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Overcoming dormancy in *Rubus sellowii* Cham. & Schlitdl. seeds, an endemic species to Brazil

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ABSTRACT: The seeds are used for conservation, genetic improvement and production of seedlings of native and cultivated species. However, seed dormancy has been a challenge, a process that prevents or delays germination, delaying or hindering their use in these applications. Thus, this study aimed to evaluate, understand and overcome, the seed dormancy in *Rubus sellowii*, a raspberry species native to Brazil. In our observations it was confirmed the existence of a rigid layer in seeds of *R. sellowii* that impedes water absorption. Seed scarification using sulfuric acid for 10 to 20 minutes increased the germination (up to 42.5% and 51.3%, respectively), and germination speed index (1.01 and 1.58), compared to no seed germination without scarification. Therefore, scarification was necessary and sufficient to overcome dormancy and allow the germination of *R. selowii* seeds. As well as in vitro experiments, the germination in substrate was favored by incubation under germination chamber conditions with day/night temperature alternation, improving *R. sellowii* seed germination. The alternation of temperature proved to be an important factor in controlling the germination of *R. sellowii* seeds.

Index terms: chemical scarification, raspberry, seed germination, temperature.

RESUMO: As sementes são utilizadas para a conservação, melhoramento genético e produção de mudas de espécies nativas e cultivadas. Porém, um desafio tem sido a dormência em sementes, processo que impede ou retarda a germinação, atrasando ou dificultando o seu uso nessas aplicações. Assim, este estudo teve como objetivo avaliar, compreender e superar, por meio de estudos de escarificação, a dormência de sementes de *Rubus sellowii*, espécie de framboesa nativa do Brasil. Foi confirmada a existência de uma camada de impedimento da absorção de água nas sementes de *R. sellowii*. A escarificação das sementes com ácido sulfúrico por 10 a 20 minutos aumentou a germinação (até 42,5% e 51,3%, respectivamente) e o índice de velocidade de germinação (1,01 e 1,58), comparado às sementes não escarificadas que não apresentaram germinação. Dessa forma, a escarificação foi necessária para superar a dormência e permitir a germinação das sementes de *R. sellowii*. Assim como nos experimentos *in vitro*, a germinação em substrato foi favorecida pela incubação em condições de câmara de germinação com alternância de temperatura dia/noite, promovendo a germinação das sementes de *R. sellowii*. A alternância de temperatura mostrou-se um fator importante no controle da germinação de sementes de *R. sellowii*.

Termos para indexação: escarificação química, framboesa, germinação de sementes, temperatura.

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INTRODUCTION

The genus *Rubus*, Rosaceae family, includes more than 600 species in 15 subgenera, distributed on all continents (Martin et al., 2013; Ward et al., 2013). They are herbaceous-shrubby plants with sexual reproduction by seeds and vegetative propagation modes with development of rhizomes and sprouts from above-ground stolons (Zasada and Tappeiner III, 2008).

Although the propagation of *Rubus* species (raspberries) used in the fruit industry is carried out vegetatively, seed reproduction is an important tool for crop breeding, which introduce new traits and produce improved cultivars (Foster et al., 2019). Great interest exists that these new cultivars have new agronomic characteristics such as resistance to pests and diseases (Graham et al., 2014; Parikka et al., 2016; Campos et al., 2023), adaptation to new geographical regions and to climate changes (Ballington, 2016), better fruit quality (Jennings, 2018) and thornless and erect-caned branches (Foster et al., 2019).

In addition, seed propagation is also useful for reforestation, as it preserves genotypic diversity within the species and increases the chances of successful restoration and reproduction of native species in the wild (Caballero et al., 2010; Ivetic and Devetakovic, 2017) compared to vegetative propagation.

