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ORIGINAL ARTICLE – Silviculture

Limonium brasiliense (Boiss.) Kuntze Seeds: Disinfestation and Conservation of Viability Under Storage

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Abstract

This study evaluated the effect of disinfestation methods and different storage environments on the germination of seeds of *Limonium brasiliense* (Boiss.) Kuntze. Seeds were disinfested using ethanol followed by sodium hypochlorite; water under agitation; and no disinfestation from two origins. Longevity was tested by storing the seeds in a cold chamber, dry chamber, and at ambient laboratory temperature. Beginning of germination (BG), percentage (%G), and mean germination time (MGT) and mean germination speed index (MGSI) were evaluated. Data were submitted to an analysis of variance, comparison of mean values and a polynomial regression analysis ($p<0.05$). In the disinfestation, there was significant difference in %G and MGT only between origins. For longevity, IG and MGSI had a positive and negative linear behavior, respectively, while %G had a quadratic behavior. *L. brasiliense* seeds disinfestation does not affect germination. Dry chamber was the most suitable storage environment.

Keywords: Germination, Plumbaginaceae, therapeutic potential, seed propagation.

1. INTRODUCTION

Limonium brasiliense (Boiss.) Kuntze (Plumbaginaceae), popularly known as 'baicuru' or 'guaicuru', is native to Brazil, occurring naturally in the states of Rio Grande do Sul (RS), Santa Catarina, Paraná, and Rio de Janeiro, in the coast, restingas, and mangrove areas. It is a perennial herbaceous plant, of approximately 40 cm in height, with verticillate leaves in rosettes, reddish and thick rhizomes, flowers with reddish corolla and bluish calyx, and dehiscent fruits (Zappi et al., 2015).

According to Moura et al. (1985), *L. brasiliense* has been used in popular medicine, especially in the Brazilian southern region, due to the therapeutic properties of its rhizome. Several components with antioxidant activity have been identified in rhizome extracts of this species, which explains its popular use (Murray et al., 2004).

Several other potentials have also been described for this species. Blainski et al. (2017) determined that rhizome extracts have antibacterial properties. On the other hand, Faral-Tello et al. (2012) demonstrated its anti-HSV-1 activity, as well as antiviral, virucide, and cytotoxic activity, and Caleare et al. (2017) reported the action of this extract in reducing the adhesion of *Porphyromonas gingivalis* cells to epithelial cells in the mouth. In addition, its potential to replace *L. latifolia*, an exotic species, as an ornamental plant has also been described, due to its architecture and to the color of its flowers (Stumpf et al., 2015).

Therefore, in order for all potentials of *L. brasiliense* to be exploited, it is necessary to know the characteristics related to the germination and conservation of its seeds. Several factors might affect the germination process, such as water availability in the environment, seed water content, temperature, light, oxygen, and other chemical compounds, presence or absence

of seed dormancy (Bewley et al., 2013), attack by pathogenic microorganisms (Carvalho & Nakagawa, 2012), storage time and conditions, and even the individual characteristics of the parent plant, which include both genetic and environmental factors (Baskin & Baskin, 2014).

It is worth noting that the presence of pathogenic microorganisms such as bacteria or viruses also affects the germination process, as these microorganisms interfere with plant metabolism via enzymes, toxins, and regulatory factors (Agrios, 2004; Carvalho & Nakagawa, 2000). Seeds can be disinfested or treated with sodium hypochlorite solution or fungicides, for example, attempting to eliminate microorganisms potentially present in them and, therefore, preserve their anatomical integrity for germination (Baskin & Baskin, 2014). Protocols involving the use of sodium hypochlorite solutions and ethanol 70% are common for seed sterilization, being successful in inhibiting the growth of bacteria and fungi (Barampuram et al., 2014; Fantinel et al., 2017). In some cases, sodium hypochlorite solutions can even increase germination by removing the seed coat without damaging the endosperm and embryo (Jesus et al., 2015).

