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Research Article
A Protocol for the Cryopreservation of *Sapindus saponaria* L. Seeds

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Abstract

Background and Objective: *Sapindus saponaria* L. is a plant species of pharmacological importance and also with the potential for the production of biodiesel. Thus, the present study developed a protocol for the cryopreservation of *S. saponaria* seeds and applied physiological and vigor characters to define the best water content for cryogenic storage, the best defrosting method and assessments on the need for treatment with cryoprotectants. **Materials and Methods:** The study was developed in two tests, the first defining the best water content for storage and defrosting method. In a second test, seeds were stored with the best water content defined in test 1, under the action or not of cryoprotectants DMSO (10%) and glycerol (10%) and defrosted the seeds applying the best defrosting method also evidenced in test 1. Evaluation of seeds was done at 60, 120 and 180 days of storage in liquid nitrogen. **Results:** The best germination performance, emergence, emergence speed index (IVE) and germination after accelerated seed aging was observed in seeds stored with 6% water content and thawed quickly in the microwave. Regarding the use of cryoprotectants, this use can be indicated or not, depending on the storage time. **Conclusion:** Seeds cryoprotected and stored for periods less than or equal to 120 days exhibited inferior performance with regard to emergence, total length and dry seedling mass.

Key words: Cryoprotectants, thawing, germination, seed water content, emergence speed index

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sapindus saponaria L. (soapberry) is a tree species native to the Americas, belonging to the Sapindaceae family. It is widely distributed across the continent, occurring in the USA, Mexico and Argentina, as well as in the Brazilian states of Amazonas, Goias, Mato Grosso and Mato Grosso do Sul, where it is used in urban landscaping and in models for the recovery of degraded areas[1-3]. This species is of great importance for the reforestation of impacted areas, as well as in toy making and its seeds are used for handicrafts and its wood explored for use in civil construction[4]. Studies have highlighted the medicinal potential of terpenes obtained from fruits of this plant[5,6], as well as antifungal potential[7-9], which has been exploited even against phytopathogens[10,11]. The pulp of *S. saponaria* fruits consists mainly of saponin and the properties of the extract obtained from the raw pulp sap are comparable to those of commercial surfactants, leading to its potential use as a biosurfactant[12]. Pelegrini *et al.*[13] attested to the potential of *S. saponaria* in obtaining biodiesel oil and Rodrigues *et al.*[14] point out this species as a fluoride air pollution bioindicator.

Despite the commercial potential of *S. saponaria*, the cultivation and conservation of natural populations are hampered by a lack of information on their biology and fruit and seed conservation, including seed behavior during cryopreservation. These studies, however, are important factors for managing the propagation and storage of raw material for agro-industrial and conservation purposes[15-17]. In this sense, cryopreservation is noteworthy as an ultra-low temperature storage process (liquid nitrogen, -196°C), considered the best procedure for the long-term preservation of germplasm[18,19]. Over the past 25 years, several protocols have been developed allowing for the cryopreservation of seeds of numerous species of ecological, economic and medicinal interest[20-22].

In the development of seed cryopreservation protocols, some factors must be carefully evaluated, with water content being the most critical, since there is a need to avoid the formation of cell ice crystals[23,24]. Other primary aspects to be established are type of thawing, which directly interferes in seed vigor and the need to use cryoprotective substances that aid in preserving tissue physiological quality[25]. Penetrating cryoprotective agents decrease the chemical or mechanical damage that freezing causes to cells[26], although, many of them are chemically toxic and can cause osmotic injuries or molecular toxicity[27,28]. Therefore, defining the seed residence time under the action of the cryoprotectant can be fundamental to minimize the damage caused by cell exposure.

Thus, given the scarcity of information about the biology and conservation of *S. saponaria* seeds and the need to create strategies that allow for future conservation of the germplasm of this species, this study developed a seed cryopreservation protocol plant using vigor characters, such as germination and emergence percentage, as well as physiological characters associated with stress, such as the synthesis of antioxidant enzymes, to define the best water content for cryogenic storage, the best defrosting method.

MATERIALS AND METHODS

Study area: *Sapindus saponaria* L. fruits were obtained in October, 2018, cryopreservation conducted until April, 2019 and germination tests conducted until June, 2019.

