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# Storage of short-lived seeds of *Inga vera* subsp. *affinis* in osmotic medium

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**Research Paper**

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## Abstract

*Inga vera* subsp. *affinis* (Fabaceae) is a tree species native to riparian forests in Southeast Brazil and is key for the restoration of deforested areas. The species produce seeds that are highly recalcitrant. Extreme sensitivity to desiccation as well as vivipary are commonly observed in mature seeds, which also tend towards polyembryony. Past research has shown that typical strategies to store seeds are inapplicable to *Inga vera* as viability is completely lost when seeds are either dried to around 28% water content (wet basis) or stored at 5°C for a few weeks. Here, we examine the feasibility of storing the seeds under hydrated conditions but at reduced water potential. Freshly collected seeds were kept under conventional storage conditions (plastic bags in cold chamber, 5°C) and in polyethylene glycol (PEG) solutions (−1.6 and −2.4 MPa) at 10°C. Seed germination was assessed after various intervals of time, until all seeds had lost viability. Before storage, seeds attained 100% germination and produced an average of 1.8 normal seedlings per seed (due to polyembryony). Storage in PEG at −1.6 MPa maintained 90% germination (radicle protrusion) and one normal seedling per seed on average for more than 200 d. Osmotic storage likely slowed down metabolism within the seed and hence consumption of food reserves. The storage time achieved has practical applications for *in situ* restoration, but cannot address *ex situ* germplasm conservation. Extending shelf life for an additional 6 months allows tree nurseries to optimize the production of seedlings so that they can be planted during the wet season.

## Introduction

Conservation of plant genetic diversity requires both *in situ* and *ex situ* approaches. *In situ* conservation, which works at the habitat level, is preferred, but protected landscapes remain threatened by several factors, such as fire, deforestation, diseases and habitat fragmentation. This vulnerability can lead to depletion of genetic diversity within populations, threatening their long-term stability survival (Faria et al., 2006; Cruz Neto *et al.*, 2014). *Ex situ* conservation can mitigate the loss of genetic diversity in fragmented habitats by maintaining reserves of germplasm in botanic gardens and seed banks. Technologies for *ex situ* conservation are readily available for species that produce seeds that survive drying to low relative humidity; once dried, these 'orthodox' seeds maintain viability when placed in freezer storage at −20°C (FAO, 2014). However, not all species produce seeds with high tolerance to desiccation.

Seeds that are sensitive to desiccation were classified by Roberts (1973) as 'recalcitrant' because they cannot survive long-term storage or subzero temperatures. The basis for the desiccation tolerance/sensitivity in seeds is associated with the overlap between embryo development and germination metabolic programs, and this can lead to the appearance of a continuous range of seed physiologies (or tolerances to drying and temperature) rather than fixed categories (Berjak and Pammenter, 1994; Pammenter et al., 2003; Bonjovani and Barbedo, 2008).

Seeds from guaba (*Inga vera*) are highly recalcitrant and, like *Castanospermum australe*, represent a rare example of recalcitrant-seeded species within Fabaceae (Marques et al., 2019). In this species, drying to water contents (WCs) below 35% (wet basis) is lethal (Bonjovani and Barbedo, 2008), as is storage for 15 d or more at room temperature (Carvalho, 1994; Bilia and Barbedo, 1997). The high metabolic activity might explain the requirement for water and short life span (Bonjovani and Barbedo, 2008). *Inga* trees are used for food, firewood, land reclamation (Pritchard et al., 1995) and medicinal purposes (Stein et al., 2010). Guaba is one of the most frequently planted tree species for ecological restoration of riparian forests (Caccere et al., 2013; Cruz Neto *et al.*, 2015). Hence, the recalcitrance of the seeds has important economic and ecological implications.

Since lower, non-lethal water potentials slow metabolic activity (Vertucci, 1989), we hypothesized that exposure of Inga seeds to osmotic media might extend shelf life (Andreo



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et al., 2006; Faria et al., 2006). Polyethylene glycol (PEG) (molecular weight = 8000) is an inert polymer that does not penetrate the cells and controls water movement between the seeds and the medium. This study aimed to find a water potential ×temperature combination that prolonged the survival of guaba seeds. In this paper, we compared the shelf life of these seeds using a commonly used practice of refrigerator storage and new methods that adjust the osmotic potential during storage in PEG solutions.

