Are Seeds of *Genipa americana* L. (Rubiaceae) Tolerance to Water Submersion?

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**ABSTRACT**

The knowledge of the germination responses of seeds from plants growing along river margins or in areas susceptible to flooding is an important factor in the adoption of restoration practices. Considering that maturation of fruits of *Genipa Americana* L. when river margins are flooded, we raised some questions: Is seed germination of this species affected by water submersion, and, do distinct seed populations present differences in germination? Seeds of *G. Americana* were submerged in water for different periods to assess the germination and growing responses, using populations from different locations. Water submersion decreased the germination percentage and the germination speed index, and increased seed mean germination time in both populations. Growth was found to be hampered for most variables in the different seed populations. The adaptation of *G. Americana* to flooding involves the seeds being tolerant to submersion, with this factor not being effective in distinguishing populations studied here.

**Keywords:** anoxia, germination, hypoxia, phenotypic plasticity, populations.
1. INTRODUCTION

The significant ecological value of riparian forests and the surrounding landscape justify the efforts for conservation and restoration of these areas (Buijsse et al., 2002; Glenz et al., 2006). Interventions are hindered by the limited basic biology knowledge of the plant species found in these areas, especially in the environmental determinants for germination (Lucas et al., 2012), with emphasis on the harmful effects of soil flooding on seed germination and seedling development (Kozlowski, 1997).

Considering the gas diffusivity, the flooding frequently is associated with the reduction of oxygen levels in soils. According to Stroo & Ward (2010) the term “anoxic” refers to conditions in which the concentrations of dissolved oxygen are between 0.1 to 0.5 mg/L. On the other hand, the term anoxia should be restricted to define the complete absence of oxygen, that is not true when active photosynthetic tissues are involved in a system where metabolic oxygen is produced (Sasidharan et al., 2017).

Many tree species naturally grow in environments subjected to flooding (flooding conditions), and may take advantage of the water for seed dispersal (Kestring et al., 2009) and break their dormancy (Lucas et al., 2012). However, environmental change from non-flooded to flooded areas can promote stress to non-tolerant species. These conditions are characterized by the increase in the levels of reactive oxygen species (ROS), enhancement of catabolic reactions, loss of developmental stability and frequently the organism death (Kozlowski, 1997; Petrov et al., 2015).

Plants that are tolerant to periodic flooding (adapted genotypes) must frequently adjust their metabolism and life cycle, through plastic responses, keeping ROS concentrations under control since they are essential for aerenchyma development, changing physiological and/or morphological processes to be tolerant to hypoxic or even anoxic conditions caused by flooding, which requires them to possess adapted genotypes (Dat et al., 2000; Petrov et al., 2015). When environmental determinants associated to plant stress responses, activate specific stress-induced genes or gene groups (co-adapted genes) in the tolerant plants, that when expressed, result in characters with adaptive value (Lynn & Waldren, 2002), increasing the chances of the organism survival despite environmental stressors.

Owing to selection processes, genotypes adapted to environmental determinants may occur in groups with no phyllogenetic relationships, may be restricted to families or genera, or may even be limited to certain populations (Daws et al., 2004; Givnish et al., 2010; Lamarca et al., 2011; Mattana et al., 2012; Santiago et al., 2015). This matter requires further studies at different levels (morphological, anatomical, physiological, molecular and other) for a better understanding of the phenotypic plasticity of responses.

Information on seed tolerances to submersion remains scarce, especially for tropical tree species. The knowledge of the germination responses of seeds from plants growing along river margins or areas subjected to flooding is relevant because these plants may potentially be important in restoring this type of habitat (Kestring et al., 2009).

Thus, Genipa Americana L. is highlighted, which is a tree species belonging to the family Rubiaceae and is commonly known as "genipapo". This species has great ecological and economic relevance, since its fruits are also consumed in natura by humans and used to produce sweets, cakes, jams, jellies, and liquors, besides being consumed by fauna. This species has dispersion by birds, mammals, reptiles, fish, as well as the hydrocornical dispersion (Strong & Fragoso, 2006; Carvalho, 2008; Lorenzi, 2008; Donatti et al., 2011). It is distributed all over Brazil and may occur in indifferent biomes. According to Carvalho (2008), it is considered a pioneer, secondary to early secondary to late. It is most frequently found in open forest areas and as secondary vegetation in floodplains situated in temporarily or permanently flooded areas, which are being recommended for recovering vegetation in riparian forests, being the individuals young and adults endowed with remarkable tolerance to the periodic flood (Santiago & Paoli, 2007; Carvalho, 2008; Lorenzi, 2008).