Water content×defrosting method (test 1): *Sapindus saponaria* L. fruits were sampled from 10 adult plants in full production, located in the municipality of Santa Helena de Goiás-GO, Brazil (17º49'05"S and 50º36'29" W). A specimen was deposited at the Goiano Federal Institute Herbarium (IF Goiano, Campus Rio Verde), under number n° 1008/2018. The seeds were manually extracted, processed and scarified with sulfuric acid P.A. for 90 min to overcome dormancy. After determining the initial water content, the seeds were dried in an induced circulation oven at 40°C to obtain seeds with four final moisture levels (8, 7, 6 and 5% b.u).

For cryopreservation, the *S. saponaria* seeds with different water contents were packed in aluminum foil and conditioned in cylindrical aluminum tubes (canisters). Subsequently, the tubes were stored in cryogenic cylinders, isolated under partial vacuum, at -196°C for 10 days. After this period, the seeds were subjected to different thawing treatments: Slow and gradual, following a sequence of steps (freezer -80°C, freezer -26±2°C, BOD 10°C and room temperature of 25°C, 1 h each step); rapid defrosting in a water bath (60°C for eight min); and microwave (at 15000 w for three min). After thawing, the seeds were washed in distilled water and subjected to tests to assess physiological quality, evaluated by means of emergence, germination (percentage) and vigor (emergence speed index-ESI, germination after accelerated aging and total seedling length).

Germination was evaluated by performing daily counts of the number of germinated seeds, using the radicle protrusion (1 cm) as a criterion. The counts began at 12 days and lasted until 35 days after sowing, carried out according to Brazil[29] and expressed as a percentage.

For emergence assessments, four replicates consisting of 25 seeds were sown in a sand bed, with an approximate depth of 2 cm, in a greenhouse, under three daily irrigations. Daily counts of emerged seedlings were performed from the 14th to the 45th day after sowing, using as criterion the number of seedlings displaying the appearance of eosinophil. The total number of emerged seedlings was expressed as a percentage, according to Brazil[29]. The size of each seedling (cm) was determined at the end of the emergence test.

In the accelerated aging test, layers of 25 seeds were placed on metal screens attached to a gerbox containing 40 mL of water at the bottom. The gerboxes were capped and kept in a BOD at 42°C for 72 hrs. After the artificial aging period, four samples of 25 seeds were evaluated by the germination test, obtaining the percentage of normal seedlings according to the methodology described by Lopes *et al.*[30].

The tests were completely randomized and conducted in a 4×3 factorial scheme (water content x defrosting methods), with four replications, with a sample consisting of 25 seeds. The data were submitted to two-way analysis of variance and the means compared by the Tukey test, at 5% significance level.

Cryoprotectant×storage time (test 2): To evaluate cryoprotectant and storage time effects on the physiological quality of *S. saponaria* seeds, the seeds were subjected to the following cryoprotection treatments: liquid nitrogen without any cryoprotectant; liquid nitrogen with the cryoprotectant glycerol (10%) and the cryoprotectant dimethyl sulfoxide (DMSO) (10%). The seeds were packed in aluminum foil and packed in cylindrical aluminum tubes (canisters). The tubes were stored in cryogenic cylinders, insulated under partial vacuum, at -196°C. The seeds submitted to the different cryoprotection treatments were sampled at 60, 120 and 180 days.

The sampled seeds were defrosted using the best method determined in test 1-water content x defrosting method and evaluated for germination (%) and emergence (%) parameters, while vigor was tested by means of seedling total length (cm) and dry biomass (g). To this end, the seedlings were taken to a drying oven, at 80°C, for 24 hrs, as recommended by Vanzolini *et al.*[31].

Seed vigor was also evaluated by the assessing enzymes belonging to the antioxidative system, namely superoxide dismutase (SOD) and catalase (CAT). To this end extracts from reserve/embryonic tissues were obtained from 0.250 g of seed fragments macerated in N_2. The powder was then homogenized in 2 mL of 50 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonic fluoride (PMSF) and 5% polyvinylpyrrolidone (PVPP) v). The homogenate was centrifuged at 12000×g, for 15 min, at 4°C and the supernatant used as an extract for the enzymatic determinations.

SOD levels were determined by adding 60 µL of the extract in 1.94 mL of a reaction mixture consisting of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, p-nitro-tetrazolium blue (NBT) 75 µM, 0.1 mM EDTA and 2 µM riboflavin[32]. The reaction took place at 25°C under 15 W lamps. After 15 min of exposure to light, the lights were turned off and the blue formazan produced by NBT photoreduction was measured using a spectrophotometer (Evolution 60, Thermo Fisher Scientific Inc., Massachusetts-USA), at 560 nm[33]. One unit of SOD was defined as the amount of enzyme needed to inhibit NBT photoreduction by 50%[34].