## Materials and methods

### Plant material

Mature fruits were collected in Ribeirao Vermelho, MG, Brazil, and taken to the Tree Seed Laboratory at the Federal University of Lavras for seed processing according to Faria et al. (2006). Seeds were extracted from fruits manually and washed under the tap to remove sarcotesta and the extremely thin seed coat leaving the bare embryo. For the sake of simplicity and to avoid confusion with the polyembryonic condition often observed in this species, hereafter embryos will be referred to as seeds.

### Water content determination

Seed WC was assessed by comparing the fresh and dry mass of seeds, with dry mass measured after heating seeds in an oven (105 ± 3°C) for 24 h according to Brasil (2009). The results are expressed as a percentage of the wet (i.e. fresh) mass basis.

### Seed storage

Two methods of storage were tested: (1) cool storage – seeds stored in plastic bags in a cool chamber at 8°C; (2) osmotic storage – seeds stored in PEG solutions. For osmotic storage, seeds were placed in plastic trays and covered to a 0.5 cm depth with solutions of PEG in water to give water potentials of −1.6 and −2.4 MPa (following the protocol by Andreo et al., 2006). The trays were covered with plastic film to decrease water evaporation. The solution was replaced to maintain a 0.5 cm level or when it was noticeably darkening. Trays were kept in an incubator at 10 ± 1°C in the dark, following the protocol by Andreo et al. (2006). Samples from both treatments were assessed for germination and WC almost monthly for a year or until germination percentage decreased to 0%.

### Germination assay

Germination tests of fresh and stored seeds used 4 replications of 25 seeds per treatment, that were rolled in wet germitest paper (JProlab, Sao José dos Pinhas, Brazil) and kept in an incubator at 30 ± 1°C (Eletrolab – EL 212/4), under constant light, according to Andreo et al. (2006). In the case of seeds with more than one embryo, germination was considered completed when root protrusion was observed for at least one embryo. A normal seedling exhibited shoot and root, with normal aspect. The final counting of germination was done 14 d after seeds were sown on the paper (Andreo et al., 2006).

### Optical microscopy

Embryonic axes were taken from each of the three sets of storage conditions about every 30 d and cut manually with a blade for microscopic analysis. The axes were stored in Karnovsky solution (Karnovsky, 1961) until the microscopic analysis procedures were performed. Lugol solution (Kraus and Arduin, 1997) was used as an indicator test for the presence of starch (Ventrella and Almeida, 2013). The specimens were observed and photographed using an Olympus CX41 microscope coupled with a digital camera Belcam DIV-3000.

### Data analysis

The experimental design was completely randomized with two factors: storage conditions (8°C in plastic bags and PEG solutions at −1.6 MPa and −2.4 MPa) and storage times (ranging from 0 to 330 d). Statistical analyses used R software (R Core Team, 2013) and were graphed using SigmaPlot. Data were analysed using general linear models with binomial error distributions. The significance of the treatments was observed using the $\chi^2$ test, and averages were compared with LSD test at 95% of confidence. WC, germination and frequency of normal seedlings with storage time and storage condition were correlated using Spearman's correlation test.

## Results

### Water content during storage

Freshly harvested seeds were pale green, as described by Pritchard et al. (1995), with 68% WC. WC did not change in seeds stored at 8°C in plastic bags (treatment 1) throughout the experiment, which ended on day 140, when seeds were dead (Fig. 1A). At 100 d, WC of seeds stored with no osmotic treatment (8°C in a plastic bag) and at −1.6 MPa PEG solution (10°C) were statistically similar (66 and 70%, respectively), the latter appearing to initially fluctuate. WC of seeds stored at −1.6 MPa declined to 62% near the 200th day of storage and thereafter remained unchanged until day 330 (Fig. 1A). Seeds stored in −2.4 MPa PEG had lower WC (53%) since the d 30, which did not change throughout the experiment, which ended on day 200 when all seeds were dead.