Notwithstanding, temperature and humidity conditions must be observed during long-term seed storage, a method commonly used for plant genetic resources conservation (Hong & Ellis, 1996), so that seeds remain viable, as each species has its particularities (Bewley et al., 2013). Regarding their moisture content, seeds are divided into two types: orthodox seeds can be dried up to 5% and 10% of their moisture content without losing their vigor, while recalcitrant seeds must be kept between 25% and 45% of their moisture content, and desiccation beyond these levels, varying according to species, can render the seeds inviable. In general, both types of seeds can be stored in cold conditions (around 5-10°C), state that reduces their metabolism, slowing down deterioration and loss of viability (Bonner et al., 2008; Carvalho & Nakagawa, 2012). Although there are no studies in the germination behavior of *L. brasiliense* regarding desiccation, a study shows that *L. aureum* (L.) Hill. can be dried below moisture contents of 5% still retaining its viability (Li et al., 2007), indicating that the genus may have orthodox seeds.

Therefore, due to the above-mentioned and to the need for studies on suitable conditions for the propagation of species, the aim of this study was to evaluate the effect of disinfestation methods and storage environments on the germination of *L. brasiliense* seeds.

2. MATERIAL AND METHODS

Fruits of 20 parent plants were collected from each *L. brasiliense* origin in the city of Torres, RS (A1), coordinates 29°20'42"S; 49°43'39"W, and at two sites (A2 and A3) in the city of São José do Norte, RS, coordinates 32°04'07"S; 52°02'25"W (A2) and 32°08'23"S; 52°04'35"W (A3). The harvesting was performed within 5 days for all parent plants, entire panicles with mature fruits were collected, and the seeds were only removed after a week in the laboratory workbench. Furnas Hill, where A1 sampling was performed, is a site characterized by receiving higher insolation in the morning and early afternoon, with clayish soil and rocky outcrops. On the other hand, the second site (A2) was shaded due to the presence of specimens of the Poaceae and Juncaceae families, which are larger sized, on a sandy yet soaked terrain (which was observed at sampling); the third site (A3) was on the margins of Patos Lagoon, characterized by sandy soil, with no accumulation of water and with vegetation primarily comprised of small-sized specimens (up to 15 cm height) from the Poaceae and Liliaceae families. The climate at the three sampling sites is classified as Cfa (according to Köppen-Geiger's classification), characterized as humid in all seasons and with a hot summer (Ayoade, 1996). The city of Torres is located in the ecoregions of Atlantic Coast Sandbanks and Sea Mountain Coastal Forest (Atlantic Forest biome), while São José do Norte is located in the South Camps (Pampa biome) (Instituto LIFE, 2015).

2.1. Seed disinfestation tests

Three seed disinfestation methods were tested and only seeds from A1 and A3 origins were used. Approximately 30 days after sampling, seeds were processed and sown on germitest paper rolls, hydrated with distilled water, 2.5-fold their mass. Treatments consisted of (1) standard disinfestation, in which seeds were submerged in 70% alcohol for one minute, followed by 2.5% sodium hypochlorite (NaClO) (i.a.) for 20 minutes and triple washing with autoclaved deionized water; (2) disinfestation with distilled water under agitation for 20 minutes; and (3) no disinfestation.

This study was conducted in an acclimatized room with a temperature of 25 ± 2 °C and 16 hours of photoperiod (white light bulbs with an intensity of 2500 Lux). The experiment was performed with a completely randomized design, in a 2 x 3 factorial arrangement, where the first factor consisted of origins (A1 and A3) and the second factor was represented by seed disinfestation treatments (standard disinfestation, water disinfestation, and no disinfestation), in four replicates with 25 seeds each.

Evaluations were performed twice a week until there were no new germinations. Seeds were considered germinated when they exhibited root protrusion larger than or equal to 2.0 mm, and regular seedlings were considered formed when

their aerial part and root system were visible to the unaided eye. The following were defined in each replicate: beginning of germination in days (BG), which corresponds to the time elapsed between sowing and the first appearance of a germinated seed; germination percentage (%G), calculated based on the percentage of seeds with root protrusion of at least 2 mm; and mean germination time (MGT). MGT calculation followed Labouriau (1983) and represents the weighed mean value between numbers of germinated seeds in each evaluation at time intervals previously defined for each count. The normality of the residues and data homoscedasticity were tested by Kolmogorov–Smirnov and Bartlett tests respectively, and were considered normal and homoscedastic. Data were then submitted to an analysis of variance, followed by a comparison of means using the LSD Fisher test ($p<0.05$). The tests were performed using Costat 6.4.

