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Seed germination performances of *Styrax* species help understand their distribution in Cerrado areas in Brazil

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Abstract

In this descriptive paper, we described germination responses of *Styrax pohlii*, *S. camporum* and *S. ferrugineus* seeds at 5, 10, 15, 20, 25, 30, 35, 40 and 45 °C. We also assessed the percentage germination (%G) of *S. pohlii* seeds with different seed water contents because, as a forest species, it seems to have recalcitrant seed behavior. Intrigued by the capacity of seeds of this species to germinate directly from puddles formed on poorly drained soils of riparian forests, where it typically occurs, we also tested the effect of de-pulping fruits on germination of *S. pohlii* seeds under hypoxia and normoxia conditions. In addition, we checked whether distinct concentrations of gibberellic acid (GA_3) could break *S. ferrugineus* seed dormancy, a typical seed behavior of Cerrado species. No germination occurred at 5, 40 and 45 °C, regardless of the species. The optimal temperature for germination was 20 °C for *S. pohlii* and 25 °C for *S. camporum*. However, *S. ferrugineus* seeds showed a very low %G, regardless of the temperature, and GA_3 could not consistently break possible physiological seed dormancy. For *S. pohlii* seeds, the higher the seed desiccation the lower the %G, and fruit pulp removal showed to be essential for seed germination. *S. pohlii* seeds germinated independently of oxygenation conditions.

Key words: hypoxia, *Styrax pohlii*, *S. camporum*, *S. ferrugineus*, temperature, desiccation seed tolerance.

Desempenho germinativo de sementes de espécies de *Styrax* ajuda a entender sua distribuição em áreas de Cerrado no Brasil

Resumo

Neste trabalho descritivo foi mostrada a resposta germinativa das sementes de *Styrax pohlii*, *S. camporum* e *S. ferrugineus* a 5, 10, 15, 20, 25, 30, 35, 40 e 45 °C. Avaliou-se também a porcentagem de germinação (%G) de sementes de *S. pohlii* com diferentes conteúdos de água, pois, por ser uma espécie florestal, suas sementes parecem ser recalcitrantes. Dada a capacidade de as sementes dessa espécie germinarem diretamente em poças d'água formadas em solos mal drenados nas florestas ripárias, onde a espécie tipicamente ocorre, também testou-se o efeito do despolpamento dos frutos para a germinação de sementes de *S. pohlii* em condições de hipóxia e normóxia. Além disso, verificou-se se diferentes concentrações de ácido giberélico (GA_3) poderiam quebrar a dormência das sementes de *S. ferrugineus*, que é característica de sementes de espécies do Cerrado. Não houve germinação a 5, 40 e 45 °C, independente da espécie. A temperatura ótima para a germinação foi 20 °C para *S. pohlii* e 25 °C para *S. camporum*. Porém, as sementes de *S. ferrugineus* mostraram %G muito baixa, independente da temperatura, e o GA_3 não quebrou consistentemente a possível dormência fisiológica dessas sementes. Para as sementes de *S. pohlii*, quanto maior a dessecação, menor %G, e a remoção da polpa dos frutos mostrou ser essencial para a germinação das sementes. As sementes de *S. pohlii* germinaram independentemente das condições de oxigenação.

Palavras-chave: hipóxia, *Styrax pohlii*, *S. camporum*, *S. ferrugineus*, temperatura, tolerância à dessecação.

1. INTRODUCTION

Seed germination performances studied under laboratory conditions are critical for the understanding of species distribution in nature. Many germination results obtained under laboratory conditions have been reported (Zaidan and Carreira, 2008) for species of the Brazilian savanna (Cerrado), which is a biodiversity *hot spot* in South America (Klink and Machado, 2005). However, few results, if any, possess relationships with data obtained in the field. On the other hand, the plant reproductive success and species distribution are intimately related because of the ability of seeds to germinate and establish plants in natural communities (Kissmann et al., 2012).

Seed germination is widely influenced by environmental resources, such as sunlight (Takaki, 2001), temperature

(Fenner, 1991) and water availability (Fenner and Thompson, 2005). Nevertheless, seed dormancy, maturity, tolerance to desiccation and age are intrinsically important (Bewley and Black, 1994; Daws et al., 2004). Requirements for germination are species-specific, but these requirements may also vary between and within populations from which seeds are selected for laboratory studies (Gutterman, 2000).

