 months of

stool field growth. Our previous experiments on the effect of LN exposure on common bean and wild tomato seeds did not show any phenotypic change in the macroscopic traits of young or adult plants grown *in vivo* (4, 5, 35, 36).

Although the effects of the cooling/warming cycle on the genome are unknown, the potential variations observed may not be due to cryopreservation per se but may be the result of the whole *in vitro* culture-cryoprotection-regeneration process (2, 12). Walters *et al.* (34) commented that variability in ageing kinetics within a seed species could not be accounted for by water content and temperature only; the effect of genotype x environment interactions during plant growth and seed formation before cryostorage is also relevant. These authors suggested that the basis for this variability was not only genetic but due to environmental factors and cautioned that seed gene bank operators could not assume that a particular accession would exhibit average deterioration kinetics.

Another important finding resulting from thermodynamic studies, which contradicts the classical rule of thumbs that "the lower the storage temperature and the moisture content of a seed, the longer the viability" is that the optimal water content for seed storage in the glassy state increases with decreasing temperature (33, 34). Clearly, the physiological status of germplasm before it is cryobanked has important implications for its long-term stability and viability.

In the gene bank context and from an agronomic point of view, the effect of LN exposure on seed viability and germination should be tested for each plant material before using cryopreservation for long-term storage. As far as we know, there is no published report on the impact of LN exposure on *S. lycopersicum* adult plants derived from cryostored seeds. The results presented in this paper confirm at the botanical phenotype level the effectiveness of *S. lycopersicum* seed cryostorage to conserve and regenerate true-to-type plants,

although experiments with higher numbers of plants are required to screen potential mutants and to study homogeneity of accession 56.

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