



## Storage temperatures for *Bowdichia virgilioides* seeds: an endangered species

Temperaturas de almacenamiento de semillas de *Bowdichia virgilioides*: una especie en peligro de extinción

Temperaturas de armazenamento de sementes de *Bowdichia virgilioides*: uma espécie em extinção

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### Abstract

*Bowdichia virgilioides* Kunth is a tree species native to the Brazilian Cerrado that has been listed as an endangered species due to its overexploitation in the last decades. Given its slow and difficult propagation in natural environments, propagation in plant nurseries appears as an interesting strategy to preserve this endangered species. Therefore, in this study we employed a wide range of temperatures (i.e. from -25°C to -196°C) to store seeds of *B. virgilioides* in the short-term, and evaluated its efficiency by assessing seed germination rate, speed index, time and synchrony, as well as seedling growth and quality. Germination percentage was only slightly negatively affected by storage time (up to 32 weeks) at 25°C. Despite that, after 32 weeks of storage, all storage temperatures resulted in similar seed germination parameters and seedling growth and quality. Overall, our results highlight that the seeds of this species can be stored in the short-term at a wide range of temperatures, facilitating large-scale propagation in plant nurseries.

**Keywords:** cryopreservation, germination, orthodox seeds, seed storage, seedling growth

### Resumen

*Bowdichia virgilioides* Kunth es una especie arbórea nativa del bosque Cerrado brasileño que ha sido considerada en peligro de extinción debido a su sobreexplotación en las últimas décadas. Dada su lenta y difícil propagación en ambientes naturales, la propagación de plantas en viveros aparece como una estrategia interesante para preservar esta especie en peligro de extinción. Por lo tanto, en este estudio empleamos un amplio rango de temperaturas (es decir, desde -25 °C a -196 °C) para almacenar semillas de *B. virgilioides* en un corto plazo y evaluar su eficiencia a través de la tasa de germinación de semillas, el índice de velocidad, el tiempo y la sincronía, así como el crecimiento y la calidad de las plántulas. El porcentaje de germinación se vio afectado ligeramente por el tiempo de almacenamiento (de hasta 32 semanas) a una temperatura de 25 °C. A pesar de eso, después de 32 semanas de almacenamiento, todas las temperaturas de almacenamiento dieron como resultado la germinación de las semillas, con crecimiento y calidad de plántulas similares. En



general, nuestros resultados destacan que las semillas de esta especie se pueden almacenar en un corto plazo en una amplia gama de temperaturas, facilitando así la propagación a gran escala en viveros.

**Palabras clave:** criopreservación, germinación, semillas ortodoxas, almacenamiento de semillas, crecimiento de plántulas

## Resumo

*Bowdichia virgilioides* Kunth é uma espécie arbórea nativa do Cerrado brasileiro que tem sido considerada ameaçada de extinção devido à sua superexploração nas últimas décadas. Por ter uma propagação lenta e dificultada em ambientes naturais, a propagação em viveiros de plantas surge como uma estratégia interessante para preservar esta espécie ameaçada de extinção. Portanto, neste estudo, empregamos uma ampla gama de temperaturas (i.e., de -25°C a -196°C) para armazenar sementes de *B. virgilioides* em curto prazo e avaliamos sua eficiência através da taxa de germinação de sementes, índice de velocidade, tempo e sincronia, bem como crescimento e qualidade da plântula. A porcentagem de germinação sofreu pequenas reduções com o tempo de armazenamento (de até 32 semanas) apenas na temperatura de 25°C. Apesar disso, após 32 semanas de armazenamento, todas as temperaturas de armazenamento resultaram em parâmetros de germinação de sementes, crescimento e qualidade de plântulas semelhantes. De forma geral, nossos resultados demonstram que as sementes dessa espécie podem ser armazenadas no curto prazo em uma ampla faixa de temperatura, facilitando a propagação da espécie em larga escala em viveiros.

**Palavras-chave:** criopreservação, germinação, sementes ortodoxas, armazenamento de sementes, crescimento de plântulas

## 1. Introduction

*Bowdichia virgilioides* Kunth (Fabaceae) is a tree species native to the Brazilian Cerrado<sup>(1)</sup>. This species is considered to be pioneer, being well adapted to poor and dry soils and burnt areas, which are typical to Cerrado<sup>(2)(3)</sup>. It bears small (3 to 5 mm in diameter) orthodox seeds that exhibit a strong integumentary dormancy that hinders its propagation in natural environments<sup>(4)</sup>. Alongside its low propagation rate in natural environments, the overexploitation of this species for pharmacological purpose resulted in a considerable reduction in its populations to critical levels, which led to its classification as an endangered species of Brazilian flora<sup>(5)</sup>.