Rubus sellowii Cham. and Schltdl. (IPNI, 2020) is a native species from South and Southeast regions of Brazil (Barcelos and Heiden 2015; WFO, 2020), with occurrence in regions with Atlantic Forest biome (IBGE, 2019). Commercial varieties of raspberries cultivated in Brazil were all introduced from Northern hemisphere species (Pereira et al., 2014; Campos et al., 2023), so, native Brazilian species as *R. sellowii* is of great interest for genetic improvement to enlarge their adaptability to other environments. *R. sellowii* was also showed high nutritional and functional potential when compared to commercial *Rubus* cultivars (Teixeira et al., 2019).

Despite this potential, few works have been carried out on understanding reproductive biology and efficient propagation methods and none with *R. sellowii*. (Heide and Sonsteby, 2011; Wada and Reed, 2011a; Wada and Reed, 2011b). In *Rubus* genus seeds, physical and physiological dormancy are reported as the main barriers for germination, including commercial raspberries cultivars (Pergolotti et al., 2023; Kim et al., 2024).

The seed coat plays an important role in embryogenesis and germination, controlling embryo development and factors related to dormancy and germination (Zurawicz et al., 2017; Jennings, 2018). Seeds of this genus have dormancy caused by a rigid and impermeable seedcoat (which is, in fact, fruit endocarp) and endogenous dormancy regulated by biochemical processes (Rahmawan et al., 2023). Physical dormancy restricts the absorption of water and mechanically impedes expansion of the embryo. This dormancy is overcome by endocarp removal or scarification, while physiological dormancy is regulated by biochemical or biophysical processes that occur during the after-ripening period (Contreras et al., 2016; Masny et al., 2022).

Rubus seeds are usually germinated in sand or soil under greenhouse conditions (Clark et al., 2007) or under *in vitro* conditions (Pergolotti et al., 2023). However, low and slow seed germination rates were commonly observed, caused by one or more type of dormancy in these species (Pergolotti et al., 2023).

In *Rubus*, there is a large variation in seed-coating thickness and structure (Wada and Reed, 2011a). Chemical scarification, with sulfuric acid, was efficient and increased *R. occidentalis*, *R. ursinus* and *R. georgicus* seed germination compared to non-scarified seeds (Wada and Reed, 2011b). These authors also showed that times of exposure of seeds to sulfuric acid to break seed dormancy vary according to species.

Thus, this study aimed to evaluate factors that aid to promote seed germination, such as chemical scarification, temperature requirements for germination and causes of seed dormancy in *Rubus sellowii*, a native species from Brazil.

MATERIAL AND METHODS

Seeds of *Rubus sellowii* were collected from plants grown in riparian vegetation Brazil, São Paulo: Itaberá, 23°50’27.0”S 49°07’26.6”W and obtained from fruit that were completely mature (purple to black color), in October

2015. Seeds were washed in running tap water to remove the fruit pulp and then dried at room temperature (± 25 °C) for seven days, stored in paper bag under refrigerator temperature at 4 °C for ten to fourteen days until its use in the experiments.

Morphological characterization of R. sellowii *seeds*

For morphological characterization, a digital camera was used (Compact USB2.0 CMOS Camera with 23.2mm DIA for Ocular Tube. ToupTek Photonics, Zhejiang, P.R.China) coupled to a stereoscopic microscope to take seed pictures and determine seed length and width in the polar and mid-length regions. Measurements were made on 30 seeds to calculate the mean. Also, measurement of five repetitions of 100 seeds was performed to determine the fresh mass value of 100 seeds, measured on a precision scale with four-decimal places (Mettler Toledo Inc., Model MS204 // A01 - Switzerland).

Rubus sellowii *unscarified seed germination in substrate*

Two hundred seeds were sown in tray containing commercial substrate (Tropstrato HT, based on pinus bark and vermiculite (Vida Verde, Mogi Mirim, Brazil)). The tray was kept in a germination chamber with 16-hour photoperiod (8 hours in dark) and constant temperature of 25 ± 2 °C for 180 days. Due the low percentage and rate of germination along this time, we adopted after 180 days the temperature alternance, with 16-hours photoperiod, 25±2 °C on light and 8 hours 15 ± 2 °C in dark, similar to described by Wada and Reed (2011a).

