CRYOSTORAGE OF SUNFLOWER SEEDS

CRIOARMAZENAMENTO DE SEMENTES DE GIRASSOL

Ludymila Brandão MOTTA[1]; José Carlos LOPES[2]; Rafael Fonsêca ZANOTTI[1]; Paula Mauri BERNARDES[1]; Josimar Aleixo da SILVA[1]

1. Programa de Pós-graduação em Produção Vegetal, Universidade Federal do Espírito Santo, Centro de Ciências Agrárias - CCAUFES, Alegre-ES, Brasil. ludymilamotta@gmail.com; 2. Professor do Departamento de Produção Vegetal, Universidade Federal do Espírito Santo, Centro de Ciências Agrárias - CCAUFES, Alegre-ES, Brasil.

ABSTRACT: Sunflower seeds present a great economic importance due to the high oil content. The aim of this study to evaluate the effect of storage sub-zero temperatures and the quick and slow thawing methods on the physiologic quality of seeds of *Helianthus annuus,* cultivars BRS 122 and BRS 324. The experiment was set up in a randomized complete design, in a factorial 2x6 (cultivars x procedures) with four replications; means were compared by the Tukey test at 5%. Were evaluated: first count and percentage of germination, mean time of germination, germination speed index, dry mass seedling, root and shoot length and stability of membranes, through electrical conductivity test solution soaking of seed. Sunflower seeds tolerate storage in sub zero temperatures up to six months; the seeds of cultivars BRS and BRS 324 122 present different answers in relation to the type of freezing and thawing.

KEY-WORDS: *Helianthus annuus*. Storage. Freezing. Cryopreservation. :Thawing.

INTRODUCTION

Plant's genetic resources can be preserved in the form of seeds, pollen, grains, vegetative bodies and field plantations. It is essential that the preservation method ensures the maximum viability and genetic stability of the accesses, and also that materials may be preserved free from pathogens, in a controlled and accessible location, in order to facilitate their multiplication and use (BENSON, 2008).

The type of *ex vitro* germplasm preservation depends on the material used, as seeds, embryos, other vegetative structures or complete individuals. The maintenance may be performed in distinct conditions, such as in field and greenhouse, *in vitro*, in culture medium, cold chambers and under cryopreservation at -150 ºC or -196 ºC (SANTOS, 2001).

Cryogenic techniques may ensure the preservation of biologic material for long periods of time, once that when the vegetable material is submitted to ultralow temperatures, the cellular metabolism is really reduced, slowing down the deterioration process. Thus, the biological material may be preserved for an indefinite time and with the maintenance of genetic stability (HARDING, 2004; BENSON, 2008).

Cryopreservation of biological materials has proved an efficient maintenance and seed explants important for endangered status (SARTOR et al., 2012; ZANOTTI et al., 2012) and for the potential use as ornamentals (WANG et al ., 2011; SURENCISKI et al., 2012), biofuel (SILVA et al., 2012; SURANTHRAN et al., 2012), food (CAMILLO et al., 2009; CEJAS et al., 2012), aromatic and medicinal (KHOLINA et al., 2012; COELHO et al., 2012).

One important factor to be considered in cryopreservation is the thawing method. The longer the thawing of the seeds occurs, the better the preservation of its physiological characteristics will be (MOLINA et al., 2006).

Preservation of seeds also is also influenced from their chemical composition. Due to chemical instability of lipids, oilseeds are deteriorated more quickly, therefore, a prolonged preservation is not recommended because oilseeds may suffer rancid effects of the fatty acids that compose the oil (MARCOS FILHO, 2005).

Therefore, preservation methodologies of phytogenetic resources require knowledge about the physiology of the material to be preserved. When it comes to seeds, studies that assess the effect of storage methods are crucial to ensure the physiologic quality and keep the viability for a longer period. Considering that sunflower seeds present a great economic significance due to the large oil contents, the purpose of this study was to assess the effects of the storage in sub-zero temperatures and the quick and slow thawing methods on the physiologic quality of seeds of *Helianthus annuus,* cultivars BRS 122 and BRS 324.

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MATERIAL AND METHODS

The experiment was performed in the Laboratory of Seed Analysis of the Department of Vegetable Production of the Center of Agricultural Sciences in the Federal University of Espírito Santo (*Laboratório de Análise de Sementes do Departamento de Produção Vegetal do Centro de Ciências Agrárias da Universidade Federal do Espírito Santo* - CCA-UFES), located in Alegre-ES.