### Germination after storage

Fresh seeds were highly viable and usually, 100% germinated (radicle protrusion) within 2 d (data not shown). Germination percentages did not decline after 30 d of storage and were similar for the different storage conditions (93, 99 and 98% at 8°C, −1.6 and −2.4 MPa, respectively). Major changes in seed quality were visible in the 8°C stored seeds after 60 d: the seeds lost their greenish colour, smaller seeds turned black and bigger seeds turned pale pink; germination decreased to 14%. In contrast, germination of seeds stored in either PEG solutions was 99%, statistically equivalent to germination of fresh, non-stored seeds (Fig. 1B). Seeds stored at 8°C did not germinate after 90 d of storage, while those stored in PEG maintained 92% (−1.6 MPa) and 80% (−2.4 MPa). After 140 d of storage, germination of seeds stored in PEG at −2.4 MPa decreased to 41%, while germination of the −1.6 MPa treatment declined slightly to 90%, which was not statistically different from fresh seeds at the 0.05 level. After 200 d of storage, all seeds stored in PEG −2.4 MPa were dead, while 88% of those stored in −1.6 MPa germinated (not different statistically from fresh seeds at 0.05 level). The smaller seeds turned black and the bigger ones stayed pale green. At 260, 300 and 330 d of storage, 45, 24 and 0%, respectively, of the seeds stored in PEG −1.6 MPa germinated (Fig. 1B).

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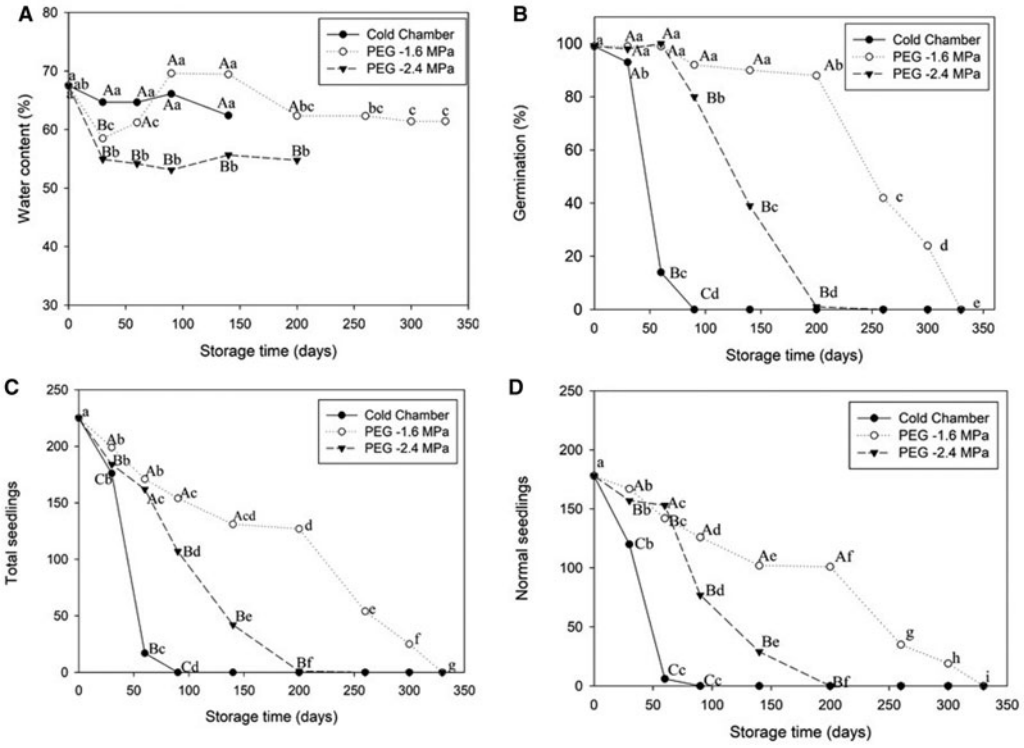


**Fig. 1.** Effect of storage time of guaba seeds on (A) WC (%, wet basis); (B) germination percentage (seed was considered germinated when a root protruded from at least one embryo); (C) total number of seedlings produced by 100 seeds; (D) number of normal seedlings produced by 100 seeds. Capital letters compare storage conditions within a given storage time and small letters compare storage times within storage condition, according to Fisher (LSD) with 5% of confidence.

Guaba seeds are polyembryonic, and in our study 100 freshly harvested seeds yielded 225 seedlings (Fig 1C) of which 178 were normal, producing both roots and shoots (Fig. 1D). Both the total number and the number of normal seedlings produced from 100 seeds declined with storage time, with fastest decline observed in the 8°C (no osmotic) treatment (Fig. 1C, D). A decline in the number of seedlings occurred at similar rates in the −1.6 and −2.4 MPa treatments until about day 60 when the −1.6 MPa treatment supported higher incidence of polyembryony. By day 200, only one normal seedling was produced per seed (on average).