2.2. Seed longevity tests

Based on a pilot study, in which it was found that the seeds of different origins assume a similar behavior after the beginning of the storage period, we opted to utilize a homogeneous sample of seeds, comprised of subsamples from the three origins (A1, A2, and A3). They remained stored in zip bags in three environments: (1) cold chamber at a temperature of 5ºC and relative air humidity of approximately 60% (CF); (2) dry chamber at a temperature of 20ºC and 45% humidity (CS); and (3) under a laboratory shelf, with no rigorous control of temperature and humidity, which varied from 15 to 28°C and from 50 to 70%, respectively. The seeds were not disinfested prior to storage or before sowing. At 90, 150, 210, 270, 395, 478, and 564 days after sampling, seeds were sown on a germitest paper roll, and maintained in an acclimatized room under the same conditions of the previous test.

The experiment design was completely randomized, in a 3 x 7 factorial arrangement, where the first factor consisted of the three storage environments (cold chamber, dry chamber, and ambient temperature), and the second factor was represented by times after fruit sampling (90, 150, 210, 270, 395, 478, and 564 days). Four replicates were used with 25 seeds each.

Evaluations were performed every four days until no germination was observed in 10 consecutive evaluations. Seeds were considered germinated when they exhibited root protrusion higher than or equal to 2 mm. The following were defined: beginning of germination in days (BG), germination percentage (%G), mean germination time (MGT), and mean germination speed index (MGSI). The latter was obtained the number of germinated seeds in each evaluation, divided by the corresponding time since sowing. Then, the result was divided by the total number of germinated seeds in each replicate, following the formula by Silva & Nakagawa (1995), modified according to Santana & Ranal (2004). The same previous tests for residues' normality and data homoscedasticity were applied, and data were considered normal and homoscedastic. Data then were submitted to an analysis of variance, followed by a polynomial regression ($p<0.05$), using Costat 6.4 and SigmaPlot 11.0, respectively.

3. RESULTS AND DISCUSSION

3.1. Seed disinfection tests

Results showed that there was no interaction between origins and seed disinfestation methods for %G, MGT, and BG. There was a significant variance in germination percentage and mean germination time between origins (Table 1).

Table 1. Germination percentage (%G), mean germination time (MGT), and beginning of germination (BG) in origins A1 (Furnas Hill, Torres) and A3 (margins of Patos Lagoon, São José do Norte), and disinfestation methods for *Limonium brasiliense* (Boiss.) Kuntze seeds.

		%G	MGT (days)	BG (days)
Origin	A1	15.14 b	16.33 b	10.25 a
	A3	36.42 a	20.87 a	7.33 a
Disinfestation method	Standard	27.98 a	19.98 a	9.50 a
	Water	27.22 a	15.61 a	7.63 a
	No disinfestation	22.15 a	20.22 a	9.25 a

Means followed by the same letter in the column inside each factor do not differ from each other using LSD Fisher test at 5% of error probability.

The occasional incidence of microorganisms in this study did not affect germination, and neither did disinfestation agents affect germination. These results differed from those obtained by Aimi et al. (2016), who found higher germination percentages in *Cabralea canjerana* (Vell.) Mart. disinfested with Maxim® fungicide (90% of germination) and sodium hypochlorite (89% of germination), significantly differing from the treatment with no disinfestation. In this case, non-disinfested seeds were more frequently attacked by fungi, especially by *Penicillium*, which negatively affected germination. On the other hand, Hennipman et al. (2017) reported that the beginning of germination in *Araucaria angustifolia* (Bertol.) Kuntze was delayed when seeds were treated with sodium hypochlorite, which caused a temporary damaging effect on germination, and this was not observed in the present study. However, the same authors also described that when the same seeds were not treated, they had nearly no germination at 12 months of storage, while treated seeds had a significantly higher percentage. The reason why there

was no germination of non-treated seeds was that they were attacked by the fungus *Schizophyllum commune*.