The design experimental used was 2x6 factors (cultivars x procedures). The seeds were submitted to the following freezing and thawing procedures (Figure 1):

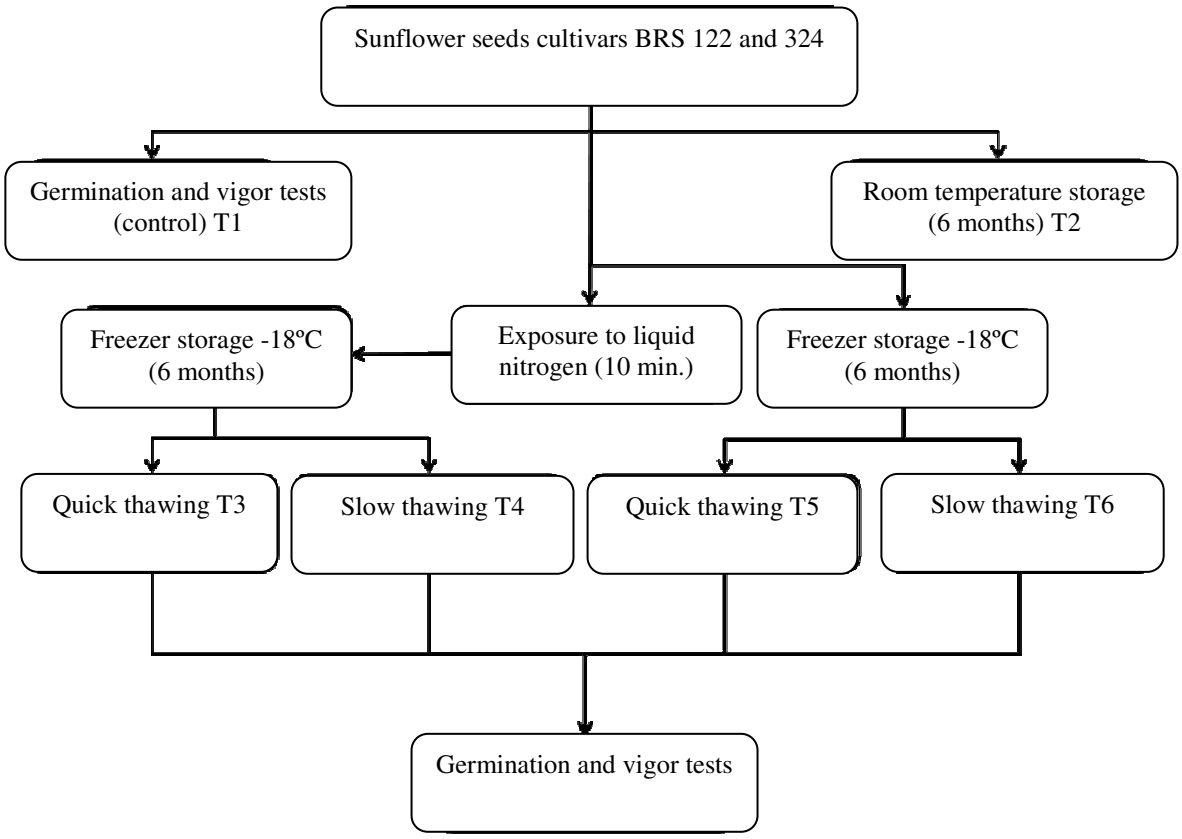

Figura 1: Methodology used to assess the physiologic qualities of *H. annuus* seeds, cultivars BRS 122 and BRS 324 stored in sub-zero conditions. T1- Control: Non-stored seeds; T2: Room temperature storage (6 months) T3: Immersion in liquid nitrogen (10 min.), -18 ºC (6 months) and water bath (37 ºC, 30 min.); T4: Immersion in liquid nitrogen (10 min.), -18 ºC (6 months) and room temperature (12 hours); T5: -18 ºC (6 months) and water bath (30 min.); T6: -18 ºC (6 months) and room temperature (12 hours).

On germination tests, four repetitions of 25 seeds were used in three 'germitest' paper sheets. The sheets was damped in distilled water volume equivalent to 2.5 times the weight of the dry papers. The seeds were manually distributed in longitudinal orientation of the rolls and kept in a germination chamber at 25±2 ºC. The analyses performed were the first count at four days and the daily count, conducted until the tenth day, as recommended for the species (BRASIL, 2009). The germination percentage was calculated, mean germination time and germination speed index (GSI). For that, it was considered as germinated seeds those which presented protrusion of primary root with length ≥ 2.0 mm. The GSI was performed by daily computing the number of germinated seeds, using the formula proposed by Maguire (1962).

