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Different Methods for Overcoming Integumental Dormancy during *in vitro* Germination of Red Araza Seeds

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Abstract

Red Araza, or Red Strawberry Guava (*Psidium cattleianum* Sabine) is a native Brazilian Atlantic Forest species of the Myrtaceae family, whose seeds exhibit integumental dormancy. Due to its importance to different industries worldwide, recent research efforts are seeking to expand this species' micropropagation processes using *in vitro* seedling germination, especially since *in vitro* micropropagation of adult plant material has, so far, been limited. This research effort evaluated different methods of overcoming integumental dormancy during *in vitro* germination of the Red Araza, so as to allow future micropropagation of the species. The seeds' emergence and vigor were evaluated based on mechanical and acid scarification, using different substrates and immersions in solutions with different levels of gibberellic acid (GA_3), and on the influence of the pre-immersion of seeds in water and sulfuric acid. The mechanical and acid scarification of the seeds, combined or separate, resulted in higher *in vitro* germination percentages and a higher germination rate index (GRI). Pre-immersion in distilled water (20 hours) also proved to be efficient for the germination of the Red Araza seed, with 76.2% of the seeds germinating and a higher speed of emergence (GRI = 0.18). When compared to a Murashige and Skoog (MS-zero) medium, sowing in a hydrophilic cotton substrate showed greater emergence and vigor, with approximately 70% of the seeds germinating. Treating the seeds by pre-immersing them in GA_3 turned out to be unnecessary. The methods used for overcoming integumental dormancy during *in vitro* germination of Red Araza seeds proved to be efficient, and could be used to develop micropropagation protocols of seminal origin for this species.

Keywords: acid scarification, Araçá, gibberelic acid, mechanical scarification, myrtaceans, red strawberry guava, tegumentar dormancy

1. Introduction

A species of the Myrtaceae family, Red Araza, or, hereinafter, Red Strawberry Guava (*Psidium cattleianum* Sabine) is native to the Brazilian Atlantic Forest, but also found in different tropical and subtropical ecosystems (Tng et al., 2015). It's an arboreal species that produces a fleshy fruit with peculiar taste, possessing a rich chemical composition (Galho, Lopes, Bacarin, & Limac, 2007), a large variety of bioactive compounds, such as phenolics and carotenoids (Silva, Rodrigues, Mercadante, & De Rosso, 2014), as well as essential oils and other volatile compounds of pharmacological interest (Marin et al., 2008). Previous studies have already determined that aqueous and ketone extracts from the Red Strawberry Guava generate various antioxidant activities, have an anti-proliferative effect on human cancer cells, and acts as an antimicrobial agent against *Salmonella enteritidis* (Medina et al., 2011).

Due to its heightened importance to the agro-industrial and pharmacological fields, many studies have been done with the intent to broaden the knowledge base concerning this species' variability and genetic

selection, its use in biotechnological processes, details regarding its ecological management and the establishment of commercial orchards/plant husbandries (Kinupp, 2011). Studies that focus on the *in vitro* propagation of Red Strawberry Guava can promote important mechanisms for the sustainable exploitation of this species; mechanisms that, up until now, have been rather scarce (Pasqual, Chagas, Soares, & Rodrigues, 2012).

To date, tests for the *in vitro* introduction and multiplication of these Myrtaceae using herbaceous branches have proved ineffective, mainly due to the high percentage of *in vitro* phenol oxidation and microbial contamination (Rodríguez, 2013; Freire, Oliveira, & Vieira, 2014). Consequently, protocols developed from *in vitro* germinated seedlings are being actively investigated as a feasible alternative to species micropropagation, especially considering that they have contributed to producing healthy explants and allowed for the continuity of *in vitro* propagation processes.

Red Strawberry Guava seeds, however, are known to show integumental dormancy and other studies about its *in vitro* germination can be developed under different conditions so as to optimize and homogenize seedling emergence (Da Silva, Perez, & De Paula, 2011). With that in mind, this study set out to optimize the *in vitro* germination of red Strawberry Guava (*Psidium cattleianum* Sabine, Myrtaceae), using different methods to overcome its inherent integumental dormancy, in order to obtain healthy seedlings that can later contribute to the development of micropropagation processes.