Pinheiro et al. (2016) tested disinfestation methods on seeds of four forest species and showed the influence of these methods on germination and on the presence of fungi. *Bauhinia forficata* Link seeds had a higher germination percentage when disinfested with 2% sodium hypochlorite for one minute, followed by rinsing with distilled water, compared to other treatments, including the control (with no disinfestation). According to the same authors, this occurred due to the reduction in the presence of fungi, especially those of the genera *Aspergillus* sp. and *Penicillium* sp., which cause seed deterioration. However, treatments with disinfested seeds of *Cedrela fissilis* Vell. and *Parapiptadenia rigida* (Benth.) Brenan did not significantly differ in germination percentage, even though there was a reduced incidence of *Penicillium* spp. On the other hand, germination percentage in *Senegalia bonariensis* (Gillies ex Hook. & Arn.) Seigler & Ebinger did not vary between treatments, and even the control, with no disinfestation, showed no incidence of fungi.

Fungi of the genera *Penicillium* and *Aspergillus* are usually associated with seeds, especially during storage, and are responsible for negatively affecting their metabolism and germination (Carvalho & Nakagawa, 2012), which are controlled by the use of sodium hypochlorite and fungicides (Baskin & Baskin, 2014).

The beginning of germination did not significantly differ from each other between the origins tested, either; however, germination percentage and mean germination time were significantly higher in A3. This means that a higher number of seeds germinated in this origin, yet over a longer period than in A1, which had a more even germination (Table 1). The characteristics of each environment where parent plants were located may have had different effects on the development of the seeds and therefore on their initial germination characteristics; they were located in two different biomes (Pampa and Atlantic Forest), which may have affected the general physiology of the parent plants, reflecting in their seeds, and their genetic background may have interfered in the results obtained.

The results obtained in the present study follow other studies that investigated seed germination in one species with different origins. Ladeia et al. (2011), for instance, obtained lower %G and GSI (germination speed index) in *Pseudobombax longiflorum* (Mart.) A. Robyns collected in Rondonópolis, Mato Grosso, compared to those collected in Cuiabá, also in the state of Mato Grosso. Similarly, Silva & Dantas (2013) reported the same beginning of germination for seeds of different *Sideroxylon obtusifolium* (Roem. & Schult.) T.D.Penn. parent plants collected in Boa Vista, Paraíba, and Juazeiro, Bahia. However, at the end of the experiment, those derived from Juazeiro had higher %G and GS (germination speed, calculated by inverting the MGT value) than those from Boa Vista.

Several studies have already proven that factors related to the origin of seeds, i.e., which directly affect the parent plant during fruit formation and ripening, also affect the physiological quality of seeds, such as carbon dioxide levels and soil moisture, competition with plants nearby, photoperiod and light quality, application of herbicides or hormones, mineral nutrition, physiological age of the plant, as well as genetic polymorphisms (Baskin & Baskin, 2014).

Also, even though the panicles were collected in a short period between each mother plant and location and bore fruits considered mature, the maturation state of the seeds may have been unequal between themselves. Indeed, it is known that even seeds from the same fruit or plant, collected at the same time, may be in different development stages (Bewley et al., 2013). Seed maturation is directly associated with fruit maturity, but is of difficult measurement and can vary greatly between species, being associated with seeds' embryo size, vigor, moisture or dry matter content, weight, size, or chemical factors (Bonner et al., 2008; Carvalho & Nakagawa, 2012). Since the seeds in this part of the study were tested just after 30 days of being collected, their maturity stage may have played a more important role in germination, because results of previous studies with *L. brasiliense* (data not published) showed that after a few weeks of storage, germination becomes similar between seeds of different origins, which could indicate the stabilization of metabolic activities resulting from the end of the stage of maturation of seeds or fruits (Marcos-Filho, 2015).

3.2. Seed longevity tests

There was interaction between time of storage and storage environments for germination percentage, mean germination time, mean germination speed index, and beginning of germination.

