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Full Length Research Paper

Cryopreservation of quina seeds (*Strychnos pseudoquina* A. St. Hil)

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This study aimed to evaluate the effects of storage in liquid nitrogen at -196°C on *Strychnos pseudoquina* A. St. Hil. seeds with moisture contents of either 6 or 7%wb subjected to quick thawing (in a water bath at 37°C for 10 minutes) and slow thawing (at room temperature (26°C±2°C) for 2 hours) and then stored for 2, 4, and 6 months. The germination percentage in cryopreserved quina seeds decreased by approximately 12.4% compared to non-cryopreserved seeds (95 to 83.22%), regardless of the moisture content. In cryopreserved seeds with 7% moisture content, the germination percentage of seeds thawed quickly versus slowly differed only for those seeds stored for 2 months; the seeds subjected to fast thawing showed lower germination percentages than those subjected to slow thawing. Quina seed cryopreservation may be viable for seed conservation as germination remained above 80% in all samples.

Keywords: Storage, Germination, Viability.

INTRODUCTION

The species *Strychnos pseudoquina* A. Hil St. (Loganiaceae) is commonly known as quina-do-cerrado (quina) and is native to the Brazilian Savannah (Cerrado). It is found in the states of Minas Gerais, São Paulo, Goiás, Mato Grosso do Sul, and Tocantins (Almeida et al., 1998). The prepared peels of quina are used medicinally to treat stomach and liver problems. Due to the regional importance of this species, it is necessary to find techniques for seed storage for long durations that do not result in a decrease of vigor or loss of the seeds' physiological characteristics.

Storage at low temperatures, including cryopreservation in liquid nitrogen, is an efficient and practical approach to conserve plant genetic resources. Medeiros and Cavallari (1992) define cryopreservation in liquid nitrogen as the preservation of biological materials at low temperatures (between -160 and -196°C), whereby all metabolic processes are essentially suspended, allowing for long-term preservation.

Although it is a relatively new science, cryopreservation protocols have been developed for many plant species, including vegetative propagated plants, grasses, ornamentals, tropical and temperate fruits, legumes, oilseeds, medicinal plants, and aromatic plants (Santos, 2001).

It should be noted, however, that cryopreservation should not be used as a replacement for traditional methods of *ex situ* conservation. Cryopreservation offers those responsible for germplasm banks an alternative in addition to existing methods for germplasm conservation. The choice of a particular conservation method depends on the desired duration of conservation,

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the species to be conserved, the part of the plant to be preserved, the availability of labor, and the financial resources available. Within the next few years, cryopreservation will probably be used more often for storage of plant materials and conservation of plant genetic resources for long durations of time (Engelmann, 2004).

According to Cunha (1996), seed water content is probably the most critical factor in successful cryopreservation; if the level is too high, instant seed death occurs during the freezing and/or thawing process.

In a germplasm bank at low temperatures, the cryopreservation process should not be the only factor taken into consideration. The seed thawing method must also be considered because the faster thawing occurs, the better the preservation of seed physiological characteristics. Thawing at room temperature can be questionable because there is a possibility of refreezing during this period. For this reason, thawing should occur in a water bath at 37°C to 40°C for 5 minutes (Molina et al., 2006).

We studied the possibility of cryopreserving quina (*Strychnos pseudoquina* A. St. Hil.) seeds with moisture contents of 6 and 7%wb and determined the most suitable thawing methods for different durations of seed cryopreservation.

MATERIALS AND METHODS

In this study, we used seeds from quina fruit collected from the Gameleira farm in the municipality of Montes Claros de Goiás - GO, Brazil (16° 06'20"S - 51° 17'11"W, 395m above sea level).

Seed processing

After harvest, the fruits were sent to the Seed Laboratory of the Federal Institute of Education, Science, and Technology of Goiás -Rio Verde Campus-GO for pulping and obtaining seeds. The seeds were treated with Thiram-Vitavax® fungicide (Active Ingredient (carboxin + thiram): 200 + 200 g/L] at a dosage of 300 ml fungicide diluted in 500 ml distilled water per 100kg of seeds to prevent contamination by fungi. The initial seed water content (on a fresh-weight basis) was then determined using the standard 105±3ºC oven method, which is based on drying the product for 24 hours in an oven, as described in the Rules for Seed Analysis (Brazil, 2009).