At the end of the germination test, the seedlings were dehydrated at 70±2 ºC for a period of 72 h. After this period, the dry seedlings were weighed in a precision scale (0.0001 g), in order to obtain the mean weight of dry mass per seedling (mg seedling^{-1}) (NAKAGAWA, 1999).

In the electric conductivity test, four repetitions of 25 seeds were weighed in precision scales (0.0001 g) for each treatment and, next, the seedlings were immersed in 75 mL of distilled water for 24 h, at a temperature of 25 °C. The conductivity reading of the damping solution of the seeds of each treatment was performed in a conductivimeter, with

results expressed in μS cm^{-1} g of seeds (VIEIRA and KRZYZANOWSKI, 1999).

The data were statistically interpreted through the variance analysis and the means were compared by the Tukey test in 5% level. The analyses were performed in the statistical program Assistat 7.6 beta (SILVA and AZEVEDO, 2002).

RESULTS AND DISCUSSION

The germination percentage values (Table 1) of the cultivar BRS 122 were higher than BRS 324 for storage at room temperature (T2) and quick freezing followed by quick thawing (T3). For the other conditions tested, regarding the germination percentage, there has been no difference between cultivars. For BRS 324, the room temperature storage presented the lowest germination percentage, therefore, regardless of the type of freezing and thawing, the storage at sub-zero temperature was effective in maintaining germination regarding the control. It is possible that the seeds BRS 324 lot presented lower vigor than the BRS 122 lot.

Comparing the methods used, in BRS 122 it was observed no differences in the first count. For BRS 324, the storage at room temperature has determined a higher decrease in this parameter. The chemical instability of lipids is one of the factors which are directly related to the reduced performance of the seeds from several species, especially oilseeds. Lipid peroxidation and oxidation stress have caused deterioration of seeds during their aging. In sunflower seeds, vigor and viability reduction during the aging is related to the decreased potential of antioxidant enzymes, and, thus, to the increase in lipid peroxidation (TORRES et al., 1997).

Table 1. Germination (%), first count and germination speed index (GSI) of sunflower seeds stored at -18 °C and subjected to different freezing and thawing methods.

Germination (%)						
Cultivares	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
BRS 122	99 aA	96 aA	98 aA	98 aA	96 aA	98 aA
BRS 324	99 aA	66 bB	93 bA	95 aA	93 aA	94 aA
CV(%)	3.24					
First count (%)						
BRS 122	98 aA	95 aA	98 aA	98 aA	96 aA	97 aA
BRS 324	95 aA	65 bB	93 bA	91 bA	93 aA	94 aA
CV(%)	3.61					
GSI						
BRS 122	12.74 aA	11.51 aB	12.21 aAB	12.15 aAB	11.92 aB	11.97 aB
BRS 324	13.25 aA	7.36 bB	11.48 bA	11.16 bB	11.46 aB	11.42 bB
CV(%)	3.13					

Values marked with the same lowercase letter are not significantly different in column, at P<0.05, Tukey's test. Values marked with the same uppercase letter are not significantly different in line, at P<0.05, Tukey's test.

GSI for BRS 122 was lower in T2, T5 and T6 if compared to the control (T1), and in T3 and T4, both methods of quick freezing, they were not different between from each other, neither in relation to the control. In BRS 324, T2, T5 and T6 were also prejudicial to the GSI, as well as T4. It was noted that T3 presented the highest GSI for BRS 324, being the only method that was not different from the control. Therefore, in addition to the pre-immersion in liquid nitrogen, the quick thawing was fundamental for the maintenance of the GSI for this cultivar.

Quick freezing may result in intracellular crystals formation, which is lethal for the seeds' cells and tissues. However, slow thawing may result in damages or cellular death, because it occurs an extreme osmotic dehydration, when the intracellular water moves outside the cell, compensating the frozen water of the extracellular components (DUMET; BENSON, 2000).

Papaya seeds quickly thawing in water bath at 40 °C, presented reduced germination power. With the use of tetrazolium salt, it was possible to notice that the seed were cracked in the section of the cotyledons evidences by clear and irregular stains in the cotyledons and embryo axis (ALTHOFF; CARMONA, 1999).