Seeds emerged from the substrate were counted at every thirty days for ten months and were considered as germinated those that showed any development above the substrate. The regression curve and the R^2 value were determined using Microsoft Excel software.

Water absorption in scarified R. sellowii *seeds*

Seeds were previously submitted to chemical and thermic scarification to evaluate seed water absorption in scarified and non-scarified seeds. So, *R. sellowii* seeds were immersed in sulfuric acid 98% for five or ten minutes (Wada and Reed 2011b, modified), a five-minute treatment in water at 98 °C (thermal scarification) (Dapont et al., 2014, modified) and a control treatment with distilled water at 25 °C (room temperature) totalizing four treatments. After treatments, acid neutralization was proceeded with the seeds of all treatments by washing in running water for five minutes and then immersed in a solution of 3 $g.L^{-1}$ calcium hypochlorite [$Ca(ClO)_2$] for five minutes, followed by soaking for a further five minutes in a solution of 3 $g.L^{-1}$ potassium hydroxide (KOH) and washed for a further five minutes in running tap water (Wada and Reed 2011b).

To determine water absorption, 80 seeds were immersed in 50 mL distilled water. To evaluate the fresh mass gains, the seeds were removed from the water with the aid of a plastic sieve and dried on absorbent paper, followed by fresh mass measurement on the same precision scale used in item 2.1, performed at intervals of 24 hours, till mass stabilization. The percentage of water uptake by the seeds was calculated based in 80 seeds on the initial fresh mass of the seeds. A completely randomized design with four repetitions of 20 seeds each, totalizing 80 seeds per treatment.

The percentage of water content in the seeds was calculated according to the following formula:

$$percentage\ (\%)\ of\ water\ absorbed = \left(\frac{fw - iw}{iw}\right) \times 100$$

Where:

fw = final weight, in grams (water absorbed after each period evaluated);

iw = initial weight, in grams, of seeds before imbibition.

For this experiment, data in percentage were used for presentation in the scatter plot. The data obtained were analyzed by the percentage of water absorbed by the seeds and the period of immersion in water after the scarification treatments. The regression curve and the R^2 value were determined using Microsoft Excel software.

Scarification effects on petri dishes germination of R. sellowii seeds

The scarification of seeds was realized using three immersion times in sulfuric acid (98% H_2SO_4): ten, twenty and thirty minutes, to verify which one would be the most effective to increase germination rate and to shorter the time required for seed germination. The control used for this experiment was the seeds without chemical scarification. Then, seeds were subjected to the same post acid scarification procedures previously described and placed for germination in polyethylene petri dishes covered with lid, lined with filter paper, moistened with distilled water (5 mL per petri dish) and kept in germination chamber with alternated temperature 16 hours at 25 ± 2 °C in light and 8 hours 15 ± 2 °C in dark (Wada and Reed, 2011a). A completely randomized design was used with four replications with 20 seeds per petri dish, totalizing 80 seeds per treatment.

Germination speed index (GSI) was also calculated (Maguire, 1962), given by the following formula:

$$GSI = \frac{G_1}{N_1} + \frac{G_2}{N_2} + \frac{G_n}{Nn}$$

Where:

G_1; G_2; G_n = number of seedlings in the first, second to n counts;

N_1; N_2; N_n = number of days after sowing after first, second to n counts.

The percentage data of germination obtained in the different treatments was plotted in a scatter plot and analyzed for the correlation between the variable percentage of germination and cultivation time. The regression curve and the R^2 value were determined using Microsoft Excel software.

The final germination data, in percentage, obtained at 63 days after scarification were transformed by the function arcsine √x/100. This data and Germination Speed Index (GSI) were submitted for the normality (Shapiro-Wilk) and homoscedasticity (Levene) tests. The analysis of variance (ANOVA) was performed, and means were compared by Tukey's test at 5% significance. The statistical software ASSISTAT 7.7 (Silva and Azevedo, 2016) was used to perform the analyses.

RESULTS

Rubus sellowii seeds are small, oblong, and have depressions in their extension. The seeds length was = between the poles = 2.04 mm (± 0.08 standard deviation); diameter on the mid-length region = 1.25 mm (± 0.12 standard deviation). Weight of one hundred seeds was 0.143 ± 0,012 g.