Considering the ecological role of G. Americana, some questions were raised: is their germination affected by water submersion and, do seeds show a different germination percentage depending on plant population? Here, we evaluated the germination response of G. Americana L. seeds after water submersion with
the aim of determining the phenotypic plasticity of responses in different populations of plants.

2. MATERIAL AND METHODS

Fruits of *Genipa Americana* L.in their final stage of maturation were collected from adult trees, in November 2013, in the municipalities of Dourados (DD) (22°13’18.54”S and 54°48’23.09”W - altitude of 400 meters) and Antônio João (AJ) (22°11’27.81”S and 55°56’52.48”W - altitude of 681 meters), Mato Grosso do Sul, Brazil. These areas are 150 km apart, both affected by the Rio Paraguai and Rio Paraná basins, and characterized by the Cerrado biome, with the municipality of Dourados also containing fragments of Atlantic forest.

From each location, named here as populations DD and AJ, intact matured fruits were manually collected from 5 to 7 different stocks. In December 2013, the pulp with the seeds was extracted from the fruits and passed through a fine-mesh sieve with the aid of running water to facilitate the extraction of the seeds. They were selected according to their integrity and standardized in size and color. After being processed, the seeds were disinfected with 3% sodium hypochlorite solution for 5 minutes and washed in running water for 3 minutes to prevent fungi proliferation.

*Genipa Americana* seeds are brown, with a wrinkled surface and have small variations in length, width, and thickness, presenting higher values in length than in width and thickness between populations. The mean mass was 43.77mg ± 0.012 for DD and 42.50 ± 0.015 for AJ, and water content of approximately 38.3% for DD and 38.5% for AJ. The mean length (mm), thickness (mm) and width (mm) of DD seeds were 6.98 ± 0.991, 1.88 ± 0.435 and 5.24 ± 0.847, respectively, and for AJ, the mean length (mm), thickness (mm) and width (mm) were 6.54 ± 0.871; 1.67 ± 0.354; 5.12 ± 0.767, respectively.

Subsequently, the seeds of *G. Americana* from both populations (DD and AJ) were placed in separate 1000mL beakers containing 500 mL of distilled water. Inside the beakers, 225 seeds from each location were subjected to the following submersion periods: 0 (T0), 16 (T16), 32 (T32), 64 (T64), and 128 (T128) days.

Dissolved oxygen was assessed at the end of each submersion period by using a HI 9828 multi-parameter water quality meter (Hanna Instruments, Woonsocket, USA) with the results being expressed in mg/L. The data were presented as mean ± standard deviation.

The seeds removed from the submersion period were separated in three repetitions per treatment and wrapped in Germitest® paper, placed in transparent plastic bags in a roll shape and kept in a BOD germinator regulated to the constant temperature of 25ºC, with a photoperiod of 12 hours for 30 days.

From the beginning of each germination test, the number of germinated seeds was assessed every two days, where seeds that were considered germinated were those presenting primary roots with length equal or greater than 2.0 mm. At the end of the germination periods, the germination percentage (%G), germination speed index (GSI) (Maguire, 1962), mean germination time (MGT) (Edmond & Drapala, 1958), and the relative frequency of germination were calculated.

Forty days after sowing, 30 seedlings from each treatment were measured with a 150-mm digital caliper (Starret®), assessing the stem diameter (SD), root length (RL), and length of the aerial part (LAP). By using an analytical digital scale (Bel Engineering®) the dried mass of the root and aerial part were assessed to determine the total dried masses (TDM).

The experiment was conducted in a completely randomized design (CRD) in split-plot design, with the plot being the populations and the subplots being the submersion times, with 3 repetitions of 75 seeds. The data were tested for error normality, homogeneity of variance and subjected to analysis of variance by the F test. The submersion times were assessed through a Scott-Knott test and the population t-test at 5% probability, by using the SISVAR statistical program (Ferreira, 2011).

3. RESULTS

The interaction between origin of seeds and submersion time was significant for all the variables analyzed, except for length of the aerial part (Table 1).