It is noted that to preserve the seeds of oleaginous species, cryopreservation is a good alternative. The seeds of *Helianthus* spp, *Glycine max, Ricinus communis* and *Arachis hipogagaea* may be cryogenically stored both in liquid and

vapor nitrogen. Seeds of *Gossypium hirsutum* maintained the highest germination percentage when cryogenically stored directly in liquid nitrogen and seeds of *Glycine max* when stored in the vapor of liquid nitrogen (ALMEIDA et al., 2010).

The percentage of normal seedlings of cultivar BRS 122 submitted to any of the methods has not differed from the controle (Table 2). There has been a difference only between T3 and T5 in relation to T4, and both methods that used the quick thawing (T3 and T5) obtained the higher percentage of normal seedlings, regardless of the quick or slow freezing. A different result was found for BRS 324, in which T2, T3, T4 and T6 were not different from the control. There has been a difference only between T1 and T4, which presented higher percentage of regular seedlings regarding T5. Comparing the percentage of regular seedlings amongst the cultivars, it is noted that BRS 122 was negativelly affected by T4 if compared to the other cultivar tested. On the other hand, for BRS 324, T5 reduced the development of normal seedlings.

For the analysis of dry mass of the seedlings, there was no interaction between factors cultivar x storage methods, therefore, the factors were tested separately (Table 2). Seedlings from the cultivar BRS 122 presented higher accumulation of dry mass if compared to BRS 324. However, the results associated to the storage methods factor show that there has been damage in the accumulation of dry mass, both for room temperature storage and sub-zero temperature storage. This shows that the seeds from both cultivars presented reduced vigor during storage.

Tabela 2. Normal seedlings (%), mean weight of dry mass per seedling (mg seedling^{-1}), length of shoot (cm) and length of root (cm) of sunflower seedlings, from seeds stored at sub-zero temperatures and under different thawing methods.

Normal plants (%)							
Cultivares	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**Média**
BRS 122	73 aAB	74 aAB	82 aA	56 bB	79 aA	65 aAB	___
BRS 324	82 aA	66 aAB	77 aAB	80 aA	59 bB	76 aAB	___
CV(%)	12.48						
Mean weight of dry mass per seedling (mg seedling^{-1})*							
BRS 122	110.0	35.0	35,0	35.0	32.5	37.5	47.5 A
BRS 324	102.5	17.5	32.5	30.0	32.5	30.0	40.8 B
Mean	106.3 a	26.3 b	33.8 b	32.5 b	32.5 b	33.8 b	
CV(%)	12.8						
Length of shoot (cm)*							
BRS 122	6.14	8.37	7.33	6.99	7.91	5.46	7.03 B
BRS 324	6.24	7.79	9.47	8.30	7.78	8.21	7.96 A
Mean	6.19 b	8.08 ab	8.40 a	7.65 ab	7.84 ab	6.83 ab	
CV(%)	18.02						
Length of root (cm)							
BRS 122	2.62 aC	6.69 aA	5.47 aAB	4.10 aBC	6.28 aA	3.03 bC	___
BRS 324	3.00 aB	3.97 bAB	4.75 aA	4.07 aAB	3.97 bAB	4.30 aAB	___
CV(%)	17.63						

Values marked with the same lowercase letter are not significantly different in column, at P<0.05, Tukey's test. Values marked with the same uppercase letter are not significantly different in line, at P<0.05, Tukey's test.
* There was no interaction between the factors.

In the length of shoot, the interaction between factors was also not significant. The cultivar BRS 324 presented higher shoot development. This fact may be related to the different size of the cultivars, once the mean height of plants of BSR 324 is higher than the mean height of BRS 122.

Regarding the storage methods factor, the growth of shoots was estimulates when the seeds were submitted to quick freezing and quick thawing, and it differed from the control.

Comparing the cultivars about the length of the root, differences were fond for T2 and T5, in which BRS 122 presented larger roots, and for T6, it was noted the opposite. Amongst the storage methods employed, it was verified that, for BRS 122 T2, T3 and T5 enable higher growth of roots, were not different amongst them and differed from T1, T4 and T6. BRS 324 had a distinct behavior, and the

only method that overcame the control was T3. The other methods showed no significant difference.

Factors such as physical and chemical properties, determination of ideal water contents, proper freezing and thawing rates are known for presenting a species-specific response; therefore, require studies and tests for each species (DICKIE; SMITH, 1995; POTTS; LUMPKIN, 1997; ENGELMANN, 2000). The present study highlights that such differences may also occur in cultivars of the same species.

Despite of the increase in the length of the aerial part and root for some of the storage methods, if compared to the control, the accumulation of dry mass in all methods was very inferior for dry mass.

