Moist Heat Overcomes Physical Dormancy at the Seed Coat Lens in *Schizolobium parahyba* var. *amazonicum*

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**ABSTRACT**

*Schizolobium parahyba* var. *amazonicum* (Huber ex Ducke) Barneby (Fabaceae-Caesalpinoideae) is a tree of the Amazon region with pioneer characteristics and large seeds with physical dormancy. Using Accelerated Ageing (AA) methodology, seeds were exposed to Moist Heat (40 °C; >97% Relative Humidity; RH) or Dry Heat (40 °C; 22% RH). Furthermore, it was also investigated dormancy release and the primary site of water entrance into the seeds. Seeds tolerate these conditions for up to six days without any reduction in vigour, however, only Moist Heat could overcome seed dormancy, allowing germination. The lens is the water-gap for primary water entry (where the palisade layer is thinner), as seeds with blocked lens did not imbibe. An unusual multi-layered hypodermis of osteosclereids in the seed coat was observed. Our results suggest that the combination of high temperature with high RH is a key factor involved in overcoming dormancy in the natural habitat of this Amazonian species.

**Keywords:** accelerated ageing, Amazon, germination, imbibition, pioneer tree.
1. INTRODUCTION

Schizolobium parahyba var. amazonicum (Huber ex Ducke) Barneby (Fabaceae-Caesalpinioideae), (called paricá in Brazil), is a tropical deciduous tree in the Amazon basin, with rapid growth. The largest trees may reach 40 m in height and 100 cm in Diameter at Breast Height (DBH) (Carvalho, 2007). Its natural occurrence is in mature or secondary forests in non-flooded areas. The light wood is primarily used in making plywood and has potential use for paper and cellulose (Souza et al., 2003) indicated for agroforestry systems (Cordeiro et al., 2015). Due to its pioneer character, this tree is recommended for ecological restoration (Carvalho, 2007; Lopes et al., 2015; Schwartz et al. 2017).

Seeds of S. parahyba var. amazonicum are flattened, oval, with a hard, water-impermeable seed coat (Shimizu et al., 2011) and are classified as having physical dormancy according to Baskin & Baskin (2014). Effective treatments to promote high and uniform germination have been performed by immersion in sulphuric acid (Leão & Carvalho, 1995), hot water (Leão & Carvalho, 1995; Shimizu et al., 2011), or mechanical scarification with sandpaper or bench grinder (Shimizu et al., 2011; Fernandes et al., 2019). Two varieties are known for the species (IBR, 2019). Seeds of the Amazonian variety (S. parahyba var. amazonicum) are smaller, with individual seeds weighing between 0.7 g and 0.9 g (Cruz & Pereira, 2014), compared to seeds of the Atlantic Forest variety known as guapuruwu (S. parahyba (Vell.) Blake var. parahyba), weighing about 1.9 g (Freire et al., 2015).

In seeds with physical dormancy, specialized anatomical structures (water-gaps) may allow the entrance of water to start the imbibition process. The hilum and hilar slit in Fabaceae (e.g., Ooi et al., 2014; Dayrell et al., 2015) or alternating temperature (e.g., Souza et al., 2012; Daibes et al., 2017; Jaganathan et al., 2019). Integument disruption by heat at 30 °C and 35 °C was detected in Mimosa calodendron Mart. ex Benth. (Dayrell et al., 2015) and in M. foliolosa Benth. (Silveira & Fernandes, 2006). When S. parahyba var. parahyba seeds were submitted to alternating (20/30°C) or constant (30°C) temperatures, the absorption of water was exclusively through the lens, a subtle depression close to the hilum (Souza et al., 2012).

Seed physical dormancy allows S. parahyba var. amazonicum to form a persistent seed bank. In the soil under rainforest conditions, seeds are subjected constantly to high temperature and moisture. Generally, dormancy release by temperature is tested with seeds in contact with a moist substrate, and any positive result is attributed to temperature. To see if only high temperature (Dry Heat) or the combination of high temperature with high humidity (Moist Heat) can overcome seed physical dormancy in S. parahyba var. amazonicum seeds, an adaptation of the Accelerated Ageing (AA) methodology was used. Additionally, the primary site of water entrance into the seeds was revealed.