There was a linear effect between the beginning of germination and the storage time, regardless of the tested environment (Figure 1). The longer the storage time, the higher was the number of days required for the germination process to begin, regardless of the conditions under which seeds were stored (Table 2). This may imply aging and slowing down of the metabolic activities of the seeds over time. This is a phenomenon common to most seeds, being observed by signs such as delayed radicle protrusion (Bewley et al., 2013), which was observed in this study through the linear increase in the time it took for seeds to begin to germinate under the three storage conditions tested.

Table 2. Means for beginning of germination (days), germination percentage (%), mean germination speed index and mean germination time (days) of *Limonium brasiliense* (Boiss.) Kuntze. seeds for each storage environment and time (days) tested.

Storage environment	Storage time (days)	Beginning of germination (days)	Germination percentage (%)	Mean germination speed index	Mean germination time (days)
Cold chamber	90	10,83	27,67	0,05342	10,83
Cold chamber	150	11,75	29,84	0,04492	11,75
Cold chamber	210	11,50	25,73	0,05317	11,50
Cold chamber	270	14,04	28,46	0,04550	14,04
Cold chamber	395	-	0	-	-
Cold chamber	478	-	0	-	-
Cold chamber	564	-	0	-	-
Dry chamber	90	8,83	24,40	0,07125	8,83
Dry chamber	150	13,21	41,55	0,04013	13,21
Dry chamber	210	13,25	26,27	0,04992	13,25
Dry chamber	270	12,25	25,85	0,04858	12,25
Dry chamber	395	13,17	23,07	0,05358	13,17
Dry chamber	478	15,50	24,15	0,04625	15,50
Dry chamber	564	14,00	24,60	0,04850	14,00
Ambient temperature	90	10,58	23,55	0,06142	10,58
Ambient temperature	150	11,75	28,34	0,05292	11,75
Ambient temperature	210	13,00	25,55	0,04842	13,00
Ambient temperature	270	11,25	22,50	0,04717	11,25
Ambient temperature	395	13,75	22,58	0,05325	13,75
Ambient temperature	478	19,00	25,00	0,04550	19,00
Ambient temperature	564	16,13	24,30	0,02788	16,13

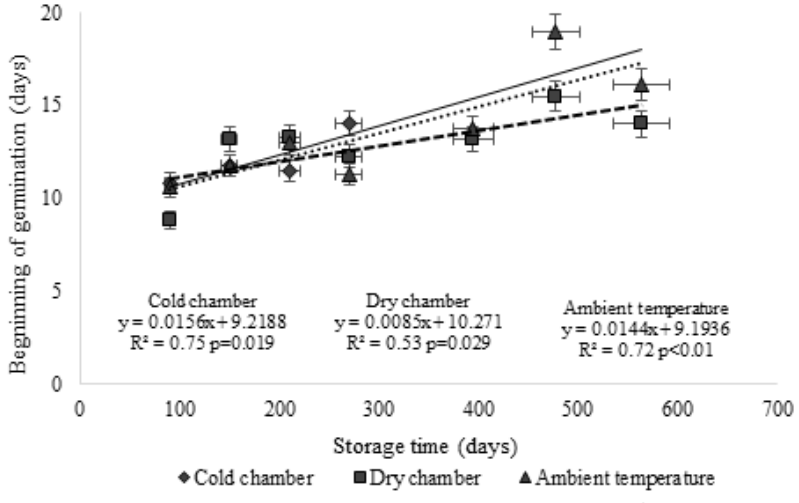

Figure 1. Beginning of germination (days) of *Limonium brasiliense* (Boiss.) Kuntze seeds under three different conditions and in different storage periods.

We can imply that, for *L. brasiliense* seeds, temperature and humidity storage conditions have little to no effect on the time they will take to begin their germination, but the time they are stored is directly and linearly linked to it.

