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Germination potential and methods for overcoming seed dormancy for domesticated and wild annatto populations after two years of storage

Wellington Ferreira do Nascimento[1*] Fabiana Gonçalves Bastos[2] Gabriel Dequigiovanni[3] Eliane Gomes Fabri[4] Maria Imaculada Zucchi[5] Charles Roland Clement[6] Elizabeth Ann Veasey[2]

[1]Centro de Ciências Agrárias e Ambientais (CCAA), Universidade Federal do Maranhão (UFMA), 65500-000, Chapadinha, MA, Brasil. E-mail: wellington.fn@ufma.br. *Corresponding author.
[2]Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Piracicaba, SP, Brasil.
[3]Centro Universitário de Cascavel (UNIVEL), Cascavel, PR, Brasil.
[4]Centro de Horticultura do Instituto Agronômico de Campinas (IAC), Agência Paulista de Tecnologia dos Agronegócios (APTA), Campinas, SP, Brasil.
[5]Agência Paulista de Tecnologia dos Agronegócios (APTA), Polo Regional de Desenvolvimento Tecnológico do Centro Sul, Piracicaba, SP, Brasil.
[6]Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brasil.

ABSTRACT: *Annatto (Bixa orellana L.) is an arboreal species domesticated in Amazonia from its wild ancestor (B. orellana var. urucurana). Bixin extracted from its orthodox seeds is a natural dye widely used in the food industry. This study evaluated methods to overcome seed dormancy and determine the germination potential, comparing domesticated and wild annatto populations. Seeds from two domesticated-type populations and two families of a wild-type population, stored for two years after field collection, were submitted to five treatments to overcome dormancy: T1 - control; T2 - mechanical scarification (with sandpaper); T3 - mechanical scarification (with sandpaper) + immersion in water at 36 ºC overnight (12 hours); T4 - immersion in water at room temperature (23 ºC, on average) for 24 h; T5 - immersion in concentrated sulfuric acid (95 - 98%) for 15 min + running water for 3 min. Highly significant differences (P < 0.001) were observed in the germination percentage of annatto seeds between wild and domesticated types, and among the treatments tested. Domesticated types showed higher germination percentage (10 - 58%) over all treatments when compared to the wild type (0 - 44%). The best treatments were those performed with mechanical scarification. Given the simplicity, we concluded that mechanical scarification with sandpaper is a good alternative to overcome dormancy of annatto seeds.*
Key words: *Bixa orellana L., B. orellana var. urucurana, domestication, mean germination time.*

Potencial de germinação e métodos de superação da dormência de sementes para populações domesticadas e selvagens de urucum após dois anos de armazenamento

RESUMO: *O urucum (Bixa orellana L.) é uma espécie arbórea domesticada na Amazônia a partir de seu ancestral selvagem (B. orellana var. urucurana). Das suas sementes ortodoxas, é extraído a bixina, corante bastante utilizado na alimentação. Este estudo teve como objetivos avaliar métodos de superação da dormência e determinar o potencial de germinação de sementes, comparando populações domesticadas e selvagens. Sementes de duas populações domesticadas e duas famílias de uma população selvagem, armazenadas por dois anos após a coleta, foram submetidas a cinco tratamentos de superação da dormência: T1 - controle; T2 - escarificação mecânica (com lixa); T3 - escarificação mecânica (com lixa) + imersão em água a 36 ºC durante a noite (12 horas); T4 - imersão em água à temperatura ambiente (23 ºC, em média) por 24 h; T5 - imersão em ácido sulfúrico concentrado (95 - 98%) por 15 min + água corrente por 3 min. Foram observadas diferenças altamente significativas (P < 0,001) no potencial de germinação de sementes entre domesticadas e selvagens, e entre os tratamentos testados. As populações domesticadas apresentaram maior porcentagem de germinação (10 - 58%) em comparação com a de urucum selvagem (0 - 44%). Os melhores tratamentos foram os realizados com escarificação mecânica. Considerando sua simplicidade, concluímos que a escarificação mecânica com lixa representa boa alternativa para superar a dormência do urucum.*
Palavras-chave: *Bixa orellana L., B. orellana var. urucurana, domesticação, tempo médio de germinação.*

Annatto (*Bixa orellana* L., Bixaceae) is an arboreal species native to Amazonia (MOREIRA et al., 2015, DEQUIGIOVANNI et al., 2018). Bixin, extracted from its seeds, is a dye widely used in Brazilian cuisine, as well as in the pharmaceutical industry for the treatment of cardiovascular and gastrointestinal diseases (LORENZI & MATOS, 2002; RUSSELL et al., 2005). Also, its seeds contain geranylgeraniol, a substance used in cancer treatment (QI et al., 2018).