Unlike orthodox seeds of most Cerrado woody species, recalcitrant seeds or desiccation-intolerant seeds are dispersed with high water content and active metabolism (Roberts, 1973). These species usually occur in humid environments, being palm trees typical examples (Roberto et al., 2011), but some species showing seeds with recalcitrant behavior also occur in dry environments (Pammenter and Berjak, 2000). Therefore, no desiccation during maturation enables recalcitrant seeds to rapidly germinate after dispersion, because these seeds do not require rehydration (Bewley and Black, 1994). This is an advantage in humid environments because these seeds are able to promptly use natural resources available. On the other hand, rapid germination may not be considered advantageous in unpredictable habitats, such as the savannas. Seeds of savanna species commonly exhibit dormancy, preventing these seeds from germinating under unfavorable conditions (Zaidan and Carreira, 2008).

Styrax L. includes species with distinct distribution in the Cerrado. *S. pohlii* A. DC. is a forest species, frequently occurring in riparian forests within Cerrado areas (Teixeira and Assis, 2005). *S. camporum* Pohl. is widely distributed in the Cerrado, and it is greatly favored by conditions at the edge of forest fragments (Habermann et al., 2011), whereas *S. ferrugineus* Nees & Mart. is well adapted to savanna-type physiognomies that exist in the Cerrado (Habermann et al., 2011). Seed germination performances of *S. camporum*, which disperses its seeds during the dry season (April – August), was studied in the field and this study indicated that its thick tegument reflects its capacity to endure long and severe dry seasons of the Cerrado areas (Kissmann et al., 2012). However, it is unclear whether *S. pohlii* seeds are tolerant to desiccation and how it germinates if fruits are not de-pulped (by animals) or if fruits and seeds fall into puddles on poorly drained soils of riparian forests. In addition, low percentage germination of *S. ferrugineus* seeds (Barbosa et al., 1985) is related to some unknown dormancy.

This is a descriptive paper, in which we characterized germination responses of seeds of these three species under a range of temperatures, under laboratory conditions. We assessed the germination performances of *S. pohlii* seeds with different water contents, and when submitted to hypoxia and normoxia, and also the effect(s) of de-pulping *S. pohlii* fruits on seed germination. Finally, we used different concentrations of gibberellic acid (GA_3) to break possible physiological seed dormancy in *S. ferrugineus*. We discuss the distribution of these species based on data obtained through field experiments (Kissmann et al., 2012) and under laboratory conditions.

2. MATERIAL AND METHODS

Plant material

Mature fruits of *S. ferrugineus* were harvested from October to December 2011 in a fragment (470 ha; 22°18'S and 47°11'W) of Cerrado *sensu stricto* (*s. str.*), which is a savanna-type physiognomy of the Cerrado *sensu lato* (*s. l.*). Fruits of *S. camporum* were harvested from April to May 2011 in a remnant (37 ha; 22°15'S and 47°00'W) of a forest physiognomy called 'Cerradão' (augmentative of Cerrado, in Portuguese), and fruits of *S. pohlii* were collected from February to March 2011 in a riparian forest fragment (32 ha; 24°00'S and 75°30'W). All these sites are located in São Paulo state, Brazil. As described, these harvests were performed after the respective dispersal time of each of the three species, and the seeds of none of the three species were stored prior to germination tests.

After harvesting, the fruits were de-pulped through friction against a 1 mm-steel sieve, under tap water, aiming to mimic natural conditions of seed dispersal, either by considering seeds after digestion or as regurgitated seeds (Kissmann et al., 2012). Part of the sample of *S. pohlii* fruits was not de-pulped in order to test the effect of pulp on seed germination. The seed water content of samples of each of the three species was gravimetrically determined (adapted from Ista, 2011) soon after the harvests.

Temperature experiments

De-pulped seeds of the three species were immersed in a solution of sodium hypochlorite (1%, v/v) for 1 min to prevent fungal infection during germination. Disinfected seeds were placed on filter paper that was wetted with distilled water within transparent plastic boxes called "gerboxes" (13×13×4 cm). Thirty seeds per gerbox (replications) were incubated under constant fluorescent light (80 μmol m^{-2} s^{-1}) within germination chambers (Eletrolab, São Paulo, Brazil), in which constant temperatures were set to 5, 10, 15, 20, 25, 30, 35, 40 and 45 °C.

At the end of the tests, non-germinated seeds were submitted to the tetrazolium test to assess their viability. For this test, seed halves were placed in a tetrazolium solution (0.5%, v/v) at pH 6.5-7.0, and incubated at 35 °C in the dark for four hours (adapted from Ista, 2011). These seeds were classified into viable or unviable seeds, according to the degree of staining of embryos.