Regarding %G throughout storage time, it had a high statistical significance value, resulting in a quadratic tendency towards the conditions 'cold chamber' and 'ambient temperature'. Germination percentage under these two conditions increased according to days after seed sampling, with maximum point calculated in 198 days for cold chamber and 287 days for ambient temperature, and decreasing values after these periods. This result might be related to the fact that, before the maximum

point, seeds were still in the final ripening phase, a process that needs to be completed before germination begins (Marcos-Filho, 2015). The low temperatures of the cold chamber seem to have slowed down the metabolism of the seeds, conserving higher %G until around day 300 of storage; while the ambient temperature showed an overall lower %G during the period, but ceasing germination only after 500 days of storage.

However, there was no significant regression adjustment for seeds stored in dry chamber, thus indicating that the germination percentage of these seeds was stable (mean value of 26.13%) throughout the storage period (Figure 2). The reduced relative humidity of the air in the dry chamber, compared to the other two conditions, may have played a role in maintaining the percentage of germination more stable during the storage, without reaching the mark of no germination during the period tested. This suggests an orthodox behavior of the seeds of this species.

Regarding MGSI, only ambient temperature (laboratory environment) had a quadratic decrease throughout storage time (Figure 3A). In other words, the longer the storage, the lower was germination speed. On the other hand, seeds kept in cold and dry chambers had no significant regression adjustment according to storage time, which was also observed for mean germination time in all storage environments (Figure 3B). This indicates that lower temperatures (cold chamber) or lower conditions of relative air humidity (dry chamber) better preserve the species' seeds regarding their MGSI, while the non-controlled temperature and humidity of the 'ambient temperature' negatively affect this index over time during storage. However, those assumptions do not apply when comparing MGT, a variable that storage time and conditions didn't seem to affect – the seeds are equally preserved in the three conditions.

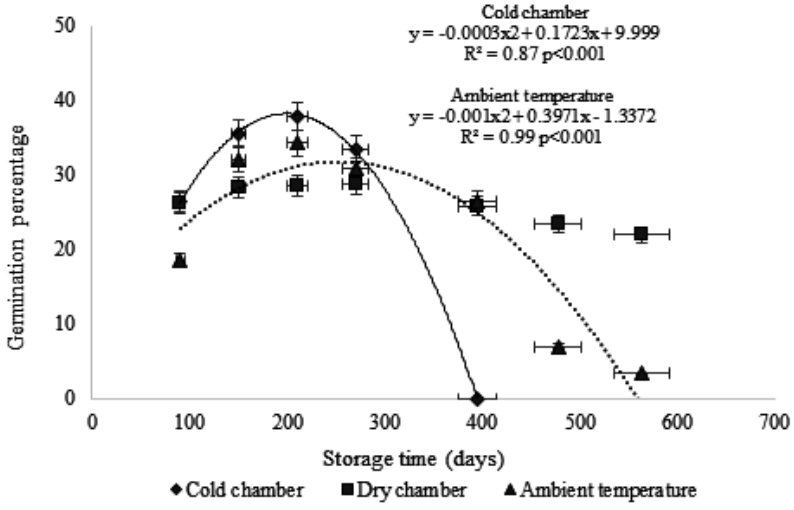

Figure 2. Germination percentage (%) of *Limonium brasiliense* (Boiss.) Kuntze seeds stored in cold chamber, dry chamber, and at ambient temperature at different times.