Developing an efficient protocol for plant propagation in nurseries is of paramount importance as part of a recovery strategy to preserve endangered species of slow and hampered natural propagation. In this scenario, seed propagation appears as the most simple and low-cost method for this purpose. Although many studies have shown simple methods to overcome its dormancy<sup>(6)(7)</sup>, no information regarding the most efficient condition for seed storage is currently found in the literature. Because plant metabolism is intrinsically dependent on temperature, lowering the temperature of storage has been long known to extend seed survival and longevity<sup>(8)(9)</sup>.

In traditional seed banks, seeds are generally kept at a temperature of 10°C and relative humidity of 40% in the medium to long-term<sup>(10)</sup>. However, this type of storage does not prevent the genetic erosion over time, especially for recalcitrant seeds, which justifies the utilization of cryogenic temperatures for the conservation of endangered species native to Cerrado, such as *B. virgilioides*<sup>(11)</sup>. In this scenario, and because seed characteristics of *B. virgilioides* imply an orthodox behavior, which typically tolerate low temperatures<sup>(9)</sup>, seed cryopreservation at ultra-low temperatures might function as an alternative for germplasm conservation of this species, ensuring its genetic stability and high physiological seeds quality<sup>(12)(13)(14)</sup>. Therefore, in this study we evaluated the longevity of *B. virgilioides* seeds by assessing germination rate, time, speed index and synchrony, following the storage of seeds at warm and ultra-low temperatures in the short-term. Additionally, we assessed seedling growth and quality through foliar chlorophyll fluorescence<sup>(15)</sup> from seeds at contrasting temperatures.

## 2. Materials and methods

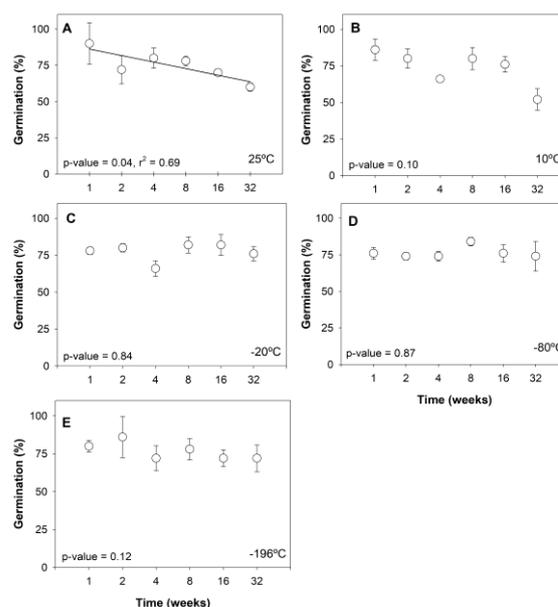
Seeds of *B. virgilioides* were collected during the summer of 2017 from 10 natural populations (i.e. 2-3 plants per population) of this species in an area

comprised of typical Cerrado vegetation located in the proximities of Bom Despacho (19° 44' 09" S, 45° 15' 07" W, 768 m above the sea level), Brazil. Fruits were harvested, packaged in paper bags and taken to the laboratory. Seeds were carefully removed from fruits by hand and immediately stored in aluminum foil bags at five contrasting temperatures: c. 25°C (room temperature), c. 10°C (in a common refrigerator), -20°C (in a freezer), -80°C (in an ultra-freezer), and -196°C (in liquid nitrogen) for 1, 2, 4, 8, 16 and 32 weeks. A set of 6 repetitions of 25 seeds each had their water content assessed after being dried in oven until constant weight, according to the Brazilian Seed Analysis Rules<sup>(16)</sup>.

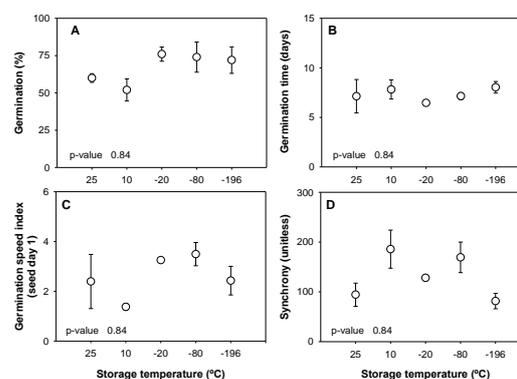
Prior to the germination tests, seeds were treated to overcome dormancy by immersion in water bath at 37°C for 3 min, followed by scarification by immersion in concentrated sulfuric acid for 10 min to overcome dormancy<sup>(17)(18)</sup>. Seeds were then placed in germination boxes containing vermiculite moistened with distilled water until the point of runoff. The boxes were placed in a growth room at room temperature (c. 25 ± 2°C) and irradiance of c. 40 μmol photon m<sup>2</sup> s<sup>-1</sup>, and seed germination was daily assessed for 15 days. Seeds were considered to be germinated after the emergence of the primary root with a length greater than 1 mm. At the end, percentage germination, germination time, speed index and synchrony<sup>(19)</sup> were calculated. In addition to assessing seed longevity and germinability, we assessed the seedlings fresh weight and the maximum quantum efficiency of photosystem II (Fv/Fm) after 15 days. The Fv/Fm was quantified between 9:00 and 11:00 AM in dark-adapted cotyledons of seedlings using an image fluorometer (FluorCam, Photon Systems Instruments, Drasov, Czech Republic)<sup>(20)</sup>.