2. MATERIAL AND METHODS

Seeds of S. parahyba var. amazonicum were collected during natural dispersal and, after manual removal of the papery endocarp, were kept in polyethylene bags (thickness 80 µm) in a cold chamber at 10 ± 1 °C. Initial seed moisture content was determined gravimetrically after drying at 105 ± 2 °C for 24 h and expressed as percentage of fresh weight, using 20 replicates of individual seeds.

2.1. Pre-treatments

Seeds were submitted to different periods of Moist Heat or Dry Heat, in addition to the control, where seeds were sown without any treatment. Moist Heat: to achieve
an environment with high humidity, the procedure for AA was applied. Replicates with 50 seeds were placed in one layer on a suspended aluminum screen in transparent germination boxes (JProlab®, 11 × 11 × 3.5 cm) containing 40 mL of distilled water, avoiding any direct contact with the water. Each plastic box was wrapped in a transparent polyethylene bag (80 µm thick) and kept at 40 ± 1 °C in a germination chamber (Fanem®, 347-CDG) in darkness, which achieves ≥ 97% Relative Humidity (RH). Dry Heat: seeds were placed under the same conditions as mentioned above, however, without addition of water into the germination boxes (resulting in 22 ± 3% RH). Seeds were exposed to these conditions for 24, 48, 72, 96, 120 or 144 h.

2.2. Germination tests

To prevent fungal contamination, all seeds were submersed in 1% sodium hypochlorite for 5 min and rinsed three times with running tap water before germination tests. Seeds were sown above vermiculite (medium granules, Terramater®), moistened with distilled water (1 g substrate: 3 g water) in transparent polyethylene trays (35 × 18 × 12 cm, Galvanotek®) containing 500 g substrate. Trays were wrapped in clear polyethylene bags (80 µm thick) to avoid desiccation. The germination test was carried out in a germination room, with a constant temperature (25 ± 1 °C) and a 12 h photoperiod, using white fluorescent light (Photosynthetically Active Radiation (PAR): 34 μmol m⁻² s⁻¹). Four replicates of 50 seeds were used for each treatment.

Radicle protrusion (≥2 mm with positive geotropic curvature) was assessed daily. Germination progress was scored over 60 days and substrate was remoistened when necessary. After 60 days, the final number of germinated seeds (radicle protrusion), hard seeds (without signs of imbibition) and dead seeds (imbibed and deteriorated) was determined and expressed as a percentage of initial total of seeds. Germination Speed Index (GSI) was calculated following Maguire (1962). After radicle protrusion, seedling development was scored as normal seedlings (elongated hypocotyls and two green cotyledons in expansion), and abnormal seedlings (little or no probability to establish in the field).

Hard seeds that had not imbibed during the first 60 days were submitted to conventional dormancy release with scarification using sandpaper (no. 120, 3M®) at the opposite side of radicle protrusion until a small part of the endosperm or cotyledons was visible. Seed germinability was tested as described above and radicle protrusion assessed daily during 60 additional days.

2.3. Primary water entrance

This was tested after 72 h under Moist Heat and Dry Heat conditions. Individual seed mass was recorded during the imbibition process after subjecting seeds to the following three treatments with four replicates of 20 seeds each: (1) hilar region including lens and micropyle area covered with instantaneous adhesive (A2 - Acrilex®) to block imbibition; (2) no blockage; (3) scarification: clipping at the opposite side of radicle protrusion with pliers to guarantee that seed coat impermeability was overcome. For imbibition, seeds were placed in transparent germination boxes (11 × 11 × 3.5 cm, JProlab®) between two sheets of moistened germination paper (250 mg, 10.5 × 10.5 cm, JProLab®) (1 g substrate: 3 g distilled water). The boxes were maintained in the germination room (see above). Imbibition was monitored every 24 h by seed weight (0.0001 g) for 14 days or until protrusion of radicle. A seed was recorded as imbibed when the amount of water uptake exceeded 50% of its initial mass. Seeds that did not imbibe during the test (hard seeds) were classified as ‘impermeable’.