The sowing in controlled germination chamber at constant 25 °C temperature showed only 13% of maximum germination after 180 DAS. Due the low percentage of seed germination, and after that, the temperature was altered from constant 25 °C day/night to 25°C (16 hours in light)/15 °C (8 hours in dark), and germination increased from 13 to 41% only after four weeks under these new conditions (Figure 1) and reaching a 49% of maximum germination at 280 DAS (Figure 1).

There was observed increased in water absorption in all treatments, whether in those without scarification or in those subjected to scarification (chemical or thermal). The highest rate of water absorption (14%) occurred in control treatment in the first 24-hours of immersion and has stabilized after the 120-hour of immersion. However, from the 24th to the 120th hour, there was an increase of less than 4%, indicating saturation in seed water absorption (Figure 2). Under scarification treatments (water 98 °C; scarification in acid for 5 and 10 min), the points of maximum absorption occurred only after 100 hours. However, this maximum absorption was fewer in the scarified seeds (around 12% in those scarified and above 30% in non-scarified seeds after 144 hours of immersion) (Figure 2). These results, associated with stereoscopy observations, indicates that the water absorption was associated with the seed coating (fruit endocarp) (Figure 3a-b) and, when it was damaged, either by acid (Figure 3c) or by hot water (98 °C), this retention capacity was drastically reduced (Figure 3) due to the degradation of this cover layer.

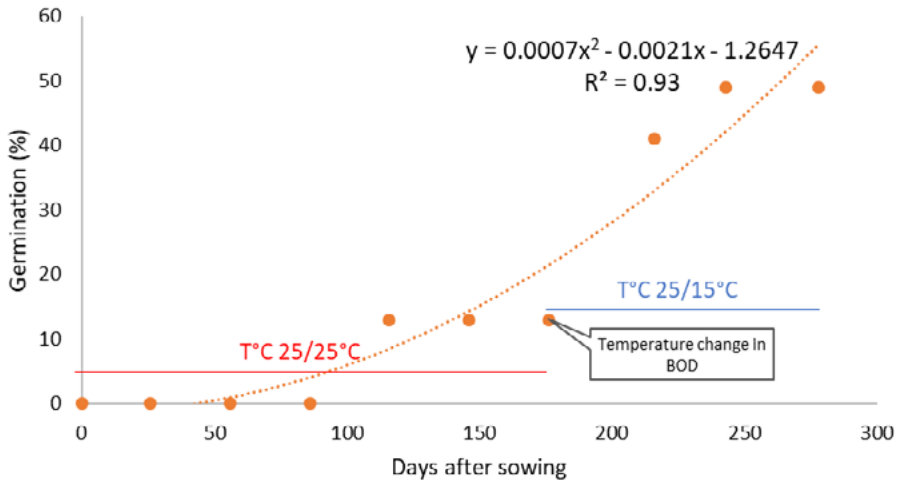

Figure 1. *Rubus sellowii* seed germination in germination conditions over 300 days after sowing.