For each of the three species, it was used a completely randomized experimental design, with six replications. The

number of germinated seeds was monitored every other day until 90 days after sowing (DAS) for *S. camporum* and *S. ferrugineus*, and until 60 DAS for *S. pohlii*. We did not monitor seeds under green light because seeds of these three species are aphotoblastic, being such behavior confirmed for *S. camporum* seeds (Simão et al., 2013). Protrusion of roots with 2 mm in length was used as evidence of germination. The percentage of germinated seeds (%G); the relative frequency of germination (RF=ni/Nt, where ni is the number of germinated seeds between times ti–1 and ti, and Nt is the total number of germinated seeds); and the mean germination time [T=(Σ(ni ti)/Σni), where ni is the number of germinated seeds in the time interval from ti–1 to ti] were calculated according to Labouriau and Agudo (1987).

For *S. pohlii* and *S. camporum* seeds, a one-way analysis of variance (ANOVA) was performed to evaluate differences in %G between 10, 15, 20, 25, 30 and 35 °C, whereas for *S. ferrugineus* the same procedure was used to check differences in %G between 10, 15, 20 and 25 °C because not all species showed germinated seeds under the nine temperatures tested. Mean values were compared by Tukey's test (p=0.05), after transforming %G into arcsin $(\%G/100)^{0.5}$.

Germination tests of *S. pohlii* seeds with different seed water contents

De-pulped seeds of *S. pohlii* were dried inside a glass desiccator containing silica gel (anhydrous calcium chloride), which was replaced whenever its color started changing from blue to purple. The target seed water content was obtained by monitoring the loss of fresh mass in seed samples. The final sample dry mass was calculated according to the following equation: $Mf=M_0\ [(100–C_0)/(100–C_f)]$. In this equation, Mf represents the final target seed mass (g), C_0 and C_f are the initial and final (target) water contents (%), respectively, while M_0 is the initial sample seed mass (Cromarty et al., 1990).

Seed water content was gravimetrically determined before and after drying samples for target values (adapted from Ista, 2011). Water content of non-dried seeds (control seeds) was 50±0.5%. After drying samples, water contents of the three seed samples were 43±0.1, 33±1.1, and 12±0.4%.

Seed samples (180 seeds) of each of the four treatments (the three seed water contents, and the control) underwent disinfestation procedures and germination tests as previously described. Six gerboxes containing 30 seeds each from each of the four treatments were put under constant fluorescent light (80 μmol m^{-2} s^{-1}) within germination chambers (Eletrolab, Brazil), under constant 25 °C.

A one-way ANOVA was used to test differences in %G between seed samples of each of the four treatments. Mean results were also compared by the Tukey's test (performed at the 5% significance level).

Germination tests of *S. pohlii* non de-pulped fruits and hypoxia effects on germination

De-pulped (seeds) and non de-pulped fruits of *S. pohlii* were placed to germinate within gerboxes, under normoxia and hypoxia conditions. Normoxia conditions was obtained by placing the seeds on wet filter paper within gerboxes, and hypoxia conditions was obtained by placing seeds on filter paper, and adding distilled water so that 2/3 of each seed was covered.

Water used for hypoxia conditions was stored in containers prior to the study. The water level in gerboxes was checked every other day, and whenever necessary water was supplemented, in a slow and gentle manner, in order to avoid bubbles and/or increase in dissolved oxygen (DO) concentration. DO concentration in gerboxes was assessed by the iodometric method (Golterman et al., 1978), and it stayed at 4.5±0.2 mg L^{-1} throughout the study, which is much lower than DO values found in floodplains of the Amazonian Forest (Bracho-Nunez et al., 2012; Furch and Junk, 1997). This procedure aimed to mimic DO concentration into puddles on the floor of swamp forests, where *S. pohlii* seeds and seedlings are found.

De-pulping, disinfection and germination procedures were conducted as already described. The gerboxes were placed under constant fluorescent light (80 μmol m^{-2} s^{-1}) within germination chambers (Eletrolab, Brazil), in which the temperature was set to constant 25 °C. Data were used to calculate the cumulative percentage germination and mean germination time (T), according to Labouriau and Agudo (1987).

A two-way ANOVA was carried out to test the effects of the two-level 'pulp' and 'oxygenation' factors, as well as their interactions on %G and mean germination time (T). The Tukey's test was performed for post hoc comparisons of mean results at the 5% significance level.

Germination tests of GA_3-treated seeds of *S. ferrugineus*

Seed samples of *S. ferrugineus* were immersed in 0, 50, 100, 150, 300 and 600 mg L^{-1} GA_3 (gibberellic acid, Gibco BRL, Grand Island, NY, USA) oxygenated solutions for 24 h, since this interval is satisfactory for the initial imbibition of *S. ferrugineus* seeds (Kissmann et al., 2012). After this procedure, these seeds were submitted to germination tests as previously described. Gerboxes were placed under constant fluorescent light (80 μmol m^{-2} s^{-1}) within a germination chamber (Eletrolab, Brazil), in which the temperature was set to constant 25 °C.