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Because flowering, fruiting and seed maturation occur during the whole year, annatto represents a profitable alternative for small farmers, providing more frequent harvests and commercialization of products derived from its seed (FRANCO et al., 2008; PORTAL et al., 2013). The annatto market accounts for approximately 90% of the natural dyes consumed in Brazil and around 70% of natural dyes consumed worldwide, mainly in the United States, Japan, and in several countries in South America and Europe (FABRI & TERAMOTO, 2015). This generated a demand for the production of seedlings, which depends on the germination of its seeds.

Despite the economic value of annatto, studies of seed germination have shown low percentages of germination in natural environments by many conventional methods (DAS et al., 2017), interfering with its large-scale commercial production (CUSTODIO et al., 2002). Seeds are the essential input for agriculture, so there is a constant interest in evaluating their quality (FERREIRA & NOVEMBRE, 2015) using germination and vigor tests to obtain information regarding the actual behavior of the seeds in the field. Although, no information is provided for annatto seeds in the Rules for Seed Analysis (BRASIL, 2009), the Instructions for Seed Analysis of Forest Species (BRASIL, 2013) provides information regarding *B. orellana* seed germination, where paper substrate is recommended, as well as a wide range of temperatures, from 20 to 35 ºC.

According to Goldbach (1979), annatto seeds, during storage, are not totally orthodox or recalcitrant. However, in a study by Mello & Eira (1995), orthodox behavior was observed in this species, and the primary dormancy imposed by the integument can be overcome by storage for one year in environmental conditions, as well as maintained or deepened by low temperatures. Also, because annatto seeds are hard, mechanical scarification treatments are recommended to overcome the dormancy of their seeds (CUSTODIO et al., 2002). To elucidate the dormancy mechanism of annatto seeds and evaluate the effect of different methods for overcoming dormancy, allied with different temperatures for germination, PICOLOTTO et al. (2013) showed that scarification with sandpaper or sulfuric acid for five minutes, and temperatures of 25 ºC or 20 - 30 ºC are recommended for this species. FERREIRA & NOVEMBRE (2015), evaluating the temperature and the substrate for the germination test of annatto seeds, concluded that the appropriate temperature range for germination of seeds is between 29.5 ºC and 31 ºC, and the ideal substrate is paper. Finally, DAS et al. (2017), analyzing the effect of different pre-treatments with sulfuric acid to accelerate and increase germination of their seeds, concluded that the use of sulfuric acid allowed an increase of germination and reduction of the average time of germination in annatto. It is worth mentioning that there is little information about the physical and biochemical processes involved in annatto seed germination and dormancy, and that the studies carried out so far have been for the germination of cultivated annatto seeds (AMARAL et al., 2009).

Although, there are no significant incentives from the industry to find populations of annatto with better germination potential, the growing importance of the crop stands out as another low-cost economic activity for Brazilian agriculture. In this context, the main purpose of this study was to determine the germination potential of cultivated (*Bixa orellana* var. *orellana* L.) and wild annatto (*Bixa orellana* var. *urucurana* L.) populations from different localities, after being submitted to different methods to overcome dormancy, aiming to determine the method that results in higher seed germination rates. Two hypotheses were tested: (I) seeds from wild populations are more resistant to the process of overcoming dormancy; (II) the mechanical scarification method and subsequent seed immersion in water at 36 ºC overnight is the most efficient. To our knowledge, this is the first study comparing the germination potential between wild and domesticated annatto seeds.