During drying, the seeds were placed in an oven at 33±3°C. The water content was obtained by monitoring the loss of mass in the seeds during drying. The seed samples with known initial masses were put on trays and placed in the oven to monitor their loss of mass at regular intervals. The final masses of the samples, corresponding to the desired moisture contents, were calculated with the equation mentioned by Cromarty et al., (1985).

Seed cryopreservation

For cryopreservation, seeds with moisture contents of 6 and 7% were separately covered with aluminum foil, packed into cylindrical aluminum tubes (canisters), and then placed into nitrogen tanks at -196ºC for 2, 4, and 6 months (Figure 1).

Seed thawing

To evaluate the effect of thawing type on germination in cryopreserved seeds after cryogenic treatment, the seeds were thawed via slow thawing at room temperature (26°C ±2°C) for a 2-hour period or via fast thawing in a water bath at 37°C for 10 minutes.

Seed physiological characterization

Before and after cryogenic treatment, the germination percentage and germination rate index (GRI) of the seeds were tested. To evaluate the germination percentage, seeds with different moisture contents were placed in a "Mangelsdorf" incubator at 30°C. The seeds were germinated on “germitest” paper moistened with distilled water: three sheets of absorbent “germitest” paper were moistened with water weighing 2.5 times their dry weight. The GRI was determined by counting the number of germinated seeds weekly until the last day of germination, and germination was determined stable 15 days after the last seed germinated. The GRI data were then calculated with the formula proposed by Maguire (1962).

Statistical analysis

The experiment was carried out in a completely randomized factorial design of 2 x 3 x 2 + 2 with four replicates of 20 seeds, each including 2 moisture contents, 3 storage durations, 2 thawing methods, and 2 additional treatments (controls; non-cryopreserved seeds with moisture contents of 6 and 7%). For the characteristic germination percentage, variance analysis was performed followed by the mean test. However, for the GRI, it was necessary to perform non-parametric analysis because normality and homogeneity assumptions were not met after log, root, and exponential transformations.