2. Materials and Methods

2.1 Obtaining the Seeds

The seeds were obtained in 2014 from ripe fruits collected from 9-year-old Red Strawberry Guava trees (26º49′06″S-50º59′29″W and 26º46′15″S-51º02′09″W) located in Caçador, a city in the southern state of Santa Catarina, in Brazil. The seeds were completely extracted from the pulp, in running water, placed on paper towels, at room temperature and without direct sunlight, and kept under these conditions for five days, until it was time to start the experiments.

2.2 Experimental Conditions

The experiments were performed according to ISTA rules (ISTA, 1999), in the state of Santa Catarina, Brazil, at EPAGRI's (Portuguese acronym for State of Santa Catarina Agricultural, Livestock, and Rural Extension Research Company) Plant Tissue Culture Laboratory. The experiments were set up in a growth chamber where the flasks were then exposed to a 16-hour photoperiod sourced by cold, white fluorescent lamps, with intensity set at 75 μmol m^{-2} s^{-1}, and a temperature of 25±2 °C. The following two types of substrate mediums were used: a cotton medium (2.05 grams $flask^{-1}$ of hydrophilic cotton moistened with 20 mL of distilled water and autoclaved for 25 minutes, at 121 °C and 1.2 atm) and a complete MS-zero medium (25 mL $flask^{-1}$) (Murashige & Skoog, 1962). The substrates were placed in closed flasks with the following dimensions and capacity: height = 95 mm, diameter = 65 mm, and a capacity of 230 mL. Operating within a laminar flow hood, a standard asepsis method was used on the seeds by immersing them for one minute in 70% v/v ethanol, followed by a 15 minutes immersion in a NaClO solution with 1.5% an active principle and containing Tween 20® detergent (10 drops L^{-1}), and washing them three times with sterile distilled water. The seeds that exhibited root protrusions equal to or greater than 2.0 mm were considered to have been germinated (Borghetti & Ferreira, 2004). Two were the factors assessed during the experiments: the germination rate index (GRI) and the germination percentage. According to Maguire (1962), the GRI was calculated using Equation (1) based on every other day assessments. The germination percentage was calculated after periods of time stipulated for each experiment.

$$GRI = (G1/N1) + (G2/N2) + (G3/N3) + (Gn/Nn) \quad (1)$$

Where,

G1, G2, G3, ... Gn = number of seeds germinated in the first, second, third and thru to the last count;

N1, N2, N3, ... Nn = number of days from the time of sowing to the first, second, third and thru to the last count.

Every time the terms standard asepsis method, cotton substrate and MS-zero substrate are mentioned in this paper, they shall be consistent with the descriptions contained in this section.

2.3 Determining Moisture Level

The seeds' moisture level was determined according to Brasil (2009); in other words, in three groups of 50 seeds, each using the oven-dried method, at 105±3 °C, for a period of 24 hours.

2.4 Mechanical and Acid Scarification of the Integument

Eight treatments were arranged in a 2 × 4 factorial design, containing two levels of mechanical scarification (sanded and unsanded seeds), four levels of acid scarification (immersion in a 9 mol L^{-1} H_2SO_4 solution for zero, five, ten, and 20 minutes) and five replications, each containing 10 seeds. For the mechanical scarification, an autoclaved metal sandpaper was used, scouring both sides of the seeds' integument adjacent to the micropyle. Subsequently, the acid scarification was performed by immersing the different seed samples in an acid solution under constant agitation. The seeds were then drained of the acid and subjected to a standard asepsis method, with cotton as the substrate. The GRI and the germination percentage were assessed, with the latter being assessed after 62 days *in vitro*.

2.5 Substrate and Gibberellic Acid (GA_3) Concentrations Tests

Using a laminar flow hood, the seeds were immersed in distilled water for 20 hours, and in a 9 mol L^{-1} H_2SO_4 solution for ten minutes, washed in sterile distilled water, and then subjected to a standard asepsis method. The treatments were arranged in a 2 × 4 factorial design, with two substrates (cotton and MS-zero medium), and four concentrations of GA_3 (Sigma ®) (0 mg L^{-1}, 250 mg L^{-1}, 500 mg L^{-1}, and 1000 mg L^{-1}). Six replications, each containing five seeds, were performed per treatment so the GRI and germination percentage could be assessed (after 80 days).