In August 2016, seeds were collected from two domesticated populations in farmers' fields in the municipalities of Paranacity (D1), state of Paraná (22°50'35.0"S, 52°05'21.8"W), and Monte Castelo (D2), state of São Paulo (21°17'30.0"S, 51°33'58.4"W). Due to the difficulty in finding wild plants with viable seeds to use in the germination tests, seeds from two families (W1 and W2), from a group of 10 families that were being used in a parallel study of annatto's breeding system, were used to represent the wild population. Its seeds were collected in October 2016 in the municipality of Corumbiara, state of Rondônia (12°59'29.7"S, 60°55'22.0"W). This population was identified on a cliff in a riverside area by local farmers, showing spontaneous regeneration. Due to the available resources and the best conditions for the development of the research, all seeds were stored in a cold chamber at approximately 10 ºC and 50 - 60% humidity until the experiment was carried out in November 2018.

Five treatments to overcome dormancy were tested: T1 – control (where seeds were not

subjected to any treatment to overcome dormancy); T2 - mechanical scarification (with sandpaper); T3 - mechanical scarification (with sandpaper) + immersion in water at 36 ºC overnight (12 hours); T4 - immersion in water at room temperature (23 ºC on average) for 24 h; T5 - immersion in concentrated sulfuric acid (95 - 98%) for 15 min + running water for 3 min. For each treatment, four replicates of 25 seeds were used. Each plot consisted of a plastic Gerbox with filter paper suitable for germination, moistened with distilled water. The different plots were randomized in a BOD-type (Biological Oxygen Demand) germination chamber at constant temperature of 30 °C (FERREIRA & NOVEMBRE, 2015). The number of seeds that germinated was counted for 30 days. A seed was considered as germinated when at least 1 cm of the primary root emerged, after which it was removed from the Gerbox and the substrate was re-wetted when necessary.

The germination percentage was estimated according to the equation $g_i = (\sum k_i = 1 n_i/N$) x 100, where n_i is the number of seeds germinated during time i and N is the total number of seeds placed to germinate. The mean germination time per seed was estimated using the equation $\bar{t} = \sum(n_i t_i / \sum(n_i)$, where t_i is the number of days between the beginning of the experiment and its i^{th} observation, with i = 4, 8, 12, 16, 20, 24, 28, 30 days; and n_i is the number of seeds germinated at time t_i (LABOURIAU, 1970).

For the statistical analysis, data were submitted to arcsine square-root transformation and subjected to analysis of variance (ANOVA) in a factorial design, where one of the factors corresponded to the type of annatto population (domesticated *vs* wild) and the other corresponded to the different treatments for overcoming dormancy. The analysis was performed with the *ExpDes* package implemented in R (R DEVELOPMENT CORE TEAM, 2019).

Highly significant differences (P < 0.0001) were observed among the methods tested to overcome dormancy. For both domesticated and wild annatto, the best treatments were T2 (mechanical scarification with sandpaper) and T3 (mechanical scarification with sandpaper + immersion in water at 36 ºC overnight (12 hours) (Table 1; Figure 1). The two domesticated types showed similar results for seed germination in treatments T2 and T3. The same was observed for the two families of the wild type (Table 1).

These results corroborate those obtained by Custodio et al. (2002), where they concluded that mechanical scarification with sandpaper promoted the highest germination percentages (40%), followed by treatment with water at 100 ºC for one minute (21%). PICOLOTTO et al. (2013), in addition to recommending mechanical scarification with sandpaper, also recommend chemical scarification with concentrated sulfuric acid for five minutes. The study by DAS et al. (2017), using the pre-treatment of sulphuric acid for different durations (5 s, 1 min, 3 min, 6 min, 9 min, 12 min and 15 min) in fresh harvested seeds and in one year-old stored seeds,

Table 1 - Mean germination percentage of domesticated (D1 and D2) and wild annatto (W1 and W2) samples of *Bixa orellana* for each method used to overcome dormancy.