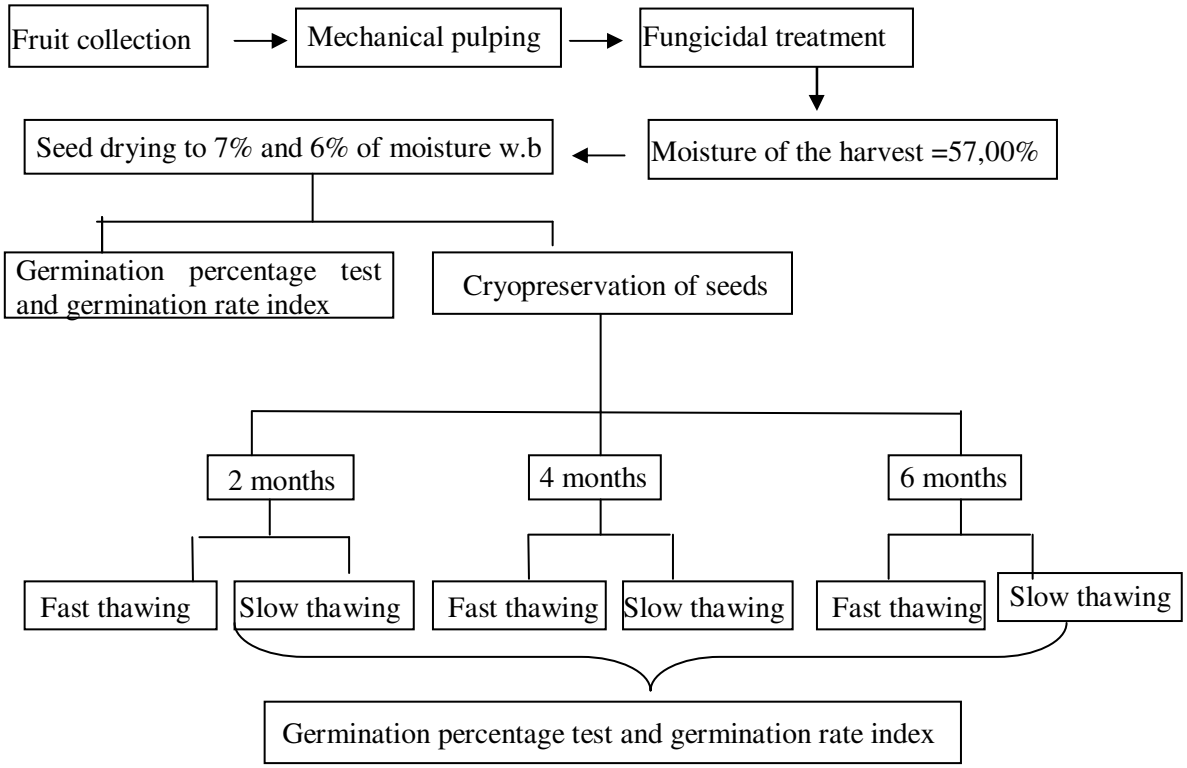

Figure 1: Methodology used to assess the germination percentage and the germination rate index of cryopreserved quina (*Strychnos pseudoquina* A. St. Hil.) seeds.

RESULTS AND DISCUSSION

After determining their fresh-weight moisture content (57.00%), seeds were subjected to drying until reaching moisture contents of 6 and 7%. These moisture contents were chosen because, to study the effects of seed conservation with low moisture content in liquid nitrogen, the seed water content must be between 4 and 7% to preserve physiological potential in liquid nitrogen (Stanwood, 1984).

According to the results, quina seeds are tolerant to dehydration. The lowest moisture content achieved in quina seeds was 6%. For cryopreservation of seeds of the genus *Coffea*, the critical level for preservation is 20% moisture content because seeds of this genus have intermediate tolerance to dehydration, i.e., they can tolerate dehydration at relatively low levels but are damaged by exposure to temperatures below zero when dry (Dulloo et al., 2009).

Considering cryopreserved seeds with 7% moisture content, the germination percentage of seeds thawed by the fast versus slow methods differed only in 2-month storage; seeds subjected to fast thawing showed lower germination percentages than those subjected to slow thawing. When seeds were stored for 4 or 6 months, the thawing method had no influence on germination. Comparing different storage durations with a given thawing method, seeds stored for 2 months and thawed quickly showed lower germination percentages than those stored for 4 or 6 months. For the slow thawing method, the germination percentages remained the same in seeds stored for 2 months and those stored for 6 months. Moreover, seeds stored for 4 months had higher germination percentages than those stored for 6 months (Figure 2).

For seeds with 6% moisture content, there was no difference between the fast and slow thawing methods, regardless of the storage time. In comparing storage durations with a given thawing method, seeds subjected to both fast and slow thawing after storage for 2 months had lower germination percentages than those stored for 4 or 6 months. In seeds stored for these durations, the germination percentages remained the same, regardless of the thawing method (Figure 3). Most studies have shown that the seed germination percentage may decrease with storage time; however, quina seeds stored for longer durations of time had higher germination percentages. Responses to storage time vary greatly among plant species. It was recently demonstrated that the germination rates for *Tuberaria macrosepala*

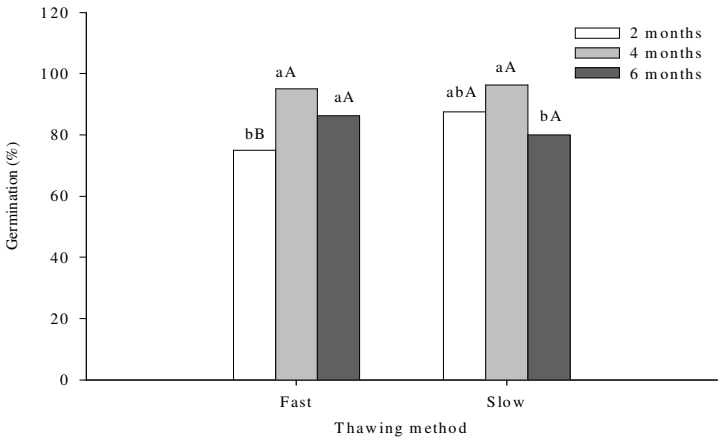

Figure 2: Germination percentage of cryopreserved quina (*Strychnos pseudoquina* A. St. Hil.) with 7% water content with different thawing durations and methods. Lowercase letters following the means denote the storage duration, and uppercase letters denote the thawing method; the values did not differ by the Newman-Keuls test (P ≤ 0.05) and the t test (P ≤ 0.05), respectively.