2.6 Influence of Water Immersion and Acid Scarification

Four different treatments were tested (T1, T2, T3, and T4), with seven replications, each containing six seeds, as described in Table 1.

Cotton was used as the substrate, and the GRI and germination percentage were assessed (70 days after sowing).

Table 1. Different treatments for the *in vitro* germination of Red Strawberry Guava seeds

Treatments	Description
T1	Control – only "Standard Asepsis Method"[a]
T2	"Water Immersion"[b] associated with "Standard Asepsis Method"
T3	"Water Immersion" + "Acid Scarification"[c] and "Standard Asepsis Method"
T4	"Water Immersion"[b] associated with "Reduced Asepsis"[d]

Note. [a] – One minute in 70% v/v ethanol, followed by 15 minutes in a 1.5% NaClO solution, with an active principal containing 10 drops L^{-1} of Tween 20® detergent. [b] – Immersion for 20 hours in distilled water. [c] – Immersion for 10 minutes in a 9 mol L^{-1} H_2SO_4 solution. [d] – Same as the standard aseptic method, but with immersion time reduced to 3 minutes in NaClO.

2.7 Statistics and Data Analysis

All tests were performed in a randomized design. The results were submitted to the Shapiro-Wilk normality test ($p < 0.05$) and for analysis of variance (ANOVA), and, using the Scott-Knott test ($p < 0.05$), their group means were later separated into qualitative variables and regression studies for quantitative variables. When outside the expected normality, the data was transformed into $\sqrt{(x+0.1)}$.

3. Results

Under the experimental conditions described, the average moisture percentage for the Red Strawberry Guava seeds was stipulated at 9.29%±0.036%.

3.1 Mechanical and Acid Scarification of the Integument

By itself, mechanical scarification, achieved by sanding or not sanding the seed integument, did not significantly change the seeds' germination percentages ($p = 0.8783$), resulting in average values of 43.0% and 43.5%, respectively (see Figure 1). However, acid scarification ($p < 0.0001$) by itself and the interaction of both acid and mechanical scarification ($p = 0.0013$) did significantly change *in vitro* germination percentages (see Figure 1). As immersion times in a 9 mol L^{-1} H_2SO_4 solution increased, mechanically scarified seeds yielded positive quadratic germination increments, while the non-scarified seeds showed positive linear germination increments. Using regression analysis (see Figure 1), optimum immersion times

for acid scarification were determined to be 20 minutes for unsanded seeds and 15 minutes for sanded seeds.

As was the case with germination percentages, only acid scarification ($p < 0.0001$) by itself and the interaction of both acid and mechanical scarification ($p = 0.0013$) yielded significant changes in germination rate indexes (GRIs) (see Figure 1). For seeds that were not mechanically scarified, results showed that germination vigor was highest when the acid scarification immersion time in a 9 mol L^{-1} H_2SO_4 solution was 20 minutes. GRIs for mechanically scarified seeds, on the other hand, did not differ ($p < 0.05$) for immersion times of five, ten and 20 minutes, which indicates that, under these conditions, a five-minute immersion is enough to obtain the highest GRI value.