CAT levels were determined according to Cakmak and Marschner[35]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2 in a volume of 2 mL. The reaction was initiated by the addition of 50 µL of the reserve/embryonic tissue extract and levels were determined by the consumption of H_2O_2 at 240 nm, for 1 min, at 25°C. The molar extinction coefficient of 36 M^{-1} cm^{-1} [36] was used to determine CAT levels, which was expressed in mmol/min/mg of protein.

The protein concentration in the reserve/embryonic tissues was determined according to Bradford[37], where the absorption spectrum of the samples was evaluated at 595 nm and the amount of proteins expressed in mg mg^{-1} of fresh mass.

These tests were also completely randomized, but in a 3×3 factorial arrangement (cryoprotection×storage periods) with four repetitions, one repetition carried out with a sample of 25 seeds for the germination, emergence and vigor tests and 0.250 g of seed fragments used for the enzyme and protein tests.

Statistical analysis: The data were submitted to a two-way ANOVA and regression analysis. The regression models were selected using the highest coefficients of determination and significance of the regression coefficients according to the t-test, at $p<0.05$. All statistical analyses were conducted with the aid of the statistical software Sisvarv.5.6[38].

The results were used in the elaboration of an initial protocol for cryopreservation of *S. saponaria* seeds (Fig. 1).

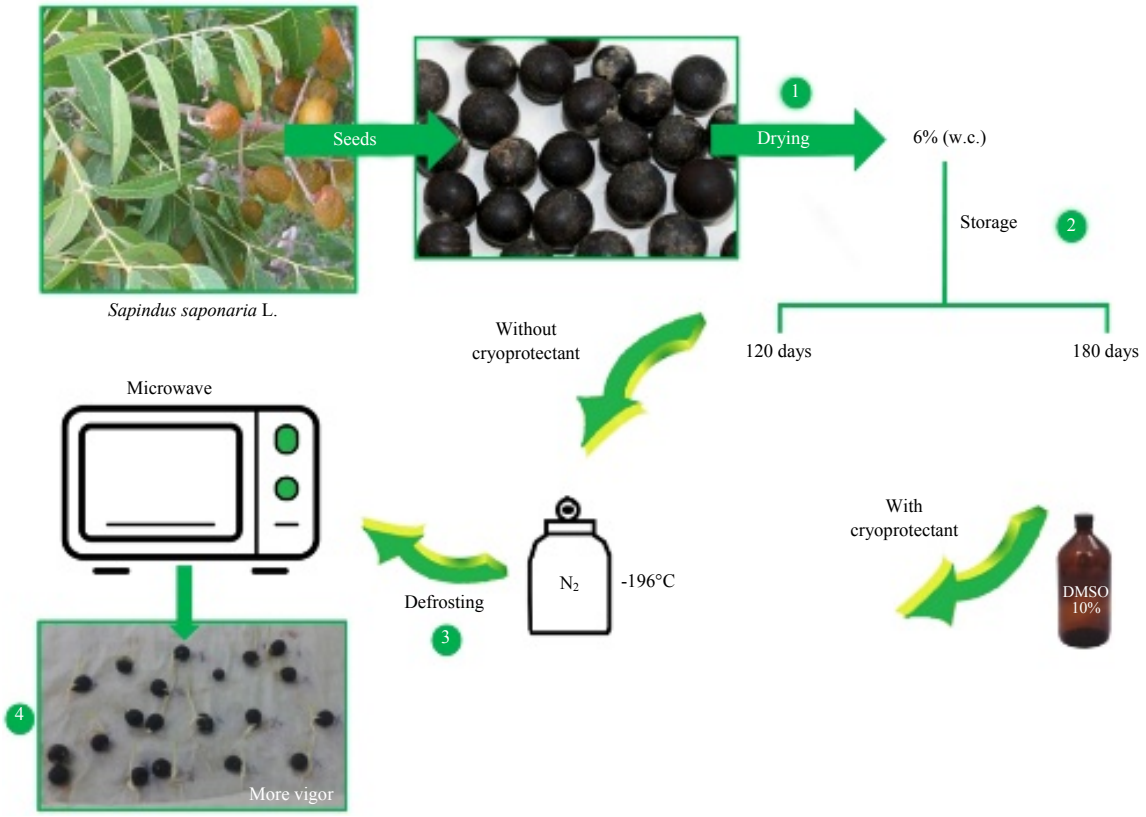

Fig. 1: Protocol for the cryopreservation of *Sapindus saponaria* L. seeds

1: The steps consist of seed drying up to 6% water content (w.c), 2: Storage in N_2 using or not DMSO (10%) as cryoprotectant, 3: Subsequent thawing in a microwave and 4: Obtaining seeds with greater vigor