A one-way ANOVA was conducted to detect significant differences in %G between seed samples treated with the six different GA_3 solutions. The Tukey's test was once

more performed at the 5% significance level to compare mean results.

3. RESULTS

The initial seed water content (after dispersion) was 50±0.5%, 11±0.01% and 18.8±0.42% for *S. pohlii*, *S. camporum* and *S. ferrugineus* seeds, respectively.

Seeds of the three species started germinating at 10 °C and no germination was observed when seeds were placed to germinate at 5, 40 or 45 °C. Besides not germinating under these temperatures, *S. ferrugineus* seeds did not germinate at 30 and 35 °C.

S. pohlii seeds exhibited the same %G when exposed to 10, 15, 20, 25 and 30 °C, but at 35 °C seeds of this species showed lower %G as compared to the other temperatures (Figure 1a). In addition, the mean germination time (T) was the lowest at 20 °C for *S. pohlii* seeds (Figure 1b). *S. camporum* seeds showed great variation in %G assessed at different temperatures, however, this species showed the best germination performance at 25 °C, although this result did not differ from %G measured at 20, 30 and 35 °C (Figure 1c). In addition, *S. camporum* seeds seemed to best distribute germination over time at 25 °C (Figure 2b,e,h,k,n,q). Most seeds of *S. pohlii* and *S. camporum* that did not germinate at temperatures ranging from 10 to 35 °C were not viable after 60 and 90 days, respectively, as evidenced by the tetrazolium test.