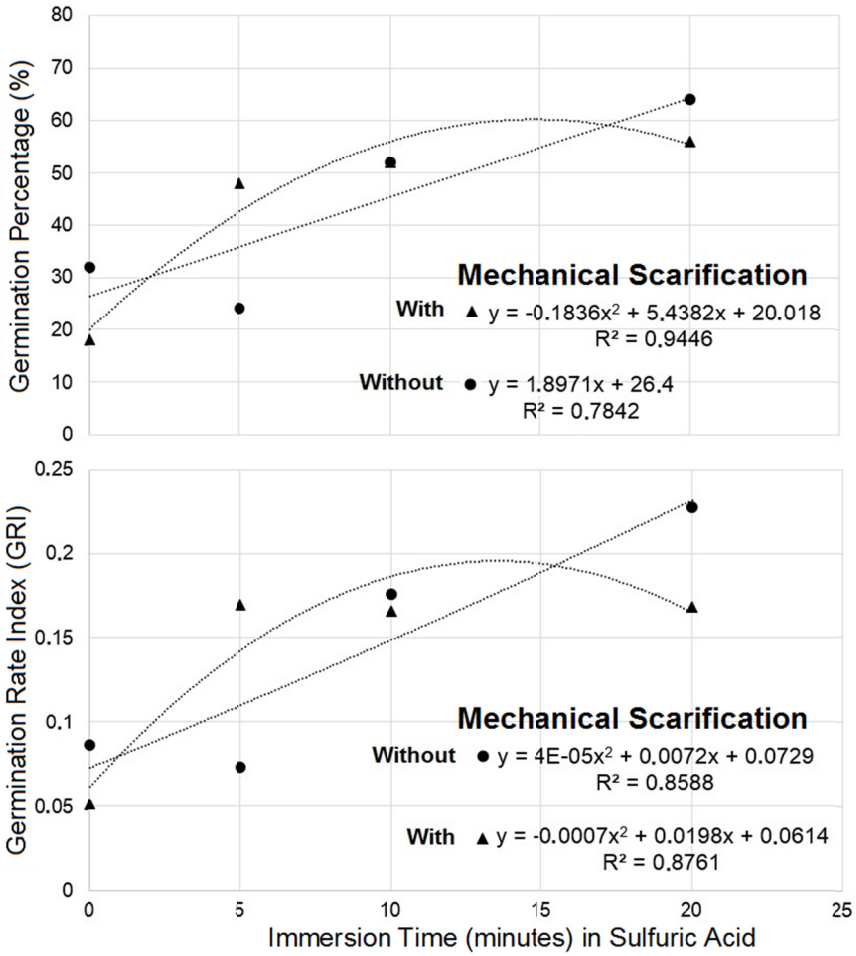

Figure 1. Germination percentages and germination rate index (GRI) for Red Strawberry Guava seeds (*Psidium cattleianum*) submitted or not to mechanical scarification (using sandpaper), and acid scarification with different immersion times in a 9 mol L^{-1} H_2SO_4 solution, 62 days after *in vitro* sowing ($p < 0.05$)

3.2 Substrate and Gibberellic Acid (GA_3) Concentrations Tests

Based on the data shown in Figure 2, one can see that the *in vitro* germination percentages of seeds placed on hydrophilic cotton were significantly higher ($p < 0.05$) than for those placed in an MS-zero medium, with averages of 70.14% and 15.28%, respectively. In spite of this, one can also see that both substrates exhibited very similar behavior. As GA_3 concentrations increased, polynomial regressions indicated a reduction in germination percentage when the concentration increased above 100 mg L^{-1}, increasing again as it went above 700 mg L^{-1} (see Figure 2). Also note that, of the four concentrations used in the experiment, the 0 mg L^{-1}, 250 mg L^{-1}, and 1000 mg L^{-1} did not differ between them and provided higher germination percentages than the 500 mg L^{-1} concentration ($p < 0.05$), regardless of the substrate used (see Figure 2).

Pertaining to GRIs, after 94 days *in vitro*, significant effects ($p < 0.0001$) were detected only with regards to the type of substrate used, showing no GRI variations for the different concentrations of GA_3 used ($p = 0.0690$) nor for the interaction between the two factors ($p = 0.9045$). Just like for the germination percentages (see Figure 2), the GRI obtained was higher for the cotton medium (on average, 7.32 times higher) ($p < 0.0001$) than for the MS-zero medium.