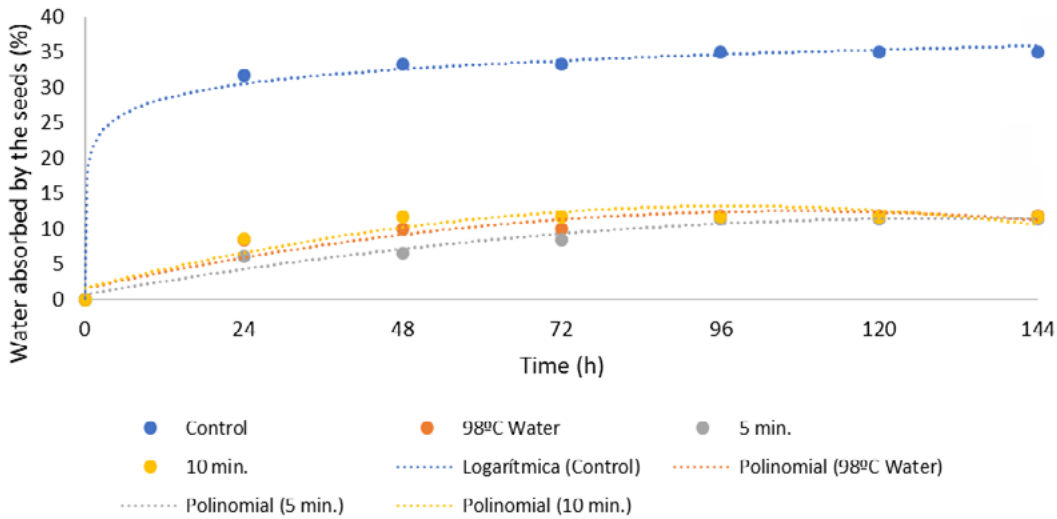

Figure 2. Percentage of water absorbed by *Rubus sellowii* seeds after chemical scarification with H_2SO_4 for 5 or 10 minutes and thermal scarification with water at 98 °C over 144 hours after immersion. Equations: Control: y = 3.0045ln (x) + 20.977. R^2 = 0.9964. 98 °C Water: y = -0.0009x^2 + 0.2036x + 1.6156. R^2 = 0.8915. H_2SO_4 05 min.: y = -0.0006x^2 + 0.1643x + 0.7451. R^2 = 0.949. H_2SO_4 10 min.: y = -0.0012x^2 + 0.2372x + 1.6482 R^2 = 0.8863.

The scarified seeds showed maximum germination after 63 days of incubation. Scarification for 10, 20 and 30 min promoted 42.5%, 51.2 and 4% germination, respectively, with no germination of seeds without scarification (Figure 4) (Table 1). Statistically, the final germination percentages using 10- or 20-min chemical scarification showed no significant difference, however, in relation to the germination speed index (GSI), the 20 min treatment showed the highest GSI value, 1.58 (Table 1), and was superior compared with the treatment at 10 min (GSI = 1.01) (Table 1).

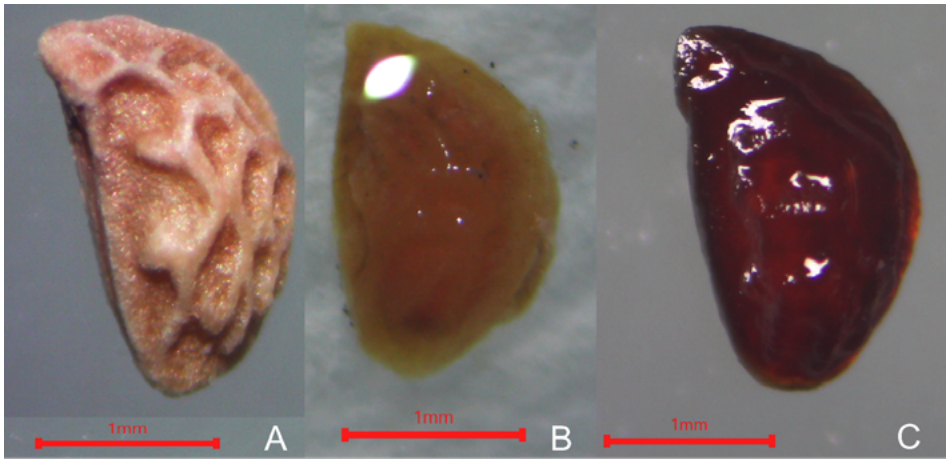

Figure 3. *Rubus sellowii* seeds before (A); twenty-four hours after water immersion (B); and seeds after 10 minutes of H_2SO_4 scarification (C). Bars equivalent to 1mm.