The experimental design was completely randomized with 5 storage temperature and 6 storage times. Germination assessments were performed in 4 repetitions of 25 seed (n = 4) and seedling characterization in 4 repetitions of all seedlings (n = 4) per storage condition and storage time. Since there was no difference in any of the evaluated parameters between storage time (see percentage germination in Fig. 1; the remaining data are not shown), we utilized data from seeds stored for the longest time (i.e. 32 weeks) (Figs. 2-4). Means were compared by the non-parametric Kruskal-Wallis test at the 95% confidence level or through linear regressions. The statistical analyses were conducted using R (version 4.0.3) and the plots constructed using SigmaPlot 14.0<sup>(21)</sup>.

**Figure 1.** Germination of *Bowdichia virgilioides* seeds stored for up to 32 weeks at contrasting temperatures. Data are mean ± SE, n = 4. A linear regression was fitted in A and the p-value is depicted within this plot. For the remaining plots, p-values for linear regressions were higher than 0.05 (see values within each plot), as well as means (n = 4) were similar between storage times according to Kruskal-Wallis test at the 95% confidence level



**Figure 2.** Germination (A), germination time (B), germination speed index (C) and synchrony (D) of *Bowdichia virgilioides* seeds stored for up to 32 weeks at contrasting temperatures. Data are mean ± SE, n = 4. The p-values for linear regressions were higher than 0.05 (see values within each plot), as well as means (n = 4) were similar between storage temperatures according to Kruskal-Wallis test at the 95% confidence level



### 3. Results and discussions

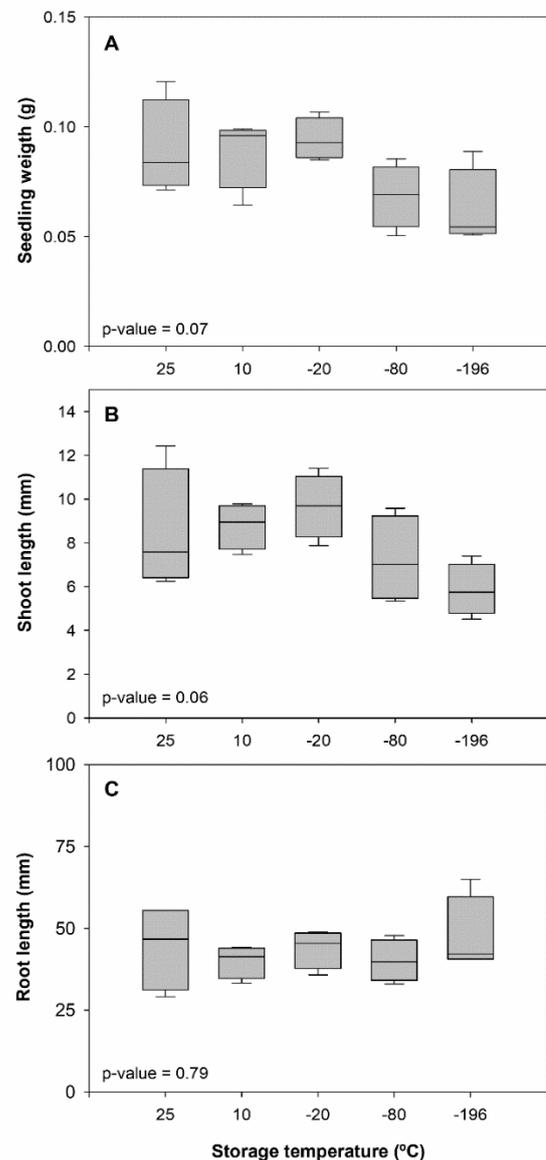
The *B. virgilioides* seed lot characterization before storage resulted in approximately 52,000 seeds per kilogram and the water contents for scarified and non-scarified seeds were 9.25% and 9.92%, respectively. Such low water content is consistent with values found for orthodox seeds<sup>(9)</sup> and in agreement with previous results obtained for this species<sup>(6)</sup>.