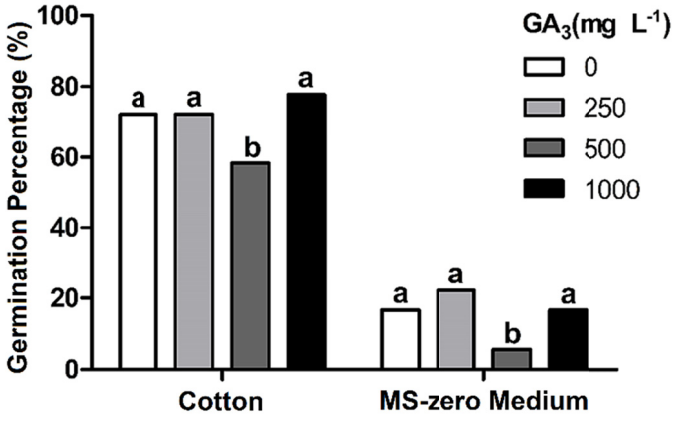

Figure 2. Germination percentages for Red Strawberry Guava seeds (*Psidium cattleianum*) submitted to different concentrations of GA_3 on a moistened hydrophilic cotton substrate and a solidified MS-zero medium (6 g L^{-1} agar), 94 days after *in vitro* sowing

Note. Original values shown; for statistical analysis, values were transformed into $\sqrt{(x+0.1)}$, ($p < 0.05$).

3.3 Influence of Water Immersion and Acid Scarification

The experiment showed that germination percentages did not vary significantly ($p = 0.0911$) as a result of the different treatments. However, when compared to the control group (T1), there was a definite increase in the number of seeds germinated when they were immersed in distilled water (T2), with 76.19% of seeds being germinated (Table 2).

Table 2. Germination rate index (GRI) and germination percentages for Red Strawberry Guava seeds (*Psidium cattleianum*) submitted to different treatments, 70 days after *in vitro* sowing

Treatments	GRI	Germination (%)
T1	0.10^{b}	52.38^{ns}
T2	0.18^{a}	76.19
T3	0.08^{b}	52.38
T4	0.14^{a}	69.05
Variation coefficient (%)	37.88	32.79

Note. Within a column, the means with the same superscript letter do not differ statistically from the Scott-Knott test at 5%. ^{ns} = not significant.

In terms of GRIs, the treatments yielded significant effects ($p = 0.0062$), with treatments T2 and T4 showing greater seed vigor (Table 2). For Treatment 2, the first germinated seed was observed on the 12^{th} day; for Treatment 4, it happened on the 16^{th} day; and, for Treatment 3, it happened only on the 20^{th} day. These results indicate that a 20-hour immersion in distilled water (done for treatments T2, T3 and T4) reduces the time needed for the onset of germination, except when associated with acid scarification of ten minutes in a 9 mol L^{-1} H_2SO_4 solution (treatment T3). In Table 2, one can see that treatment T2 yielded a GRI almost twice that of the control group (T1). The table also shows that, when acid scarification was used (T3), the GRI decreases significantly (approximately 55%) when compared to treatment T2.

4. Discussion

The acid scarification of Red Strawberry Guava seeds with H_2SO_4 proved to be more effective in increasing *in vitro* germination percentages and GRIs than mechanical scarification (Figure 1). Da Silva (2009) had already tested different H_2SO_4 immersion times in *ex vitro* germination experiments with the same species and, as was the case in this study, he noticed a quadratic germination decreasing trend when the seeds were subjected to immersion times greater than 15 minutes.

It is well known that *P. cattleianum* seeds have an impermeable integument due to its rocklike consistency (*testa petrea*) (Cisneiro, Matos, Lemos, Reis, & Queiroz, 2003), which is, in part, responsible for the seed's low imbibition rate and consequent integumental dormancy (Da Silva et al., 2011). That fact led to the reasoning that mechanical or acid scarification helps to 'injure' the seed's integument, making it easier for water and gases to enter the embryo, which, in turn, favors its germination (Bertalot & Nakagawa, 1998). Using that reasoning, this experiment found that increasing immersion times in a 9 mol L^{-1} H_2SO_4 solution contributed to reducing the time needed for germination of the seeds. However, when the seeds had already been sanded and were subjected to acid scarification for periods greater than 15 minutes, there was a reduction in their germination percentages and GRIs (Figure 1). This could be related to the possibility that the seed integument was previously deteriorated, in which case, the immersion in acid for periods longer than 15 minutes may have caused chemical or physiological damage to the embryo, resulting in a lower germination capacity.

C. C. Baskin and J. M. Baskin (2014) claim that acid scarification immersion times for seeds with integument dormancy should be carefully tested, since even periods slightly longer than the ideal time may damage the embryo and prevent it from germinating. Furthermore, these authors also say that the addition of concentrated acids may change the pH levels of the solutions surrounding the embryo and, because of the specific pH levels required for germination, it would interfere with the germination process.