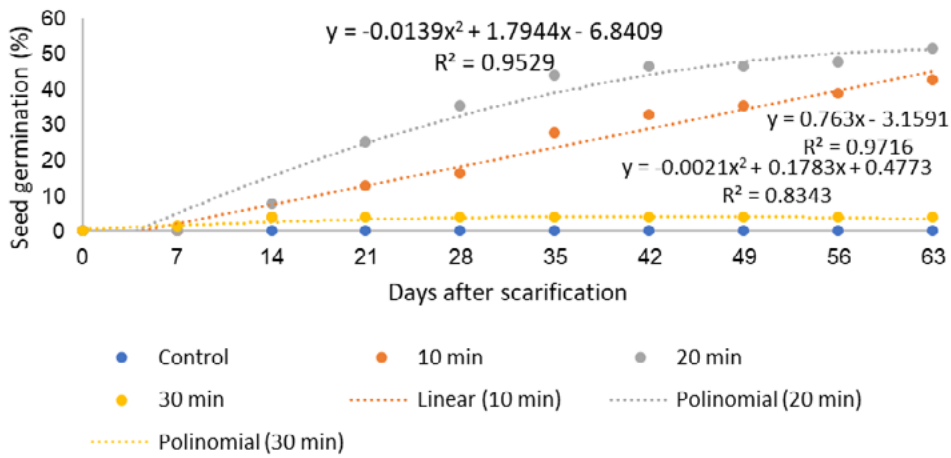

Figure 4. Germination of *Rubus sellowii* seeds over 63 days after H_2SO_4 scarification treatments. Equations: H_2SO_4 10 min.: $y = 0.763x - 3.1591$. $R^2 = 0.9716$. H_2SO_4 20 min.: $y = -0.0139x^2 + 1.7944x - 6.8409$. $R^2 = 0.9529$. H_2SO_4 30 min.: $y = -0.0021x^2 + 0.1783x + 0.4773$. $R^2 = 0.8343$.

Table 1. Germination (%) and Germination Speed Index (GSI) of *Rubus sellowii* seeds 63 days after H_2SO_4 scarification treatments.

Treatment	Germination (%) (63 days)	GSI (63 days)
Control	0 b	0 c*
10 min. H_2SO_4	42.5 a	1.0116 b
20 min. H_2SO_4	51.25 a	1.5825 a
30 min. H_2SO_4	3.75 b	0.2613 c
CV (%)	28	39.51

*Equal letters in the column show no statistically significant difference in the 5% Tukey's test. CV: coefficient of variation.

DISCUSSION

The weight of *R. sellowii* seeds observed in this work (0.143 ± 0,012 g / 100 seeds) is in the range with that reported for other species of the genus. Wada and Reed (2011a) reported 100-seed weight ranging from 0.09 g (*Rubus odoratus*) to 0.86 g (*R. chamaemorus*) in eight *Rubus* species and correlate the seed weight with the thickness and hardness of their endocarps; those with the lowest weight have lowest thickness and hardness endocarp, which this last, probably, is the case of *Rubus selowii* seeds due it low seed mass. This fact was also efforted by actual results, which seeds required short times of scarification that resulted in significant increases of germination of seeds.

Seeds sowing under growth chamber conditions at constant 25 °C along light and dark periods showed low percentage of seedling emergence, since after 180-d cultivation period. After this time, the temperature of growth chamber was changed for 25 °C for 16-h in the light and 15 °C for 8-h in the dark, according the recommendation of Wada and Reed (2011a). After four weeks of cultivation under these new conditions with low temperature under night conditions, the germination percentage increased rapidly and reached a maximum of 50% at 280 days (Figure 1). This study with *Rubus sellowii*, together with others previously conducted with other *Rubus* species (Wada and Reed, 2011a), concluded that besides the presence of a hard and impermeable seed coat (physical dormancy), there is also a seed dormancy associated with seasonal temperature variations (Zasada and Tappeiner III, 2008). These results showed that dormancy of seeds in *Rubus* is more complex than only the presence of a rigid endocarp and suggests some correlation with temperature alternation under day/night conditions. However, additional studies with temperature are required for clarify this response in *Rubus* seeds.