Another vigor test used was the electrical conductivity test (Table 3). It was performed only in seeds that were stored.

Tabela 3. Electrical conductivity (µS cm g^{-1}) in sunflower seeds stored at sub-zero temperatures and under different thawing methods.

	Electrical conductivity (µS cm g^{-1})					
Cultivares	**T1***	**T2**	**T3**	**T4**	**T5**	**T6**
BRS 122	___	113 bAB	107 bAB	128 aA	99 bB	101 bAB
BRS 324	___	213 aA	152 aBC	132 aC	159 aBC	172 aB
CV(%)	9.94					

Values marked with the same lowercase letter are not significantly different in column, at P<0.05, Tukey's test. Values marked with the same uppercase letter are not significantly different in line, at P<0.05, Tukey's test.

Comparing the storage methods for BRS 122, there was a difference only between T4 and T5. The other methods were not different amongst them. Therefore, there was a higher leaching in electrolytes in the method using quick freezing and slow thawing. Cells submitted to a quick freezing, enough to enable intracellular freezing, are far more sensible to slow thawing than to quick thawing. During the slow process, there is a time for slow crystals to aggregate and to build large crystals (recrystallization), thus leading to cellular damage and consequent death (FARRANT et al., 1977; SEKI; MAZUR, 2008).

The storage at room temperature contributed for an accelerated loss of vigor of seed of the cultivar BRS 324. Seeds' membranes of BRS 324 have become less adamant in storage at room temperature, and the storage methods used were effective in prolonging the vigor of seeds during the storage period. In addition, quick thawing was more drastic when seeds were exposed to liquid nitrogen (T3). It is noted that, amongst the methods using slow thawing, the one which used the quick freezing (T4) contributed more to maintaining the integrity of membranes and, consequently, the vigor of seeds, than the method using slow freezing (T6).

The membranes are the first structures that damage due to freezing (HALLGREN; OQUIST 1990; ODLUM; BLAKE 1996). Once the stress increases the permeability of the membrane, this results in pouring of ions, amino acids and other components of the cells. The leaching of electrolytes through the membranes, therefore, may be used to measure the tolerance to stress and predict the mortality of seedlings (STEPONKUS, 1984; ZWIAZEK; BLAKE 1990). The loss of the natural semi-permeability of membranes may lead to inactivation of enzymes, disturbance of proteins metabolism, damages to the selective transportation of membranes and cessation of mitochondrial breathing (LARCHER, 2004).

Lower deterioration of sunflower seeds was indicated by the level of peroxides, for the storage in liquid nitrogen for three months, if compared to the cold chamber (-20 ºC). It was also noted that sunflower seeds may be dried up to 3.2% of their water contents in silica gel or drying chamber and stored at -20 °C or -196 °C, with no loss of germination and vigor (JOSÉ et al., 2010).

CONCLUSIONS

Sunflower seeds tolerate the storage at sub-zero temperatures up to six months, whereas the seeds of the cultivars BRS 122 and BRS 324 present different responses regarding the type of freezing and thawing.

For the cultivar BRS 122, a higher percentage of normal seedlings was obtained with methods that used quick thawing (T3 and T5), regardless if it was used quick or slow thawing.

For BRS 324, the quick freezing with slow thawing provided higher percentage of regular seedlings if compared to the slow freezing and quick thawing (T4).

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RESUMO: As sementes de girassol apresentam grande importância econômica devido ao grande conteúdo de óleo. Objetivou-se neste trabalho avaliar o efeito do armazenamento em temperaturas sub-zero e os métodos de descongelamento rápido e lento sob a qualidade fisiológica de sementes de *Helianthus annuus* cultivares BRS 122 e BRS 324. Utilizou-se o delineamento experimental inteiramente casualizado, num fatorial 2x6 (cultivares x procedimentos), com quatro repetições; as médias foram comparadas pelo teste de Tukey em nível de 5%. Foram avaliados: primeira contagem e porcentagem de germinação, tempo médio de germinação, índice de velocidade de germinação, massa seca de plântulas, comprimento de raiz e parte aérea e estabilidade das membranas, através de teste de condutividade elétrica da solução de embebição das sementes. As sementes de girassol toleram o armazenamento em temperaturas sub-zero até seis meses; as sementes dos cultivares BRS 122 e BRS 324 apresentam respostas diferentes em relação ao tipo de congelamento e descongelamento.

PALAVRAS-CHAVE: *Helianthus annuus*. Armazenamento. Congelamento. Criopreservação. Descongelamento.

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