Immediately after exposure to Moist Heat and during the imbibition test, the seed coat, micropyle, hilum and lens were evaluated with a stereo microscope (Leica S8APO, Leica Microsystems®, Germany) and images were taken with a digital camera (DFC295, Leica Microsystems®, Germany). Sections of dry seed coats were made with a cryostat (CM1950, Leica Microsystems®, Germany) and during imbibition, manually with a razor blade. Images of seed coat features were taken with the same digital camera connected to a light microscope (DM750, Leica Microsystems®, Germany).

2.4. Data analysis

Two-way Analysis of Variance (ANOVA) (p < 0.05) was used to test the effects of Dry and Moist Heat treatments on germination release during time (0, 24, 48 and 72 h). As ANOVA indicated significant difference, the influence of Moist Heat exposure during time was evaluated by regression. Percentages of seed imbibition after 72 h following blockage,
non-blockage of hilar region and scarification were analysed by ANOVA, and subsequently a Tukey’s test at 0.05 significance level for comparison among means (SISVAR®) was used. A paired t-test between the results of radicle protrusion and normal seedling development was performed to certify that the apical meristem was not affected by the stress conditions.

3. RESULTS AND DISCUSSION

Seed moisture content was 4.8%, indicating that the seed lot was stored under adequate conditions to maintain seed quality. This was also shown by 89% radicle protrusion obtained for the control after scarification.

3.1. Effects of dry heat and moist heat on dormancy release

Interaction between treatments (Motist and Dry Heat) with the different time periods of exposure (0, 24, 48 and 72 h) was significant ($p < 0.0001$). Time of exposure had no significant effects within Dry Heat on seed dormancy release ($p = 0.9881$), as seeds showed germination below 1%. Consequently, the percentage of hard (not imbibed) seeds after Dry Heat was high ($\geq 98\%$). After the first germination test, all the remaining hard seeds were scarified and showed high germination performance in a second germination test (between 87.5 and 95.5%; $p = 0.385$).

Moist Heat showed significant differences over the time of exposure ($p < 0.0001$). In this way, a regression analysis was done. After only 24 h of Moist Heat, seed coat dormancy was released in 30% of the seeds, visible by imbibition and subsequent germination. By prolonging exposure time, dormancy release increased in an exponential model ($R^2 = 99.69\%; p < 0.0001$) and radicle protrusion reached more than 60% after 120 and 144 h (Figure 1). Consequently, seeds with no imbibition and apparently dormant ranged between 27.5 and 66.0%. After scarification and a further 60 days of germination, final percentages of radicle protrusion ranged between 80.5 and 94.5%.

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Moist Heat (or Accelerated Ageing) is normally applied as a vigour test to distinguish between seed lots of different qualities. Protocols for several agricultural or horticultural species are available (see references in Marcos, 2005). According to the species, a temperature between 37 °C and 45 °C is applied during 48 h and up to 144 h. These stressful conditions may accelerate some chemical reactions, including seed respiration and/or may initiate deterioration processes. After AA, seed vigour is assessed by comparing germination percentage, germination speed or abnormal seedling development. Seed lots with high vigour will have better performance than those with low vigour. Our data show that seed vigour was not reduced in periods of up to six days of stress, thus vigour test by AA in *S. parahyba* var. *amazonicu* seeds should be done with temperatures higher than 40 °C.

Stress caused by Moist Heat did not reduce further plant development in *S. parahyba* var. *amazonicu*, as there was no difference between radicle protrusion and normal seedlings in all tested periods (Figure 1; minimum $p$ value in the paired t-tests was 0.463). Abnormal seedlings, in very low percentages, were only detected after exposure of 120 and 144 h (3% and 4%, respectively). After Moist Heat, seed mortality ranged between 1.5–10.5% (Figure 1). Seed performance advanced with Moist Heat as shown by the GSI, which increased exponentially with exposure time ($R^2 = 96.27\%; p = 0.005$; Figure 2).

Two earlier studies applying Moist Heat for physical dormancy release had no success (0% germination) in *Operculina macrocarpa* (L.) Urb., where this treatment was applied for 72 h (Medeiros et al., 2002: species...
authors according to JBRJ, 2019), and in *Parkia discolor* Spruce ex Benth., where several periods of incubation of up to 48 h of Moist and Dry Heat (from 40 to 70 °C) were compared (Pereira & Ferreira, 2010). However, some examples of physical dormancy release after similar treatments with high RH as in this study were cited in Baskin & Baskin (2014): *Albizia julibrissin* Durazz. (40 °C/4 h), *Ceratonia siliqua* L. (40 °C/48 h), *Hymenaea courbaril* L. (40 °C/48 h), *Parkinsonia aculeata* L. (35 °C/14 d). In all examples, exposure time at 40 °C was limited to 48 h maximum. A longer period, similar to this study, was applied only with a lower temperature (35 °C) in *P. aculeata*.