RESULTS

Effects of test 1: The germination percentage was affected by the defrosting method and also by the water content (w.c.). Germination percentage and emergence of *Sapindus saponaria* L. seeds maintained under cryogenic storage for 10 days, using different water levels (8, 7, 6 and 5% W.C.) and thawed quickly in a microwave and water bath and gradually. The worse germination means were observed for the seeds thawed in a water bath (58.50%), with the means observed for microwaves and gradual thawing, respectively, of 63.75 and 61.50% (Fig. 2a). When comparing water contents within the defrosting methods, higher germination means were observed for 6% (w.c) in the microwave (75%) and water bath (68%) treatments. In the gradual thawing treatment, no difference was observed for the germination of cryopreserved seeds with different water contents.

Seed emergence percentage was also affected by the defrosting method, but only affected by the moisture content of the seeds defrosted in the microwave. The highest emergence percentages were observed in the seeds thawed in the microwave (34%), with the means observed in the thawing treatments in the water bath and gradually, respectively, of 27.50 and 28.25% (Fig. 2b). For seeds thawed in the microwave, water content maintenance at 6% (w.c) did not affect the emergence of cryopreserved seeds as much (55% emergence).

Emergence Speed Index (ESI), Germination after accelerated aging (%) and Total length (cm) (c) of *Sapindus saponaria* L. seedlings kept under cryogenic storage for 10 days, at different water contents (8, 7, 6 and 5% W.C.) and thawed quickly in a microwave and water bath and gradually. In addition to the emergence, ESI was also affected by the defrosting method, so seeds thawed in the microwave displayed higher mean vigor (0.27), followed by those thawed in the water bath (0.21) and gradually (0.20) (Fig. 3a). Comparing storage water contents within the defrosting treatments, the 6% (w.c.) content was the most efficient in preserving vigor in seeds thawed quickly in microwave, but for those thawed in a water bath, the best content was 7% (w.c.). For seeds thawed gradually, no difference was observed in the ESI averages for the different water contents.

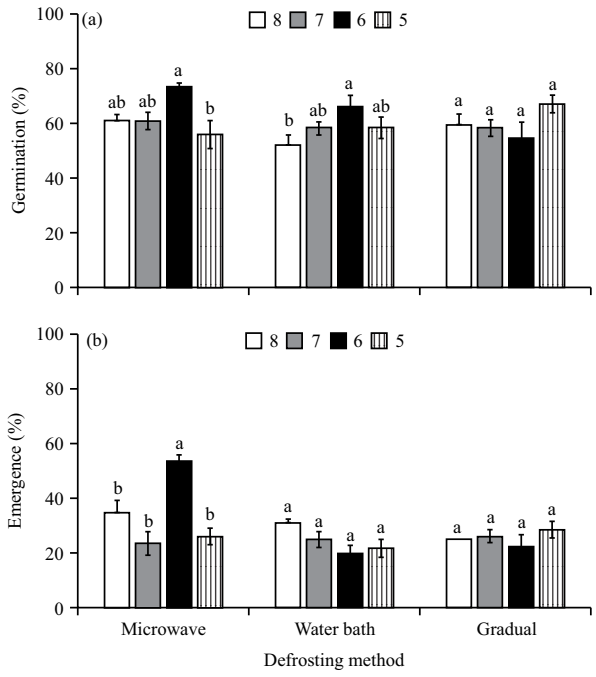

Fig. 2(a-b): (a) Germination percentage and (b) Emergence of *Sapindus saponaria* L. seeds

Means followed by the same letter do not differ by the Tukey test at 5% significance. The letters inside the circles compare seed thawing methods and those above the bars compare seed water content

In general, *S. saponaria* seeds that passed the accelerated aging test displayed drastically reduced germination, but the microwave defrosting treatment also proved to be superior to other treatments in maintaining the physiological quality of these seeds, with a germination percentage of 23.5%, while the other treatments resulted in 19.75% (water bath) and 15.5% (gradual defrosting) germination percentages (Fig. 3b). Water content did not affect germination in these last treatments, but in the rapid microwave defrosting the highest means (37%) was observed in the cryopreserved seeds containing 6% water content (w.c).