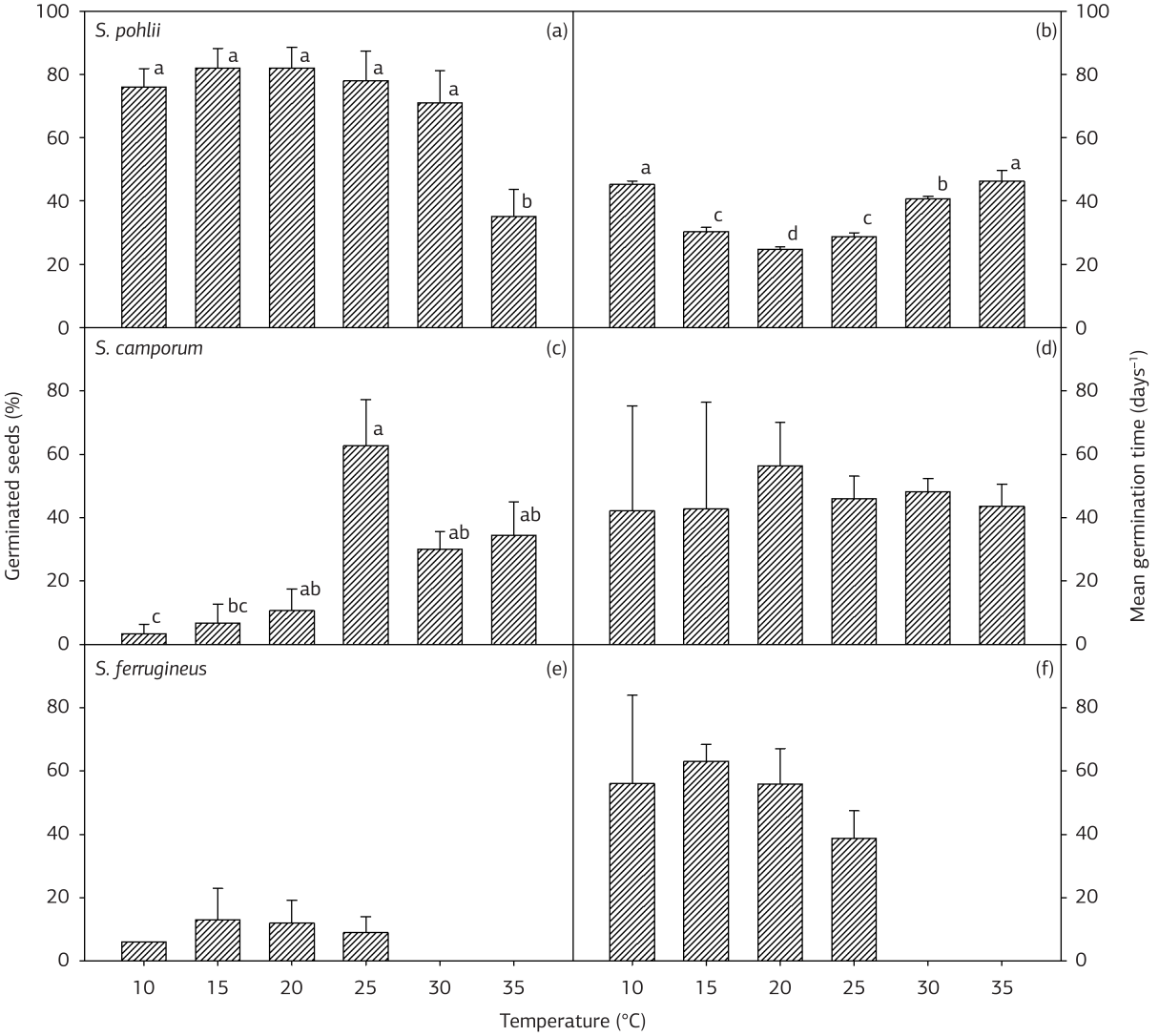

Figure 1. Percentage germination (a, c, e) and mean germination time (b, d, f) of *S. pohlii* and *S. camporum* seeds at 10, 15, 20, 25, 30 and 35 °C, and *S. ferrugineus* seeds at 10, 15, 20 and 25 °C, under fluorescent light (80 μmol m^{-2} s^{-1}). Columns represent mean values (n=6) and vertical bars are S.D. Different letters show significant differences (p=0.05) between treatments, and the absence of letters indicates a lack of significant differences between treatments.