### *Correlations among germination metrics and storage conditions*

Table 1 shows Spearman's correlation coefficients for response variables germination, total seedlings, normal seedlings, WC and storage conditions (8°C, −1.6 and −2.4 MPa in this order) and storage period (0, 30, 60, 90, 140, 200, 260, 300 and 330 d). Storage duration was the most significant factor affecting germination, total seedlings and normal seedlings (correlations of −63, −72 and −68%, respectively). WC positively correlated with germination, total and normal seedlings (33, 36 and 31%), meaning that higher germination performance occurs in seeds stored at higher WC.

### *Food reserves*

Micrographs show that fresh seeds have high starch content (Fig. 2), characterizing such seeds as amylaceous. As the storage time progresses, the starch content decreases, consistent with previous observations of guaba seeds (Faria et al., 2006).

**Table 1.** Spearman's correlation (level of significance) between storage condition, WC, storage time, germination, total seedlings and normal seedlings

| | Storage condition | Water content | Storage time |
|---|---|---|---|
| Germination | 37% (0.0034) | 33% (0.0107) | −63% ($6.83 \times 10^{-8}$) |
| Total seedlings | 31% (0.0167) | 36% (0.0049) | −72% ($1.10 \times 10^{-10}$) |
| Normal seedlings | 39% (0.0020) | 31% (0.0143) | −68% ($1.92 \times 10^{-9}$) |

## Discussion

Longer shelf life of seeds requires reduced metabolic activity of both plant and fungal cells (Parisi et al., 2013) to reduce the rate of deterioration (Andreo et al., 2006; Cardoso et al., 2012). Metabolic activity decreases with decreasing water potential and temperature (Walters et al., 2001) and greater storage potential can be achieved if these stresses do not damage seed cells. It is not possible to dry desiccation sensitive seed to very low levels, since this will damage or even kill them. Guaba seeds do not survive WCs below 35% (Bonjovani and Barbedo, 2008). Hence, optimizing water supply using an osmotic medium, like solutions of PEG, is an interesting strategy because it may avoid big changes in WC of the seeds that could lead to deterioration, germination or death.

The WC of fresh guaba seeds was 68%, similar to that reported by Andreo et al. (2006) and Bonjovani and Barbedo (2008) and also similar to seeds stored at −1.6 MPa (Fig. 1A). Neither fungal proliferation nor initiation of germination was observed during

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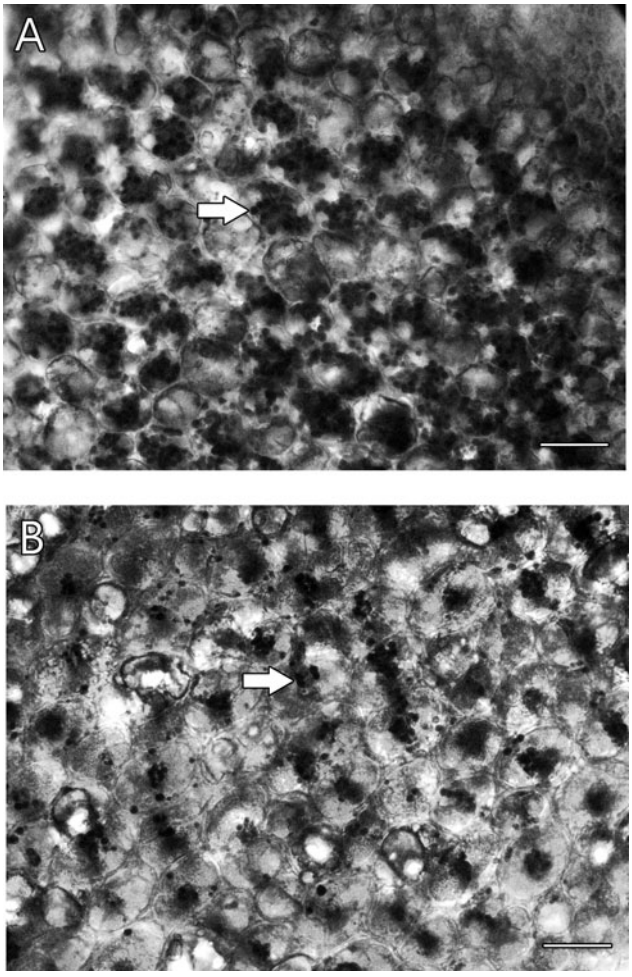