Total seedling length, however, was not affected by the thawing treatments, nor by seed water contents (Fig. 3c).

Effects of test 2: The germination of the seeds treated with the cryoprotectant DMSO (10%) decreased linearly throughout the storage time, with a germination percentage at 60 days of 87%, decreasing to 62% at 180 days of storage (Fig. 4a), although at 120 days, seeds treated with this cryoprotectant showed the highest germination percentage (71.25%). An opposite effect was observed for seeds treated with glycerol (10%) and for untreated seeds, where the

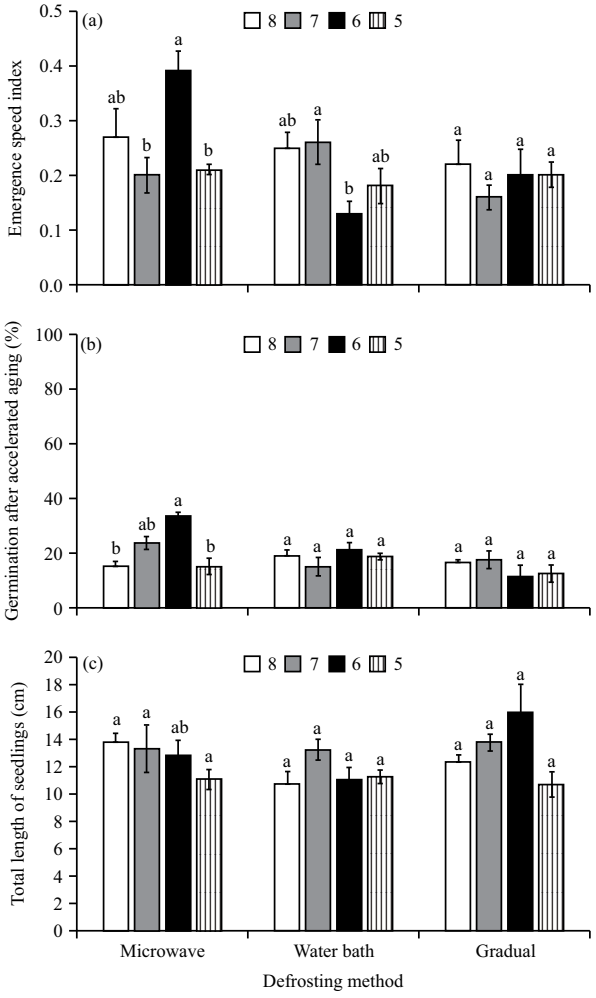

Fig. 3(a-c): (a) Emergence speed index (ESI), (b) Germination after accelerated aging (%) and (c) Total length (cm) of *Sapindus saponaria* L. seeds

Means followed by the same letter do not differ by Tukey's test at 5% significance. The letters inside the circles compare the seed thawing methods and those above the bars compare water content

germination percentage at 180 days was higher than that observed for seeds treated with DMSO (10%), of, respectively, 70 and 71%.

When analyzing seed emergence, however, seeds not treated with cryoprotectant displayed a dramatic decrease in emergence. The mean percentage at 60 days was of 35% and of 13.75% at 180 days of storage (Fig. 4b). Thus, the cryoprotectants assessed herein were proven efficient in preserving the emergence of *S. saponaria* seeds that must be stored for long periods, with emergence percentages for the seeds treated with both DMSO (10%) and glycerol (10%) close to those observed at 60 days of storage, of 33.75 and 35%, respectively, at 180 days and 35 and 30% at 60 days.