Emerged seedlings under substrate demonstrated slow shoot development after emergence (Figure 5a). Some seedlings were therefore removed from the substrate for root evaluation, and their roots were almost three times the length of the shoots (Figure 5b). This fact can be attributed to the developmental biology of plants of this genus, which have a perennial root system from which shoots grow in annual or bi-annual branches (Snir, 1988; Zasada and Tappeiner III, 2008; Jennings, 2018). These branches (canes) are renewed from the buds from the perennial root system.

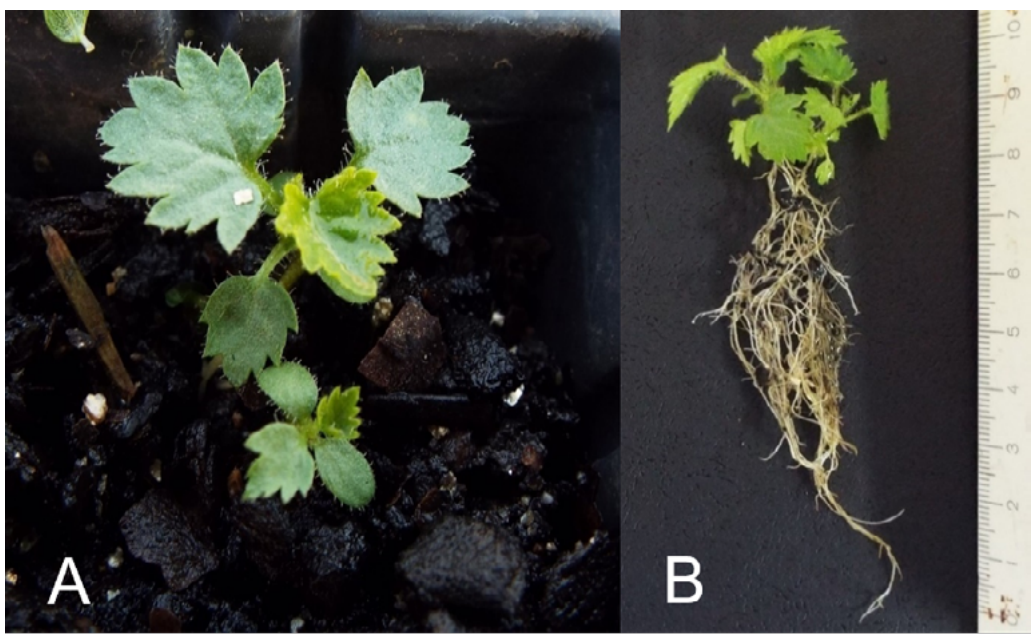

Figure 5. Emerged *Rubus sellowii* seedling, approximately 80 days after sowing, in tray containing substrate based on pine bark and kept in greenhouse conditions (A). *R. sellowii* seedling roots 110 days after sowing (30 days after emergence) (B).

The water absorption curve by *R. sellowii* seeds after 120 h immersion in distilled water did not result in a typical three-phase imbibition curve (Figure 2). In the literature, there is a lack of information about the analysis of water absorption in *Rubus* seeds, but similar difficulties for establishing imbibition curves were reported by Diez et al. (2013) for *Rubus glaucus* showing no water absorption by seeds in imbibition tests. In addition, recent studies showed that the triphasic imbibition curve in seeds, rarest occurs in native and wild species (Pereira et al., 2022), such as *Rubus sellowii* used in actual study.

The rapid and limited increase in seed weight shown at the first 24-h of immersion of untreated seeds (Figure 2) was attributed to the rehydration of the seed coat (fruit endocarp), that was confirmed by visualization using stereoscopy (Figure 3B), and was not due to really water uptake by the seeds, which was have a rigid and impeditive layer, such as demonstrated generally for the genus *Rubus* (Wada and Reed, 2011a and b). This fact can be efforted by the different behavior in the gains of seeds weight related to the different scarification treatments (Figure 2). Seeds submitted to chemical or thermal scarification showed minor weight increases (almost 12%) when compared with seeds without scarification (control), which resulted in a 35% weight increase. The scarification damaged the seed coat (Figure 3C) and reduced the capacity to water absorption by this tissue. The presence of this hard coating has been reported limiting water uptake and gas exchange in *Rubus* species and was reported as one of the cause of seed dormancy different *Rubus* species (Jennings, 1988; Wada and Reed, 2011a).