It has been shown that physical dormancy may be overcome in nature by high temperatures, e.g., in dry summers, after fire, and with alternating temperatures; however, most of the studies on dormancy release after dry heat used temperatures higher than in this study (ranging from 50-150 °C, see the review of Baskin & Baskin, 2014). Thus, it may be that higher dry temperatures could release seed dormancy of *S. parahyba* var. *amazonicum*.

Using alternating temperature to overcome dormancy depends on the amplitude of the temperature fluctuation and may be in tune with a specific environment, for example, forest gap size for *Heliocarpus donnellsmithii* Rose in Mexico (Vázquez-Yanes & Orozco-Segovia, 1982). Temperature is probably the factor involved in releasing physical dormancy in *S. parahyba* var. *parahyba*, a forest gap species from the Brazilian Atlantic Forest (Souza et al., 2012). According to these authors, alternating temperature 20-30 °C (12/12 hours) overcame dormancy faster than constant high temperature at 30 °C; however, final germination (about 80%) was the same under both treatments; at constant 20 °C, only 2% germination was achieved.

Release of physical dormancy may have different mechanisms according to the conditions of their natural occurrence (Abudureheman et al., 2014). For *S. parahyba* var. *amazonicum*, the key factor to overcoming seed coat impermeability was the combination of constant high temperature with high humidity. Temperature alternation was not evaluated in the present study with *S. parahyba* var. *amazonicum* as this tropical pioneer tree occurs only in regions with high precipitation, where seasonal variations in temperature and humidity are less pronounced. In addition, this species is not recorded in tropical savannah areas (for example, in the Cerrado) (JBRJ, 2019).

### 3.2. Primary water entrance

After Moist Heat with no blockage at the hilar region, 39.1% of seeds were imbibed and had radicle protrusion. Seeds blocked in this region did not imbibe and, consequently, did not germinate. All mechanically scarified seeds imbibed (100%), and 96.1% had radicle protrusion. Significant differences were obtained between the three treatments ($p \leq 0.001$).

In *S. parahyba* var. *amazonicum*, the hilum is positioned between the micropyle and the lens (Figure 3a). After Moist Heat, the seed coat showed cracks and was detached at the lens (Figure 3b). Covering the hilar region including the lens resulted in no imbibition. Similar results were obtained by Souza et al. (2012) with *S. parahyba* from the Atlantic Forest, recently renamed *S. parahyba* var. *parahyba* (JBRJ, 2019). Souza et al. (2012) showed that the primary site of water entrance was at the lens, as seeds could only imbibe if the lens was maintained unblocked, even with blocking at the hilum or extra-hilar region. Thus, the present study confirms that both varieties of *Schizolobium* have the lens as the natural water-gap as in other species of Fabaceae, for example, *Delonix regia* (Bojer ex Hook.) Raf. (Jaganathan et al., 2017). However, a different natural water-gap (and also more than one) was documented in the same family,

![Figure 2. Germination Speed Index (GSI; Maguire, 1962) of *Schizolobium parahyba* var. *amazonicum* seeds after different periods of exposure to Moist Heat (40 °C; RH ≥ 97%) with standard deviation. Data represent means of four replicates of 50 seeds.](image)
for example, *Peltophorum dubium* (Spreng.) Taub., showed the lens, micropyle and hilum as primary water entrance; while in *Mimosa bimucronata* (DC) O. Kuntze seeds, the water-gaps were the micropyle and the lens (Geisler et al., 2017).

During imbibition the outer layers of the seed coat increase in volume, turn transparent with a gelatinous consistency, pull apart and are completely released when seeds are fully imbibed (Figure 3c), revealing underneath a brown expandable, coriaceous tissue.