With respect to the *in vitro* germination substrates, this research effort found that sowing seeds in cotton increased the rate of germination and the final number of germinated seeds when compared to the MS-zero medium solidified with agar. Passos, Tavarez, and Alves (2007) also used moistened hydrophilic cotton for the germination of Sabia (*Mimosa caesalpiniifolia*) seeds and obtained a higher germination percentage with this substrate than with any of the others that were tested. In this study, the difference in how *in vitro* germination was affected by the different substrates became evident during experiment evaluations. On the 56th day after the *in vitro* sowing, approximately 40% of the seeds placed in the cotton substrate had already germinated, whereas none had germinated in the MS-zero medium.

Unlike what was observed in this study, other authors obtained good *in vitro* germination percentages for myrtaceans, using saline culture mediums such as MS-zero, LPm and WPM. Cid, Machado, Carvalheira and Brasileiro (1999), for example, obtained good germination percentages for the *Eucalyptus* spp. in an MS-zero medium; similar results were obtained for the Cagaita (*Eugenia dysenterica*), using the same medium and yielding almost 90% germination (Martinotto et al., 2007). Rodríguez (2013) obtained a 68.00% germination result for the Red Strawberry Guava (*P. cattleianum*) using an LPm medium (Von Arnold & Erikson, 1981). Also, Souza, Fior, Souza and Schwarz (2011) achieved an average of 70% *in vitro* germination when placing Guabijuzeiro (*Myrcianthes pungens*) seeds in a WPM medium.

Several substrate factors may affect a seed's germination quality. Since water and oxygen are essential to many metabolic processes that occur during a developing embryo's germination (Taiz & Zeiger, 2013), one of these factors is the substrate's ability to maintain good water availability and aeration for the seed (Popinigis, 1985).

According to Gulliver and Heydecker (1973), up to a certain limit, the more water available to the seeds, the greater the speed of imbibition and absorption, resulting in faster germination of the seeds. Carvalho and Nakagawa (2000) also claim that water is the most significant factor influencing the germination process, especially considering that the embryo's development and growth depend on water imbibition by the seed and its subsequent absorption by the tissues, mechanisms that enhance breathing and other metabolic activities necessary to achieve seedling emergence and root protrusions.

The positive influence of high water availability in the substrate had already been established for the germination of different species, such as the white spruce (*Picea glauca* [Moench.] Voss.) (Downie et al., 1998), the *Mesua ferrea* (Joshi, Phartyal, Khan, & Arunkumar, 2015), and also myrtaceans, such as the Guavira [*Campomanesia adamantium* (Cambess.) O. Berg.] (Dresch, Scalon, & Kodama, 2011), the Brazilian Strawberry Guava (*Psidium guineense* Swartz) (Gonçalves et al., 2009) and the Uvaia (*Eugenia pyriformis*) (Scalon & Jeromine, 2013).

However, the high salt concentration of germination substrates and the solidification of the same using agar, for example, have been reported as negative factors to the seedling emergence process (Grattapaglia & Machado, 1998). The solidification of the substrate increases the colloidal state of the same (Stoltz, 1971) and, as well as saline ions, increase the effectiveness of intermolecular interactions with water, which increases the osmotic pressure of the medium where the seeds are inserted (Doneen & MacGilliwray, 1943). These factors increase the retention of water by the substrate, reducing the water absorption/imbibition by the seed, and directly affecting their physiological responses (Carvalho & Nakagawa, 2000).

Accordingly, in this study, the difference in the availability of water between the cotton and the MS-zero mediums is believed to have contributed to the better germination percentages observed in the moistened cotton substrate (Figure 2).