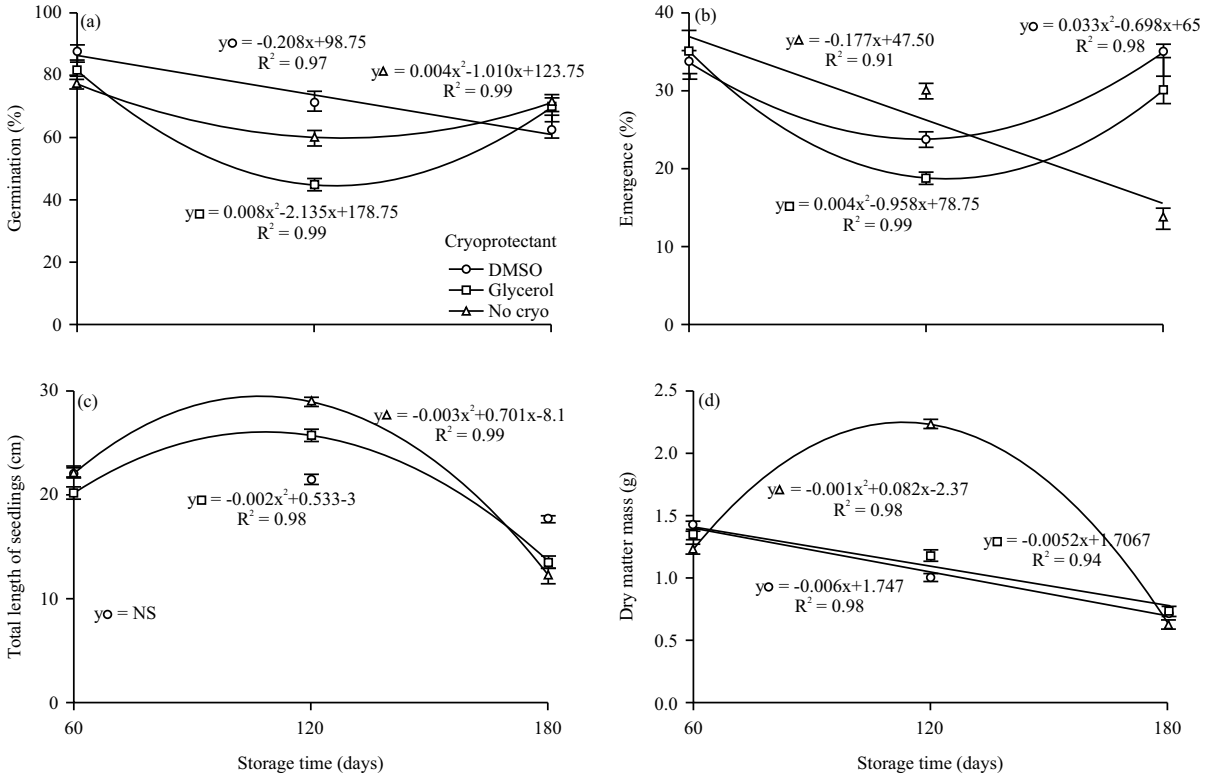

Fig. 4(a-d): (a) Germination, (b) Emergence (%), (c) Total length (cm) and (d) Dry mass of *Sapindus saponaria* L. seedlings stored for 60, 120 and 180 days without cryoprotection and treated with DMSO (10%) and glycerol (10%)
NS: Not significant by the F-test at a 5% probability

The storage time did not affect the length of the seedlings developed from seeds cryoprotected with DMSO (10%), while seeds not cryoprotected or treated with glycerol (10%) displayed reduced seedlings size when stored for longer than 120 days (Fig. 4c). Thus, at 60 days of storage, seedlings emerged from seeds treated with glycerol (10%) presented an average of 20.17 cm and untreated seeds presented 22.21 cm, while at 180 days of cryopreservation these seedlings presented, on average, 13.50 and 12.25 cm, respectively.

A linear reduction in the dry mass of seedlings treated with different was noted, from 1.42 and 1.35 g, respectively for seedlings treated with DMSO (10%) and glycerol (10%) at 60 days of cryopreservation to 0.72 and 0.73 g for 180 days of storage. On the other hand, an increase in biomass was observed, consistent with the observed increase in total length of seedlings emerged from non-cryoprotected seeds at 120 days (2.23 g), although the increase in the storage time of these seeds reduced the observed biomass at 180 days to values lower than those observed for the treated seeds (0.63 g) (Fig. 4d).

SOD levels in the seeds cryopreserved without any cryoprotectant was not influenced by the storage time. However, seeds cryoprotected with glycerol (10%) showed a linear increase in this enzyme throughout the evaluated times, increasing from 2.86 U SOD at 60 days to 3.46 U SOD at 180 days. The seeds cryoprotected with DMSO, on the other hand, presented high SOD levels at 120 days of storage (3.73 U SOD), reducing to 3.16 U SOD at 180 days (Fig. 5a).