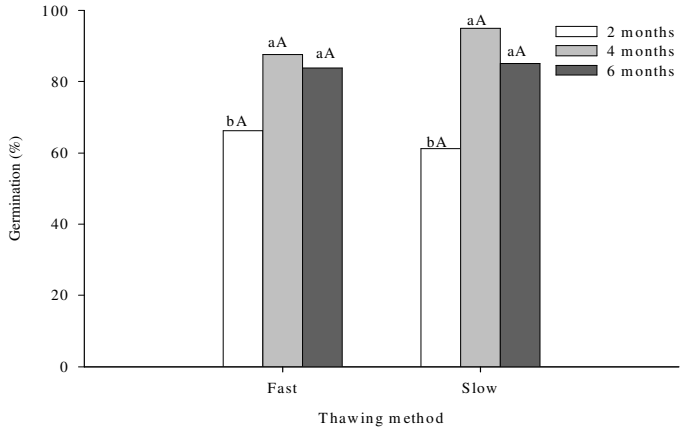

Figure 3: Germination percentage of cryopreserved quina (*Strychnos pseudoquina* A. St. Hil.) seeds with 6% water content under different thawing durations and methods. Lowercase letters following the means denote the storage duration, and uppercase letters denote the thawing method; the values did not differ by the Newman-Keuls test (P ≤ 0.05) and the t-test (P ≤ 0.05), respectively.

(Cosson) Willk, a species of the Mediterranean flora family Cistaceae, were 70 and 57% when cryopreserved for 1 and 2 months, respectively (Chaneze et al., 2010).

In this work, in general, there was no difference between the thawing methods tested for quina seeds. This finding makes the procedure more practical because after the seeds are removed from liquid nitrogen, they can be thawed at room temperature before germination. Seeds of *Camellia sinensis* were rapidly thawed (water bath at 37°C for 2 minutes) after cryopreservation in liquid nitrogen, and none germinated. These seeds are considered recalcitrant because they did not survive desiccation or cryopreservation (Kim et al., 2002).

In general, when comparing the germination of cryopreserved seeds with 6 and 7% moisture contents, the seeds with 7% moisture content showed better results, regardless of the thawing duration and method. In quina seeds with moisture contents below 7% that were immersed in liquid nitrogen at -196C, the germination percentages were reduced in the cryopreserved seeds, suggesting a negative response to cold in seeds with this moisture content (<7 %). Martins et al., 2009), studying

Table 1: Mean germination rate index (GRI) of quina seeds stored in liquid nitrogen at -196ºC in relation to the moisture content, the storage duration, and the thawing method used.

Moisture content (%)	GRI
7	0.6240 a
6	0.3848 b
Storage duration (months)	
2	0.3693 a
4	0.4215 b
6	0.4150 ab
Thawing method	
Fast	0.6018 a
Slow	0.4078 a

Lowercase letters following the means denote the moisture content, storage duration, and thawing method; the values did not differ by the Mann-Whitney test ($P \leq 0.05$).

the physiological behavior of yellow poui seeds with different moisture contents preserved in liquid nitrogen at -196ºC, found that these seeds can be dehydrated to a low moisture content (4.2%) and then be stored in liquid nitrogen for at least 360 days.

Cryopreserved seeds with a 7% moisture content demonstrated higher germination rates than did seeds with 6% moisture content. Cryopreserved seeds with 6% moisture content had slower seedling establishment rates (Table 1). Quina seed cryopreservation may be viable for seed conservation because the germination rate remained above 80%. The effects of cryopreservation, in terms of the germination percentage, vary greatly among plant species. For coffee, the most current protocol, developed by EMBRAPA, shows that germination percentage was reduced by 30% compared to non-cryopreserved seeds after a 2-year storage period. This value was considered promising for defining a protocol for seed preservation in germplasm banks. Other results also illustrate that cryopreservation tolerance varies between species and that intra-specific variation exists. In pomelo (*Citrus grandis* (L.) Osbeck), seeds preserved with 5-9% moisture contents had germination percentages varying from 22 to 86% (Bin et al., 2010). In passion fruit, *Passiflora edulis* S., the germination percentages of cryopreserved seeds also vary according to species; 50 to 95% germination was obtained for purple passion fruit, while 85 to 94% germination was obtained for yellow passion fruit (Meletti et al., 2007).

CONCLUSIONS

It is possible to store quina seeds in liquid nitrogen at -196ºC because seed viability remains high.
The thawing method did not influence the germination percentage or the germination rate of quina seeds.
The seed viability remained above 80% under storage durations of 4 and 6 months.
Cryopreservation may be a method for quina seed preservation in *ex situ* germplasm banks.

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