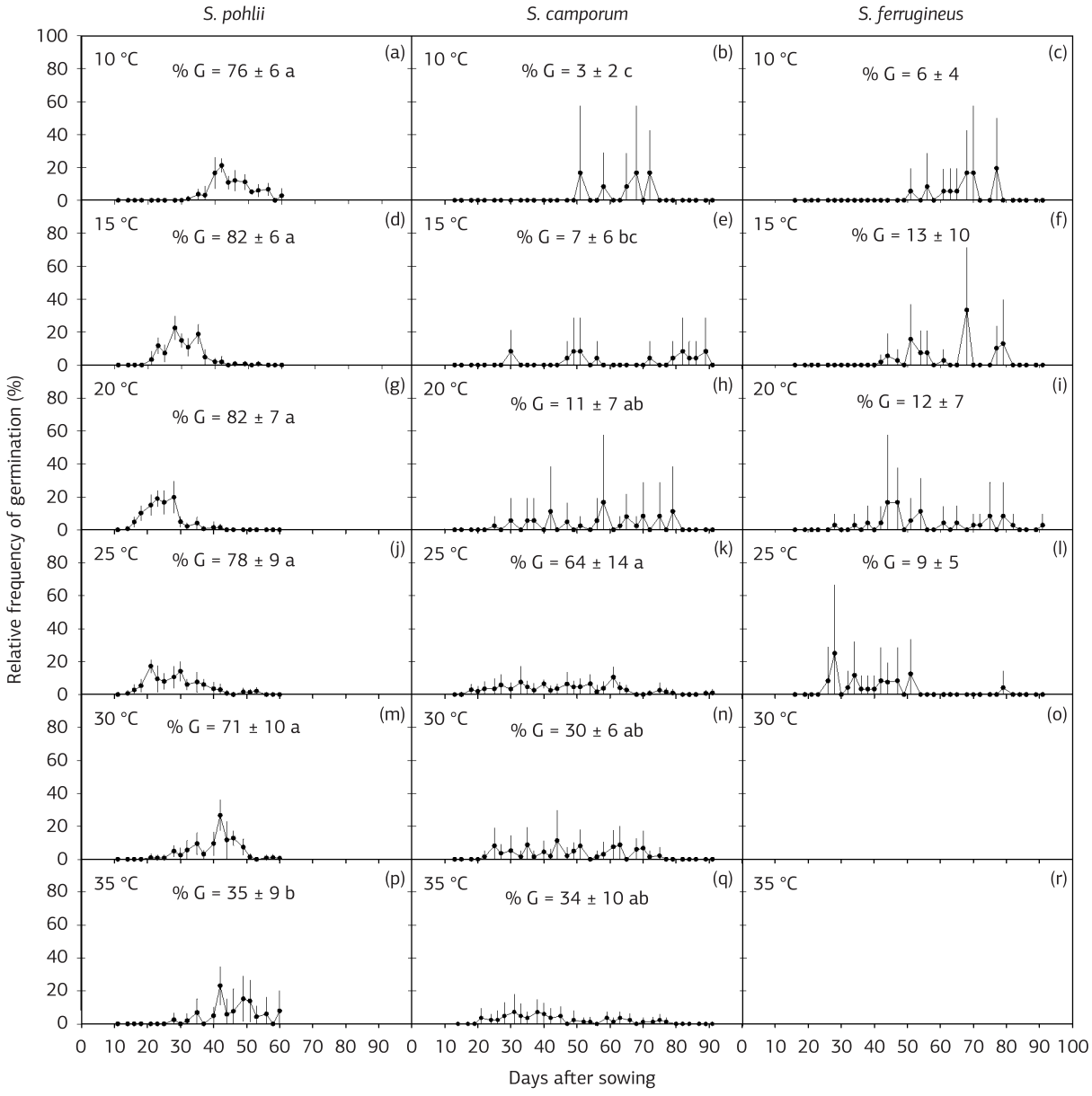

Figure 2. Relative frequency of germination of *S. pohlii* and *S. camporum* seeds at 10, 15, 20, 25, 30 and 35 °C, and *S. ferrugineus* seeds at 10, 15, 20 and 25 °C, under fluorescent light (80 µmol m^{-2} s^{-1}). Dots represent mean values (n=6), and vertical bars are S.D. %G = percentage germination ± S.D.; Different letters show significant differences (p=0.05) in %G between temperatures tested for each species.

S. ferrugineus seeds showed conspicuously low %G (Figure 1e) and similar T at 10, 15, 20 and 25 °C (Figure 1f). Compared to seeds of *S. pohlii* (best %G = 82±6.5% at 20 °C; Figure 1a,b) and *S. camporum* (best %G = 64±14 at 25 °C; Figure 1c,d), *S. ferrugineus* seeds, in general, exhibited less than half of %G values of the other two species, and after 90 days, 50% of these seeds remained viable.

Seed germination of the three species was well distributed over time and no synchronization/concentration of this response was observed, even at temperatures that returned the highest %G (Figure 2). At such temperatures, *S. pohlii* seeds started germinating within 14 days (Figure 2g), while *S. camporum* and *S. ferrrugineus* seeds started germinating within 20-25 days (Figure 2k,f).

The seed water content negatively affected %G of *S. pohlii* seeds. The higher the seed desiccation the lower the %G, but seed samples with 12% water content were still able to germinate (Figure 3). Seeds with 43% and 32% water content exhibited germination peaks earlier than seeds with 50% water content (Figure 3).

S. pohlii non de-pulped seeds showed great fungal infection, which led to 100% mortality. On the other hand, the pulp removal resulted in 83% germination, regardless of oxygenation conditions (Figure 4). Interestingly, seeds of this

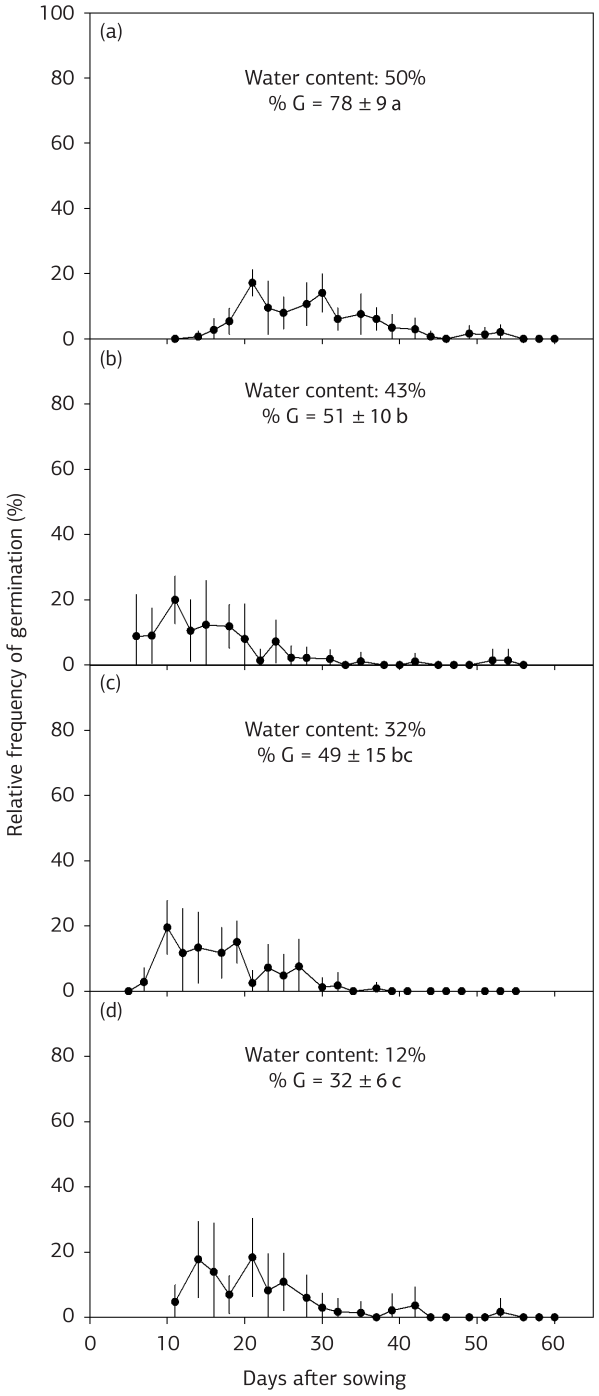

Figura 3. Relative frequency of germination of *S. pohlii* seeds, at 25 °C and under fluorescent light (80 µmol m^{-2} s^{-1}), in response to seed water contents of 50%, 43%, 32% and 12%. Dots represent mean values (n=6) and vertical bars are S.D. %G = percentage germination ± S.D.; Different letters show significant differences (p=0.05) in %G between treatments.