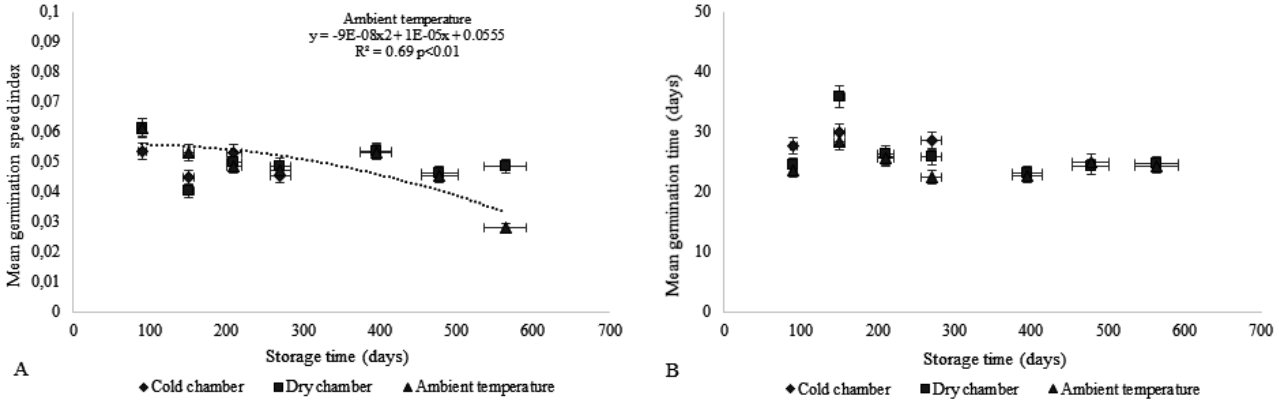

Figure 3. Mean germination speed index (A) and mean germination time (B) of *Limonium brasiliense* (Boiss.) Kuntze seeds under three conditions and different storage times.

These data indicate that it is possible to store *L. brasiliense* seeds for a longer period, without affecting MGSI under the conditions of cold and dry chambers, and without affecting MGT under the three storage conditions. This allows the storage of *L. brasiliense* seeds until they are sown at a site with more favorable environmental conditions (referring to sowing in the field) or even to create a germplasm bank that preserves certain genetic features in one batch of seeds (Bewley et al., 2013). Similar to the present study, the storage in 'ambient' and 'fridge' temperature of *Caesalpinia pyramidalis* (Benth.) Brenan seeds for up to nine months had little effect on its germination percentage and mean germination time. When stored under ambient temperature and in a refrigerator, there was increased GSI (Antunes et al., 2010). However, a different result was found by Batista et al. (2011), who studied *Cedrela odorata* L. and found that seeds stored at ambient temperature sharply reduce their germination percentage throughout storage, reaching zero in the fourth month, while those in the refrigerator maintained this variable stable until the ninth month tested.

Chaves et al. (2012) observed a small variation in %G of *Jatropha curcas* L. seeds stored for 12 months under different conditions: at ambient temperature, in a cold chamber, and in an acclimatized chamber. On the other hand, GSI had significantly lower values only in the sixth month tested. Zonta et al. (2014) studied *J. curcas*, testing different temperatures and packages in a 450-day storage, observed that seeds stored both at ambient temperature and in a refrigerated room at 18-20°C, 10-12°C or 5-7°C had a decrease in %G until the end of the study period. Something similar occurred in our study for 'ambient temperature' and 'cold chamber' conditions.

In short, the origin of *L. brasiliense* seeds affects their germination – at least in the initial 30 after harvest –, corroborating the findings in other similar studies. Also, disinfestation utilizing ethanol and sodium hypochlorite, a common method to sterilize seeds, does not affect germination; the seeds were anatomically damaged by either chemical to the point of affecting their ability to germinate. In this study, the presence of microorganisms, even without utilizing the method, did not inhibit or slow down germination, but disinfestation could still be safely utilized to prevent potential contamination during the germination period. During the storage period, however, disinfestation was not deemed necessary, and storing the seeds in a dry chamber, an environment with reduced humidity and controlled temperature, maintained the viability of the seeds for a greater period.

Finally, the data obtained in this study are essential to determine how the germination of *L. brasiliense* seeds might be affected by common procedures conducted both in the laboratory and in the field, e.g. seeds submitted to different disinfestations, as well as storage times and conditions. It is an initial study, which might help to define parameters for future tests intended for the propagation of species that have important proven bioactive activities.

4. CONCLUSION

- The disinfestation of *L. brasiliense* seeds with ethanol and sodium hypochlorite does not affect germination and could be utilized to avoid contaminations that can interfere with the analysis of the seeds.
- The longevity and the conservation of variables regarding the vigor of the seeds are affected by time and conditions of storage, and the dry chamber environment conserved the viability by 80% of the initial value after 18 months, indicating that this is the most adequate environment among those tested in this study for storage of *L. brasiliense* seeds.

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Claudimar Sidnei Fior: Conceptualization (Supporting), Data curation (Supporting), Formal analysis (Equal), Methodology (Equal), Writing – review & editing (Supporting), CRediT Taxonomy.

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