Percentage germination only decreased with time at 25°C. At all other storage temperatures evaluated in this study, germination remained similarly high up to 32 weeks (see p-values > 0.05 from B to E in Figure 1). Overall, germination started three days after sowing and reached high levels (i.e. always above 67% and approximately 75% when all data was taken together). Germination speed index, time and synchrony did not decrease with storage time for any of the storage conditions (data not shown). Because, germination decreased with time at 25°C, we decided to utilize the data obtained after 32 weeks of storage to compare the storage treatments as regarding their ability to preserve seed germination, and we observed that all storage temperatures resulted in similar percentage germination, germination speed index, time and synchrony (see p-values > 0.05 for all parameters in Figure 2).

Taken together, these results highlight that the seeds of this orthodox species can be stored at an extremely wide range of temperatures in the short-term, but lower temperatures (from 10°C to -196°C) seem to be more effective in maintaining seed viability for longer periods. Additionally, we observed that ultra-low temperatures, at which seed metabolism is completely suspended<sup>(14)(22)</sup>, are not necessarily needed to maintain seed longevity in the short-term; however, further studies are necessary to assess whether this can be an interesting alternative in the medium to long-term.

It is noteworthy that seeds of *B. virgilioides* were frozen without the utilization of any cryoprotectant<sup>(23)</sup>, and despite that, they were able to germinate as fast and efficiently as seeds stored at warmer temperatures. It is likely that the low water content prevented the formation of intracellular ice crystals that can damage the endomembrane system and cause the semi-permeability loss and cellular compartmentalization<sup>(24)</sup>. Further studies assessing storage of *B. virgilioides* seeds in the long-term might provide researchers with the information on whether the use of cryoprotectants will be necessary under this condition.

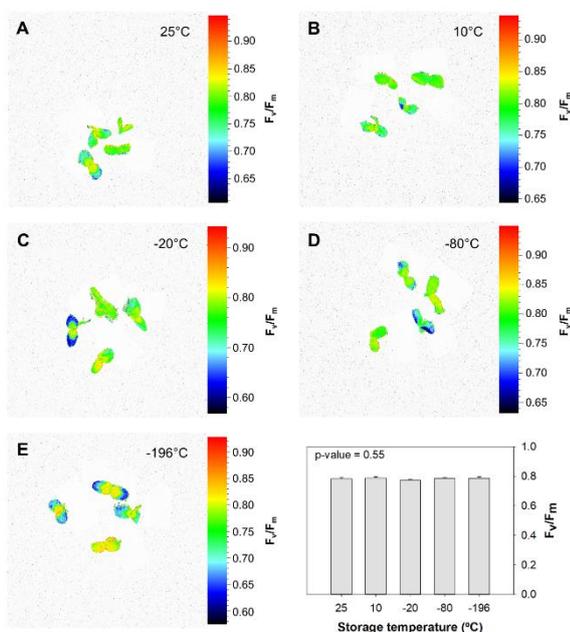
**Figure 3.** Total weight (A), shoot length (B) and root length (C) of *Bowdichia virgilioides* seedlings germinated from seeds stored for 32 weeks at contrasting temperatures. The two extreme lines of the boxplot (whiskers) show the 10 and 90% percentiles; the two bounds of the box, the 25 and 75% percentiles, and the center thick line, the median. Means ( $n = 4$ ) were similar between storage temperatures according to Kruskal-Wallis test at the 95% confidence level



Alike all seed germination parameters, seedling growth was similar in spite of the storage time (data not shown) and the storage temperature (see p-values > 0.05 for all parameters in Figure 3). Seedlings were on average c. 0.07 g and exhibited a c. 8 mm shoot length and c. 45 mm root length. The seedlings size is generally used to identify the most vigorous seeds<sup>(25)</sup>, and these data show that seed vigor of *B. virgilioides* was not negatively affected by

storage time nor storage temperature. Data on  $F_v/F_m$  of seedling cotyledons (Figure 4) demonstrate no damage at the leaf level (i.e. all values are around 0.78, after seed storage for 32 weeks for all storage temperatures), and are in agreement with healthy seedlings<sup>(26)</sup>.

**Figure 4.** Maximum quantum efficiency of the photosystem II ( $F_v/F_m$ ) of cotyledons of *Bowdichia virgilioides* seedlings germinated from seeds stored for 32 weeks at contrasting temperatures. Representative images for each storage temperature are shown in A-E and means are shown in F. Data are mean  $\pm$  SE,  $n = 4$ . Means ( $n = 4$ ) were similar between storage temperatures according to Kruskal-Wallis test at the 95% confidence level



## 4. Conclusions

Our findings demonstrate that although germination percentage slightly decreases with time at 25°C, seeds of *B. virgilioides* can be efficiently stored at a wide range of temperatures in the short-term and still maintain high germinability, synchrony and short germination time, as well as the production of vigorous seedlings. Additional studies, however, are necessary to determine whether lower temperatures, including cryopreservation, would be a better alternative for seed storage in the medium to long-term, given the decrease in germination percentage at room temperature.

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## Author contribution statement

All authors contributed equally to this study.

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