![Figure 3](image-url)

**Figure 3.** Seeds of *Schizolobium parahyba* var. *amazonicum*: (a) front view of the hilar region; (b) front view of the hilar region after Moist Heat for 72 h showing seed coat detached at the lens and with cracks; (c) outer layer of seed coat (palisade cells), with gelatinous consistency; releasing during imbibition; (d) lateral view of the seed revealing the hypodermis with detail of the expandable, coriaceous surface, after palisade layer was partially released; (e) longitudinal section of the dry seed coat showing a thin cuticule, the palisade layer, multi-layered hypodermis of osteosclereids, followed by the mesotesta with no intercellular space and the endosperm; arrow points to the light line; (f) longitudinal section showing variations in thickness of tissue layers of the seed coat and the endosperm; (g) transversal section at the embryo axis detailing the different seed coat layers. Abbreviations used in microphotographs: ct1, first cotyledon; ct2, second cotyledon; ea, embryonic axis; end, endosperm; hi, hilum; le, lens; mi, micropyle; ms, macroscleireids; mt, mesotesta; ost, osteosclereids; rm, root meristem; sm, shoot meristem.
which accompanies the volume increase during imbibition (Figures 3c and 3d), until perforated by the radicle. Peeling off the outer layers may increase the water absorption area. When observed with a stereo microscope, the underlying brown tissue seems to be porous (Figure 3d).

Longitudinal sectioning of a dry seed revealed the outer layer of the seed coat to be single-layered exotesta of macrosclereids (palisade layer) with tightly packed elongated cells, with a light line in the middle third, followed by a multi-layered hypodermis of osteosclereids (also called Hourglass Cells (HGCs); Figure 3e). The mesotesta has no intercellular space, is located below the hypodermis (the layer with dark brown coloration) and is also expandable, with clear distinctions between the other seed coat layers and different coloration towards the endosperm (Figures 3e, 3f and 3g). The thickness of the seed coat layers varies: the multi-layered hypodermis of osteosclereids is thicker near the embryo axis; the palisade layer is thinner close to the lens; and the mesotesta was thicker at the lateral sides than on the flat sides (Figure 3f and 3g). This “fragile” areas near the lens, with decreased macrosclereid size has also been reported for *Senna multijuga* (Rich.) H.S. Irwin & Barn. (Rodrigues-Junior, et al. 2014).

Using the anatomical study of Souza et al. (2012), with var. *parahyba*, the two varieties can be compared. In seeds of both varieties, a thin cuticle covers the testa, and there is one layer of thick-walled columnar cells, tightly packed and columnar palisade cells. This layer is thinner near the lens as the cells are shorter, indicating the weakest point in the seed coat. There is a light line in the middle third of the palisade layer in both varieties. Souza et al. (2012) did not observe osteosclereids in the seed coat of *S. parahyba* var. *parahyba*, while in this study, a mesotesta with thick cell walls and no intercellular space below a multi-layered hypodermis of osteosclereids could be observed. This is unusual, as several studies describe the hypodermis in the seed coat of Fabaceae as a single layer of osteosclereids (for example, Costa et al., 2011; Rodrigues-Junior, et al., 2014; Robles-Díaz et al., 2016; Geisler et al., 2017; Jaganathan et al., 2017; Teixeira et al., 2018). In this way, the multi-layered hypodermis of osteosclereids seems to be a useful characteristic to distinguish the *S. parahyba* var. *amazonicum* from the Atlantic Forest variety.

In histochemical observations of var. *parahyba*, the macrosclereids (palisade layer) stained negative for lignin and suberin, positive for cellulose, and above the light line was positive for callose reaction, and the sclerenchymatous tissue described below the palisade layer was negative for lignin (Souza et al., 2012). In the Amazonian variety, we noted a high capacity to expand after release of the palisade layer, normally not known for sclerenchymatous tissue; therefore, we speculate that the tissue is collenchymatous and possibly contains pectin and hemicellulose.

4. CONCLUSIONS

High temperature in combination with high relative humidity (Moist Heat) released seed dormancy in *S. parahyba* var. *amazonicum*. In its natural habitat, this combination is suggested to be a key factor involved in dormancy release.

In the tested seeds, the water-gap for primary water entry is the lens, a subtle depression near the hilum and opposite the micropyle. During imbibition, the palisade layer increases in volume, turns transparent with a gelatinous consistency, pulls apart and is released completely.

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