Regarding CAT, reserve/embryonic tissues of non-cryoprotected seeds CAT levels are also not affected by storage time. A linear behavior in relation to time was observed in seeds cryoprotected with glycerol (10%), similarly to what was observed for SOD, although this behavior decreased over time. At 60 days, the mean value of this enzyme in tissues was 11.5 µmol/min/mg protein and at 180 days, 4.19 µmol/min/mg protein (Fig. 5b). In the cryoprotected seeds with 10% DMSO, the behavior was similar to that observed for SOD, that is, high concentrations observed at 120 days of storage (12.89 µmol/min/mg of protein), with a reduction of these values at 180 days (4.18 µmol/min/mg protein).

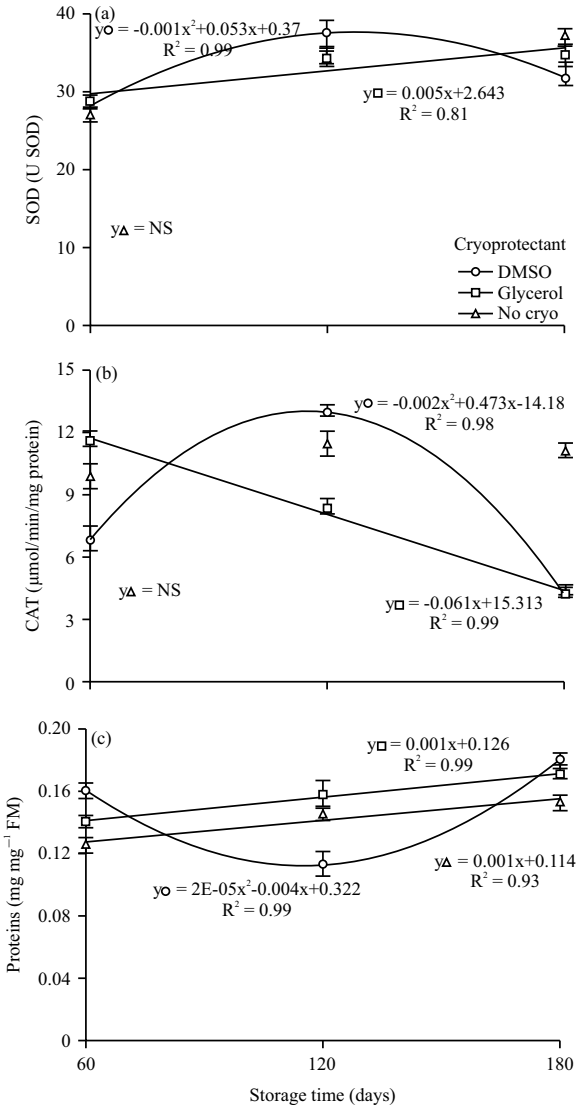

Fig. 5(a-c): (a) Superoxide dismutase (U SOD), (b) Catalase (µmol/min/mg protein) and (c) Total proteins (mg mg^{-1} of fresh mass) in the reserve embryonic tissues of *Sapindus saponaria* L. seeds stored for 60, 120 and 180 days without cryoprotection and treated with DMSO (10%) and glycerol (10%)
NS: Not significant by the F-test at 5% a probability

The protein concentrations in the reserve/embryonic tissues of seeds cryoprotected with glycerol (10%) and those stored without cryoprotectant increased linearly throughout the storage period, with concentrations at 60 days of 0.14 and 0.12 mg mg^{-1} fresh mass and at 180 days, of 0.17 and 0.15 mg mg^{-1}, respectively (Fig. 5c). The seeds treated with DMSO (10%), however, displayed reduced protein concentrations after 120 days of storage (0.11 mg mg^{-1} fresh mass), increasing at 180 days (0.18 mg mg^{-1} fresh mass).

The tests performed herein enabled the development of a protocol for cryopreservation of *S. saponaria* seeds (Fig. 1). This protocol indicates seed drying up to 6% (w.c.) and subsequent storage in N_2, although this storage must be carried out taking into account the time required for storage. If the seeds must be stored for less than or equal to 120 days, the use of cryoprotective solutions is not recommended, but if they must to be stored for periods close to or equal to 180 days, the use of DMSO (10%) is indicated , to minimize cryogenic effects. Regarding seed defrosting, quick defrosting in a microwave is recommended. This sequence allows for more vigorous *S. saponaria* seeds.