species were able to germinate even when partially covered by a water layer (hypoxia), showing the same germination performance as compared to normoxia conditions (Figure 4).

S. ferrugineus seeds treated with 100 mg L^{-1} GA_3 showed 17±11% germination, which was 6% higher than the result

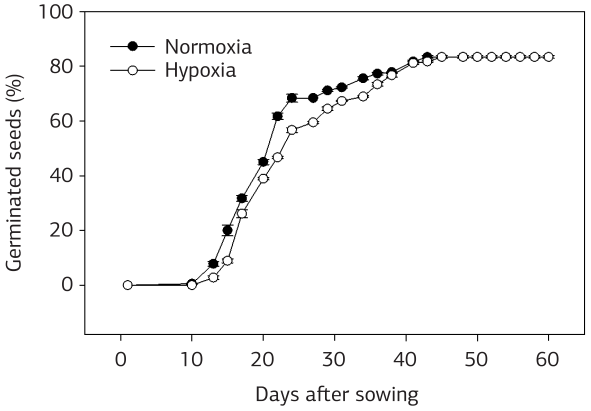

Figure 4. Cumulative percentage germination of *S. pohlii* seeds, at 25 °C and under fluorescent light (80 µmol m^{-2} s^{-1}), submitted to normoxia and hypoxia conditions. Dots represent mean values (n=6) and vertical bars are S.D.

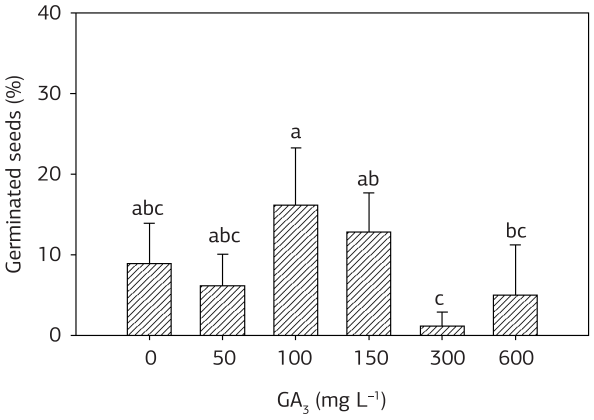

Figure 5. Percentage germination of *S. ferrugineus* seeds, at 25 °C and under fluorescent light (80 µmol m^{-2} s^{-1}) in response to GA_3 treatments. Columns represent mean values (n=6) and vertical bars are S.D. Distinct letters show significant differences (p=0.05) between treatments.

exhibited by control seeds (%G = 9±5%). On the other hand, besides being extremely variable, GA_3 did not consistently improve %G (Figure 5).

4. DISCUSSION

Limiting temperatures for seed germination, or 'cardinal temperatures', define in a species-specific manner the range of temperature at which germination is possible, allowing inferences about the origin of species (Labouriau, 1983). Seeds of *S. pohlii* and *S. camporum*, which are typical of forest physiognomies of the Cerrado, germinate in a wide range of temperatures (Figure 1a,c) in relation to seeds of *S. ferrugineus* (Figure 1e), which is a typical savanna species. Considering the optimal temperature for germination as the one that promotes the highest %G within the lowest mean

germination time (Bewley and Black, 1994), our results showed that the optimal temperature for *S. pohlii* was 20 °C (Figure 1a,b), and for *S. camporum*, it was 25 °C (Figure 1c,d). The ability of these seeds to germinate within a wide range of temperatures may represent a competitive advantage in Cerrado areas. In fact, seeds of these three species buried in different physiognomies of the Cerrado areas showed prompt germination after 60 days (Kissmann et al., 2012). However, the germination performance of *S. ferrugineus* seeds is particularly intriguing. Under field conditions (Kissmann et al., 2012), 40% of seeds of each of the three species had germinated after 60 days, but in the present study *S. ferrugineus* seeds showed 10% germination, regardless of temperature (Figure 1e). We did not test alternate temperatures (e.g. diurnal/nocturnal), but all these observations suggest possible effects of thermal amplitude occurring in soil seed banks, which could break any seed dormancy, as generally suggested by Zaidan and Carreira (2008). Moreover, in the present study, around 50% of *S. ferrugineus* seeds remained viable, as evidenced by the tetrazolium test, reiterating that these seeds show some type of dormancy.