**Fig. 2.** Micrographs of guaba embryonic axes stained with Lugol's solution for starch. (A) axis from fresh seeds; (B) axis from seeds stored for 280 days in PEG −1.6 MPa. Arrows indicate starch granules. Bars = 10 µm.

storage, although these are frequent problems in hydrated storage of recalcitrant seeds, according to Berjak and Pammenter (2013). The high moisture levels used in our study remained slightly below the threshold for germination (Bradford, 1995).

Lowering the moisture to 53% at −2.4 MPa did not appear to initially damage seeds (99% germinated after 60 d of storage), but did appear to cause more rapid deterioration than seeds stored at the higher water potential. Shifts in the longevity of seeds in hydrated storage have been previously noted (Ibrahim et al., 1983; Walters et al, 2001), and were interpreted as hydration-induced changes in metabolism and structure of cells (Vertucci and Farrant, 1995). The active metabolism is probably responsible for death due to shortage of food reserves (Fig. 2) or oxidative activity (Leprince *et al.*, 1999; Roach et al., 2010), altering the energy balance within cells (Pammenter and Berjak, 1994; Faria et al., 2006). At water potentials between 1.5 and −4.0 MPa, macromolecular surfaces are hydrated and water occupies spaces among the macromolecules (Bonjovani and Barbedo, 2008) and contributes to the osmotic responsiveness (cell swelling and contractions). According to Marcos Filho (2005), water potentials that avoid stripping water from macromolecular surfaces will favour the conservation of seeds that are highly sensitive to desiccation.

Results showed that storage at −1.6 MPa and 10 ± 1°C extended survival times of guaba seeds compared to storage at lower or higher water potentials (Andreo et al., 2006; Faria et al., 2006). Successful storage of guaba seeds for 200 d (Fig. 1) is the longest storage period reported for this species, as far as we know. Other studies showed a faster decline in survival or did not test the duration that survival could be maintained: 50% of germination after 10 days of storage in glass containers covered with Parafilm at 5°C (Faria et al., 2004); 94% after 30 days in PEG solution (−1.7 MPa) at 5°C (Faria et al., 2006); 75% after 90 days in PEG solution (−1.6 and −2.4 MPa) at 10° C (Andreo et al., 2006); 85% after 45 days in plastic bags at 8°C (Bonjovani and Barbedo, 2008); 100% of germination after 65 days in plastic bags at 7°C (Parisi et al., 2013). Additional studies to elucidate the relationship between the mobilization of food reserves and viability lost during seed storage will contribute to the understanding of the interaction between metabolism and water potential as well as inform about supplementing reserves such as with nutrient-enriched media.

Methods that prolong the shelf life of guaba seeds is a significant accomplishment for seedling production in nurseries. Guaba is a key species for the restoration of deforested areas in Southeast Brazil, and sufficient seedlings at the right developmental stage are needed for planting during the wet season that extends from October to March. The phenology of the plant currently makes it difficult for restoration work because the fruits ripen in December through February (Andreo et al., 2006; Stein and Fu, 2007) and 4–6 months are needed to produced seedlings for planting (Davide and Faria, 2008). Hence, seedlings are ready for planting in the dry season (April–August), when field survival tends to be much lower compared to seedlings planted during the rainy season. Maintaining seedlings longer in nurseries until the wet season increases the costs of production and decreases seedling quality by interfering with the proper balance between the root and shoot. Thus, extending the shelf life of guaba seeds to 6 months bridges the time to the wet season and optimizes seedling production in tree nurseries by sowing the seeds closer to the wet (planting) season.

## Conclusion

Guaba seeds are highly recalcitrant and usually survive <2 months in storage. Here we show that storing seeds in a PEG solution at −1.6 MPa maintains germination percentage statistically equivalent to fresh seeds for up to 200 d in storage. We believe this water potential is low enough to prevent seeds from completing germination but high enough to prevent stress-induced metabolism that damages the cells. The extension of the shelf life, the longest reported for the species as far as we know, is useful for ecological restoration projects.

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