The oxygen dissolved in water at T0 was 5.38 for DD and 5.19 for AJ and decreased as the submersion time of seeds increased, reaching 0.05 mg/L for the seeds from Dourados (DD) and 0.02 mg/L for the seeds from Antônio João (AJ) (Figure 1A).
The seeds from AJ germinated while submerged; however, this was only observed in the 128-day treatment (19 germinated seeds). Furthermore, these seeds formed seedlings of approximately 20 mm within the water and, afterwards they kept growing in the Germitest®.

The germination percentage showed high values for seeds from the populations DD and AJ, ranging from 88% to 100% during the 128 days (Figure 1B). Regarding to the germination speed index, there was a drop in the DD values, with the highest value observed for the control treatment (8.5) and the lowest value for the 64-day treatment (4.29). However, for the AJ population, a steady increasing trend was observed for this variable, with the highest speed being observed in the 128-day treatment (11.64) (Figure 1C).

For the mean germination time, the lowest values were observed for DD, in the shortest submersion times (T0, T16, T32) (mean of 10.15 days) and for AJ, the lowest values were observed at the greatest submersion times (T64 and T128) (mean of 8.5) (Figure 1D).

Seed germination from the DD population started on the 2nd day after sowing for T0, on the 6th day for T16, and on the 7th day for T32. The germination percentage was highest for DD, with values ranging from 88% to 100% during the 128 days.

Table 1. Mean Square residue on the analysis of variance for speed germination index (GSI), mean germination time (MGT), germination percentage (%G), root length (RL), length of the aerial part (LAP), stem diameter (SD), total fresh mass (TFM) and total dried mass (TDM) in function to origin of the seeds (O) and time of submersion (T).

<table>
<thead>
<tr>
<th>CV (%)</th>
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<tr>
<td></td>
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<td></td>
<td>9.20*</td>
<td>0.41*</td>
<td>26.18ns</td>
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<tr>
<td></td>
<td>3.88ns</td>
<td>1.20*</td>
<td>37.82*</td>
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<tr>
<td></td>
<td>35.66*</td>
<td>53.11*</td>
<td>113.21*</td>
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Where * and ns significant to 5% probability by the F test and not significant by analysis of variance, respectively; CV = Coefficient of variation.

Figure 1. (A) Dissolved oxygen content (mg/L), (B) Germination percentage, (C) Germination speed index (GSI) and (D) Mean germination time of *Genipa americana* L. seeds from different origins (DD – Dourados, MS; AJ – Antônio Joao, MS) after different periods of submersion. Capital letters compare the same locality at different submersion times and lowercase letters compare the same submersion time at different locations by the Scott-Knott test (P ≤ 0.05).
T16 and T32, on the 8th day for T64, and on the 10th day for T128. Regarding to AJ population, the germination started on the 4th day after sowing for T0 and T64, on the 6th day for T16 and T32, and for T128, the germination started while still in water. Therefore, treatments with greater submersion times presented slower germination for DD.

In general, DD and AJ populations presented a different distribution of relative frequency of germination in the different experimental periods (Figure 2). The unimodal pattern with a higher frequency percentage ranging from 8-14 days was observed in the DD population, whereas in AJ, the distribution was predominantly polymodal regardless of the submersion time. As the submergence time increases, a greater uniformity in the germination of DD seeds was observed, with 70 to 80% relative frequency at 16 and 32 days of submersion, respectively. It was possible also observed that with the increase of the submersion period from 16 to 32 days; the highest relative frequency was reached in a shorter period of time (eighth day). Correlating the Figure 2 with Figure 1, it can be observed that the higher availability of oxygen observed at time zero (T0) apparently did not affect the relative germinative frequency for both populations.

Regarding the morphological parameters, LAP decreased as the submersion time increased regardless of the populations. Seedlings of DD presented lower LAP (Figure 3A and B). The RL showed increase for both DD and AJ, with the highest values being observed.
for the 128 and 64 days submersion treatment. It is noteworthy that from 32 days of submersion, there was no significant difference in the root length of DD seedlings. For AJ, the RL did not vary significantly from the 64 days of seed submersion (Figure 3C). The stem diameter showed a decrease in the values as the submersion period of seeds increased (Figure 3D).

The TDM practically did not change with the submersion time for DD seedlings. However, there was a decrease in TDM in AJ seedlings (Figure 3E).

4. DISCUSSION

The submergence of *G. Americana* seeds does not prevent the germination, although it has caused a small reduction for seeds from DD. It should be emphasized that the seeds from DD present a high percentage and germination speed in a lower average time when not submerged or when the submersion time was up to 32 days because in times of superior submersion there is a reduction of germination potential. On the contrary, the percentage of germination of the seeds of AJ oscillated as a function of submersion times. However, when submerged by 64 and/or 128 days, they had a higher percentage and germination speed in a smaller average time.