H_2SO_4 treatment damages the rigid endocarp structure, the main physical barrier to germination in *Rubus*. There was observed that 20 min treatment resulted in the highest germination speed index (GSI). However, the final germination rate, in both 10- and 20-min treatments, was statistically similar, close to 50%, and the same values observed in greenhouse conditions (280 days after sowing without scarification (data not shown)) and in germination under chamber conditions after setting temperature alternance (Figure 1).

The cause of other 50% of seeds that are not germinated remains unsolved but may be related to the moment of collecting and seed homogenization, where a part could still not be fully mature physiologically. It is already known that in wild genotypes, flowering and anthesis occur unevenly, which result in different levels of seed maturity at the collection time (Burghardt et al., 2015).

Wada and Reed (2011b) correlated the need for longer seed immersion-period in H_2SO_4 with endocarp thickness for increase its germination in *Rubus*. In *R. hoffmeisterianus*, a species with thinner endocarp (0.087 mm), 30 min exposure to H_2SO_4 provided a 22% reduction in endocarp thickness and a considerable increase in germination rate from 0% (without scarification) to 99%. *R. occidentalis*, a thick-endocarp species (0.176 mm), required exposure to H_2SO_4 for three hours to increase its germination from 0% (without scarification) to 63%. Another similar works with seed scarification using H_2SO_4 showed increases in seed germination rate of *Rubus* different species. Peacock and Hummer (1996) reported a 26% germination rate of *R. chamaemorus* after 30-min of H_2SO_4 scarification, while seeds of *R. occidentalis* scarified with H_2SO_4 for 0.5, 1 and 2 hours presented 0, 12 and 20% germination, respectively.

To *R. sellowii* seeds, acid exposure times beyond 20 min seems to be excessive, with acid going beyond the endocarp layer and damaging the seed internally (Figure 4). In some cases, long immersion times in H_2SO_4, for some *Rubus* species, cause irreversible embryos injuries. For *R. ursinus* and *R. georgicus*, Wada and Reed (2011b) reported that increases H_2SO_4 immersion from three to three and a half hours, seed viability was 80% lost and, when seeds were scarified for four hours in sulfuric acid, there was 100% seed viability loss.

The results found in the present study, associated with these aforementioned authors, showed a large genotype-dependence of the response to scarification protocol to obtain high germination rates, and were especially correlated with structural composition and thickness of the endocarp of *Rubus* seeds. It is possible that the weight of 100 seeds can be used as a good indicator to define the time required for H_2SO_4 scarification, as the weight is directly related to the thickness of the endocarp (Wada and Reed, 2011a). For *R. occidentalis* seeds, which have heavy and thick coat (0.176 mm) and a seed weight of 0.188 g.100 seeds^{-1}, three hours of acid scarification were required to promote maximum germination of 63% (Wada and Reed, 2011b), while under shorter times, the germination rate was only 20% (Peacock

and Hummer, 1996). The *R. sellowii* seeds evaluated in actual study required only 20 minutes of H_2SO_4 scarification to promote the highest and fastest germination rate observed (50%). Therefore, *R. sellowii* can be classified as having a light seed with thin coat. This observation was supported by the decreasing in germination rate of *R. sellowii* seeds scarified with H_2SO_4 for 30 minutes, when compared to those scarified for 10 and 20 minutes.

CONCLUSIONS

Rubus sellowii seeds has dormancy caused by the rigid endocarp covering the seeds. Chemical scarification using H_2SO_4 for 10 to 20 min resulted in overcome dormancy and germination of 50% of the seeds after 63 days cultivation. Similarly, this study suggests that reduced night temperature (15 °C) compared to temperature under light conditions (25 °C) are required for seed germination, but additional studies are required to confirm this hypothesis. This information is valuable for the conservation and propagation of the species and allows the species to be used in breeding programs with other *Rubus* species and cultivars.

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