Seeds are considered dormant when they fail to germinate under favorable conditions and also if germination takes longer than four weeks (Baskin and Baskin, 1998). At 20 and 25 °C, *S. ferrugineus* seeds started germinating after 20 days, and kept germinating until 90 days (Figure 2i,l). Barbosa et al. (1985) reported dormancy in *S. ferrugineus* seeds, but these authors did not specify the dormancy type. Seeds of *S. ferrugineus* exhibit fully developed embryos at the dispersal time (data not shown) and have water-permeable seed coat (Kissmann et al., 2012), excluding the possibility of morphological or physical dormancy, respectively.

Gibberellin solutions are commonly used to induce germination in physiologically dormant seeds, acting both on the endosperm degradation (Karssen et al., 1989) as well as on the expansion of embryo cells (Bewley and Black, 1994; Roberto et al., 2011). Gibberellic acid increased %G in *S. ferrugineus* seeds from 9% (control) to 17% (100 mg L^{-1} GA_3; Figure 5). Nonetheless, the effects of GA_3 on the releasing of seeds from physiological dormancy vary greatly according to the deepness of dormancy (deep, intermediate or non-deep; Baskin and Baskin, 2004). Therefore, our data confirmed that *S. ferrugineus* seeds are naturally dormant, as generally suggested by the literature (Barbosa et al., 1985; Zaidan and Carreira, 2008), but we were not able to demonstrate that GA_3 overcome such dormancy, which would, then, be an indication of physiological dormancy. Perhaps other types of gibberellin should be tested.

Seeds of each of the three species exhibited well-distributed germination over time (Figure 2), and this behavior was also confirmed for *S. camporum* seeds (Simão et al., 2013). The slow and non-synchronized seed germination may represent a strategy, which is suitable for unpredictable environments or seasonal climates, since this behavior prevents seeds from germinating after any particular situation (Ferreira and Borghetti, 2004). Despite differences in seed coat thicknesses of these species (*S. camporum* >> *S. ferrugineus* > *S. pohlii*; Kissmann et al., 2012), each of these species are harmoniously adapted to its respective habitats, and their fruit dispersion times seem to be adjusted to the soil water availability (Kissmann et al., 2012). For *S. pohlii* seeds, different germination performances at distinct seed water contents (Figure 3) reinforce such dependence on soil water availability. Seeds of this species are dispersed with high water content (50±0.5%), and exhibit the highest %G under such conditions (Figure 3a). However, despite the low %G, the seeds are still able to germinate with 12% water content, suggesting the intermediate behavior of *S. pohlii* seeds (Ellis et al., 1990).

S. pohlii fruits are small drupes (5 mm in diameter), and this trait together with high seed water content represent limitations for wind dispersal. Thus, *S. pohlii* seeds are probably dispersed autochorically or zoochorically. Zoochory is crucial for seeds requiring pulp removal to germinate, as we observed for *S. pohlii*. Fruit pulps may limit germination because of germination inhibitors commonly present in the pulp (Yagihashi et al., 2000), or because it may offer a moist and nutritious medium for fungal infection, or even because it may represent a physical barrier for seed germination. We observed that *S. pohlii* pulp provided conditions for fungal infection, leading to 100% seed mortality. In addition, fleshy drupes of *S. pohlii* fruits are described as an important diet for birds in forest remnants (Zaca et al., 2006). As *S. pohlii* seeds exhibit thin seed coat and fast imbibition (Kissmann et al., 2012), as well as high %G after pulp removal, as evidenced by the present study, it seems reasonable to suggest that seeds of this species is zoochorically dispersed.

Because *S. pohlii* seeds are dispersed in the rainy season, when the soil is usually flooded, the capacity of these seeds to germinate in hypoxia conditions (Figure 4) shows an important advantage of this species in flooded environments. These seed germination responses, together with the fast recovery capacity of gas exchange rates observed in *S. pohlii* plants after flooding periods (Kissmann et al., unpublished data), and the high specific leaf area of *S. pohlii* plants in riparian forests, leading to advantageous photosynthetic performances in such shaded environments (Habermann and Bressan, 2011) may explain the successful occurrence of this species in riparian forests (Teixeira and Assis, 2005).

In this descriptive study, we used important data on seed germination performances of three *Styrax* species obtained under laboratory conditions and complemented with germination data of these three species obtained in the field (Kissmann et al., 2012), which constituted useful information to discuss the distribution patterns of these congeneric species in Cerrado areas in Brazil.

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