Submergence may have negative effects on seed germination and seedling growth (stem diameter and length of the aerial part). Thus, many terrestrial species have high germination rates in unsaturated soils, but their seeds fail to germinate in water or saturated soils, and/or rapidly lose viability in these media due primarily to oxygen starvation, or inducing anaerobic decomposition (Kozlowski, 1997; Parolin, 2001). However, *G. Americana* seed in both populations were exposed to oxygen restriction conditions and had no loss of viability. This probably occurred because its metabolism was adjusted to endure the submersion period into which they are subjected (Voesenek & Bailey-Serres, 2013).

This study suggests that seeds of *G. Americana* are tolerant to long periods of submersion since the figure 3.

(A) and (B) Length of the aerial part (mm); (C) Root length (mm), (D) Stem diameter (mm) and (E) Total dried mass (mg) of *Genipa americana* seedlings L. from different origins after periods of submersion different. Capital letters compare the same locality at different submersion times and lowercase letters compare the same submersion time at different locations by the Scott-Knott test (P ≤ 0.05).
germination percentage was greater than 80% even after 128 days of seed submersion. This may be related to the maturation period and fruit dispersal traits of the species, which occur in the beginning of the rainy season (Vieira et al., 2006), causing the seeds of *G. Americana* to be submerged until water levels fall. Thus, Segundo Melo et al. (2015) seem reasonable to state that seeds of *G. Americana* have high capacity of germination, even when submerged, since the seeds maintain viable embryos. In this context, the type of reserves the seeds hold has an important role in their response to submersion, as well in strategies of escape that the seedlings present to multiple unfavorable conditions (Melo et al., 2015; Lucas et al., 2013).

The differences related to seeds origin for germination speed index (GSI), length of the aerial part (LAP), root length (RL) and total dried mass (TDM) (Figures 1 and 3) and the distinct distribution of relative frequency (Figure 2) discarding the effect of vigor or collect time (high germination percentage and seeds collected at the same month) can be attributed to the gene pool of the populations.

Seeds from different environments may have different responses, which are related not only to local environmental conditions (temperature, light, soil, etc.) and the determinants of the physiology of species but also to genetic variations between the populations (Botezelli et al., 2000). This information corroborates the results observed in this work, where the seeds from different locations presented germination potential and seedling initial growth, in different periods of submersion, even though they were from different populations in the same biome.

Differences in gene expression between plants populations are observed when they are subjected to unfavorable condition (Santiago et al., 2015) since phenotypic plasticity involves both adaptive and non-adaptive germination responses against environmental variations (Gianoli & Gonzalez-Teuber, 2005; Ghalambor et al., 2007).

The results of this study suggest that the submersion was not a unfavorable condition for the seedlings of *G. Americana*, considering that the species is adapted to environments subjected to periodic flooding (Carvalho, 2008; Barbosa et al., 2007).

The acquisition of adapted genotypes occurs either under stress or when favoring optimal development, through natural selection processes (de Jong, 2005). The fact that the other-plants of *G. Americana*, from which the seeds were obtained, occur in similar environments, suggests to both plant populations, groups of genes able to induce a germination response even after the seeds are exposed to long periods of hypoxia/anoxia. The similarity between the sites of natural occurrence of the mother-plants explains the similar germination responses (Santiago et al., 2015), with this factor being fundamental, considering the acquisition and maintenance costs of the adapted genotype (Valladares et al., 2007).

The differences observed between the TDM of DD and AJ did not identify or distinguish populations. More effective germination responses that show plasticity may involve both morphological and physiological characters, and reflect the environmental heterogeneity in either genotype expression or acquisition (Gianoli & Gonzalez-Teuber, 2005).

The environment may also induce germination response sat the molecular level, such as modifications of DNA, RNA, or protein that can affect gene expression (epigenetic modifications), which can be inherited (Schlichting & Wund, 2014).

5. CONCLUSION

Nevertheless, it seems reasonable to state that the adaptation of *G. Americana* to flooding involves the seeds being tolerant to submersion, with this factor not being effective in distinguishing populations studied here. Novel studies involving exposing *G. Americana* plants to different environmental determinants are required to better clarify not only possible intrapopulation variation but also the adaptive characters of the germination responses observed.

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