This study also indicated that different concentrations of gibberellic acid (GA_3), in which the Red Strawberry Guava seeds were immersed, were not efficient enough to promote an increase in germination percentage, when compared to the control group. It's possible that this lack of efficiency occurred because the GA_3 didn't properly stimulate the supply of nutrients to the embryo, or didn't contribute to the seed's production of endogenous gibberellins, a plant hormone that plays an essential role in the germination process. Other research efforts for the same species showed conflicting results regarding the effects of GA_3 solutions on germination. Rodríguez (2013), for example, not only noted that there was no significant difference in germination when using GA_3 concentrations of 0 mg L^{-1}, 10 mg L^{-1}, 20 mg L^{-1} and 50 mg L^{-1}, but also found that the control group had a higher germination percentage. Tomaz et al. (2011), on the other hand, observed that, when previously immersed in a 500 mg L^{-1} GA_3 solution, a higher number of Red Strawberry Guava seeds successfully germinated. Interestingly, the concentration used by Tomaz et al. (2011) is the same that, in this study, resulted in lower germination percentages and vigor.

Exogenous applications of GA_3 don't always have the expected effect on germination (Kermode, 2005) because it's dependent on other factors such as the endogenous concentration of abscisic acid and other inhibiting compounds present in the seed (Taiz & Zeiger, 2013; Carvalho & Nakagawa, 2000; Khan, 1971), or whether or not they are associated to beneficial microorganisms (Dalal & Kulkarni, 2015). In addition, seed responses to exogenous applications of GA_3 are quite specific, varying among different species of the same genus, or even within the same species (Kumar et al., 2012). As an example, for the genus *Psidium*, immersion in a GA_3 solution promoted a significant increase in germination percentages of *P. guajava* seeds, when compared to the control group, to the group submitted to acid scarification with HCl and H_2SO_4 treatments, and even to the group that got immersed in distilled water (Chandra & Govind, 1990). In contrast, germination of *P. guineense* seeds was not stimulated by GA_3 solution immersions, yielding lower germination percentages than both the control group and the group immersed in distilled water (Dresch, Scalon, Neves, & Masetto, 2014).

In terms of GRIs, results obtained in this study indicated that pre-immersion of the Red Strawberry Guava seeds in water for a period of 20 hours (T2 in Table 2) increases their germinating vigor, with GRI values almost double that of the control group. Other research efforts also showed that pre-immersion in water decreases the time to the onset of germination in many other species, for instance, the *Tamarindus indica* (Azad, Nahar, & Matin, 2015), the *Acrocomia aculeata* (Rodrigues Junior et al., 2013), and the *Acacia origena* (Aref, Atta, Shahrani & Mohamed, 2011). Acid scarification after the period of pre-immersion in water (T3 in Table 2), however, significantly reduced both the GRI and the *in vitro* germination percentages. Such results may have been caused by acidity induced chemical and/or physiological damages to the embryo, which would decrease the seed's germination capacity. Similar results were attained by Tavares, Lucca Filho, and Kersten (1995) when they immersed Guava (*Psidium guajava*) seeds in a H_2SO_4 solution, yielding a significant reduction in GRI, when compared to the control group.

It is important to note that acid scarification of seeds may trigger oxidative processes and deregulate pH levels in the regions surrounding the embryo (C. C. Baskin & J. M. Baskin, 2014). This, in turn, may have negatively interfered in the germination process of Red Strawberry Guava seeds, possibly reducing physiological responses that affect the embryo's development.

5. Conclusion

Results led to the determination that immersion of non-mechanically scarified Red Strawberry Guava seeds in a 9 mol L^{-1} H_2SO_4 solution for 20 minutes yields higher GRIs and *in vitro* germination percentages. When the seeds are mechanically scarified, 5- and 15-minute immersions in a 9 mol L^{-1} H_2SO_4 solution yielded better results for GRI and germination percentages, respectively. The experiments also revealed that pre-immersing the seeds in solutions of GA_3, with concentrations ranging from 0 mg L^{-1} to 1000 mg L^{-1}, was irrelevant. Also,

sowing the seeds in a moistened hydrophilic cotton substrate is more efficient and promotes higher rates of emergence and vigor, when compared to a complete MS-zero medium. Furthermore, pre-imbibition of the Red Strawberry Guava in distilled water for 20 hours increases both the *in vitro* germination speed and percentages.

In conclusion, this research effort showed that optimization processes for the *in vitro* germination of Red Strawberry Guava seeds are efficient, and can be used to obtain healthy *in vitro* seedlings, making it possible to further develop micropropagation protocols of seminal origin for this species.

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