DISCUSSION

The microwave defrosting method proved to be more efficient in maintaining the physiological quality of *S. saponaria* seeds, maintaining higher germination, emergence and ESI than seeds subjected to other thawing treatments. These results confirm the negative effect of slow thawing for *S. saponaria* seeds, despite the orthodox pattern presented by the seeds.

Seed water content proved to be a decisive factor in maintaining the physiological quality of cryopreserved *S. saponaria* seeds and this study recommend maintaining this content at 6% (w.c.) when applying microwave defrosting. High water content can be harmful to seed cryopreservation, as this may culminate in the formation of ice crystals in the embryo[39,40]. Nucleation of intracellular ice can disrupt cell membranes, so low levels of tissue moisture become essential for success in cryopreservation[40].

The use of DMSO (10%) in seeds stored for 180 days improved seed emergence and ensured greater total seedling fulfillment, with non-cryoprotected seeds exhibiting greatly affected emergence by long-term cryogenic storage. This is due to the fact that cryoprotectants cause osmotic tissue dehydration, leading them to a vitreous state, which reduces damage to cells during freezing and/or thawing[41,42]. However, when assessing seeds stored for less than or equal to 120 days, it was conclude that the ideal for maintaining seedling emergence, growth and biomass accumulation is storage without the use of cryoprotectants. In fact, the use of cryoprotectants can alter the metabolic process of seeds[43] and culminate in germination or emergence losses, so the toxicity induced by the protector may not compensate the loss of vigor (see Best[44]).

The behavior of the assessed antioxidant system enzymes and proteins observed at 120 days of storage, for seeds treated with DMSO (10%), was contrary to that observed at

180 days. Thus, these seeds displayed the lowest SOD and CAT values at 180 and the highest at 120 days, while presenting the highest estimated protein concentrations at 180 and the lowest at 120 days of storage. These data corroborate the recommendation to use DMSO as a cryoprotectant if *S. saponaria* seeds must to be cryopreserved for long periods, close to or equal to 180 days, reinforced by the evidence that non-cryoprotected seeds presented the highest SOD and CAT values at 180 days of cryopreservation. However, if these seeds must be cryopreserved for shorter periods, equal to or less than 120 days, cryoprotection with DMSO is not recommended, since increases in antioxidant system enzymes in the reserve/embryonic tissues signal the occurrence of oxidative damage. Because of this, these enzymes are used as important biochemical stress markers[45], acting as primary defenses against free radicals[46].

On the other hand, seed protein content is considered a germination determinant, since these compounds are the main responsible for imbibition. Protein-rich seeds generally absorb water more quickly, as they are a highly hydrophilic substance, unlike seeds rich in lipids. *S. saponaria* seeds, when preserved for long periods (180 days), if cryoprotected with DMSO (10%) displayed the highest protein concentrations, while those not cryoprotected presented the lowest concentrations.

Thus, this study recommend *S. saponaria* seed cryopreservation for storage periods less than or equal to 120 days without the use of cryoprotective solutions. Thus, given the need to cryopreserve *S. saponaria* seeds, they must be preserved at 6% (wc), without cryoprotection for storage periods less than or equal to 120 days or cryoprotected with DMSO (10%), for periods close to or 180 days and thawed quickly in a microwave.

CONCLUSION

This study proposes a cryogenic storage protocol for *S. saponaria* seeds, indicating an orthodox behavior pattern for these seeds, better tolerant to cryopreservation at 6% water content. For storage for periods less than or equal to 120 days, the use of cryoprotection is not indicated, while for longer storage periods, close to or equal to 180 days, the use of DMSO (10%) can improve physiological seed quality. On the other hand, regarding defrosting, *S. saponaria* seeds of submitted to rapid defrosting in a microwave suffered less effects than seeds submitted to a water bath or thawed slowly.

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SIGNIFICANCE STATEMENT

This study discovered the seeds of *S. saponaria* should be cryopreserved with 6% of the water content and the use of DMSO (10%) as a cryoprotectant can help the long-term storage of these seeds. This study can be beneficial for fruit producers of this species, which currently do not have any technique for seed storage. This study proposes an initial protocol for cryopreservation of *S. saponaria* seeds. Researchers can use this protocol as a starting point for improvement.

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