Treatment	----------Types of annatto (domesticated and wild)----------					
Methods[1]	D1 (%)	D2 (%)	Average (%)	W1 (%)	W2 (%)	Average (%)
T1	10 b[2]	10 b	10 b	0 b	0 b	0 b
T2	48 a	68 a	58 a	51 a	36 a	44 a
T3	50 a	48 a	49 a	50 a	21 a	36 a
T4	21 ab	11 b	16 b	0 b	2 b	1 b
T5	20 ab	6 b	13 b	0 b	0 b	0 b
Average	30	29	29 A	20	12	16 B

[1]T1 - Control, T2 - Mechanical escarification with sandpaper, T3 - mechanical scarification with sandpaper + immersion in water at 36 ºC overnight (12 hours); T4 - immersion in water at room temperature (23 ºC, on average) for 24 h; T5 - immersion in concentrated sulfuric acid (95 - 98%) for 15 min + running water for 3 min.

[2]Means followed by the same lower-case letters in a column and capital letters on the last line (among averages) do not differ significantly in the Tukey test at 5% probability.

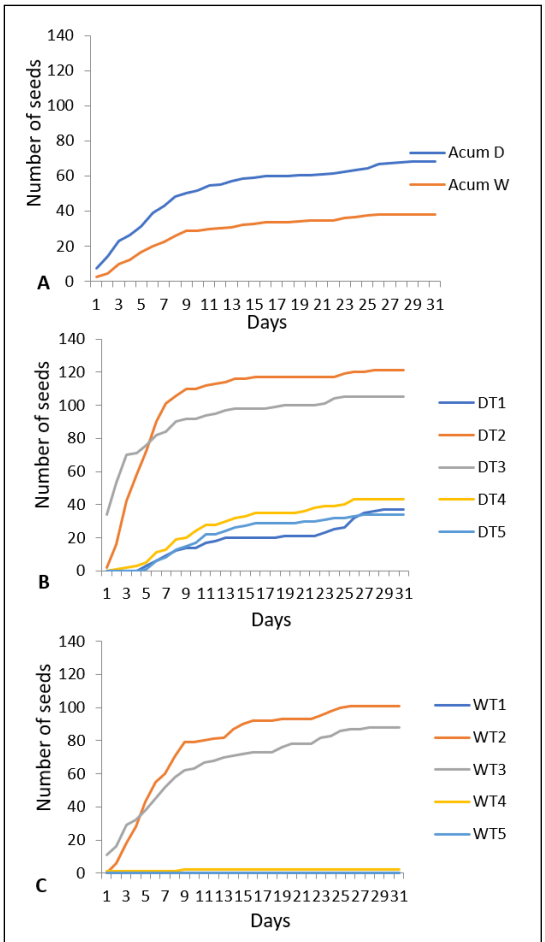

Figure 1 - Mean accumulated (Acum) number of seeds germinated for domesticated (D) and wild (W) annatto (A), for domesticated types with five treatments to overcome dormancy (DT1 to DT5) (B), and for the wild type with five treatments to overcome dormancy (WT1 to WT5) (C).

observed that the use of sulfuric acid increased germination and reduced the average germination time in annatto; although, they also observed some damage caused by sulphuric acid leading to a lower percentage of germination. The one year-old seeds showed a percentage of germination a little higher than the fresh seeds, although non-significant. Our treatment with sulphuric acid for 15 min resulted in lower germination percentage in the domesticated samples and no germination in the wild ones. The fact that our seeds were kept for two years in storage, at lower temperatures (10 °C) and humidity (around 50 or 60%), could have interfered in this result. It is worth mentioning that sulfuric acid can result in a high rate of abnormal seedlings and dead seeds when exposed to prolonged periods, above 15 min (YOGEESHA et al., 2005).

Highly significant differences ($P < 0.0001$) were also found in the germination potential of annatto seeds between wild and domesticated types. On average, domesticated annatto showed a higher percentage of seed germination (39%) than wild annatto (16%) (Table 1). When comparing both domesticated and wild samples against treatments, no significant result was found. The best treatments (T2 and T3) for overcoming dormancy in the domesticated samples (58% and 49%) were also the best treatments for the wild samples (44% and 36%). The average germination time was faster in the domesticated types with the best treatments (T2 and T3), with D2 being faster than D1. For wild annatto, W1 was faster than W2 in the two best treatments (T2 and T3) (Figure 2). Several factors may explain the differences in germination between individuals and populations; however, considering that W1 and W2 came from the same wild population, we suggested an intrapopulational genetic difference in the germination of wild annatto seeds.

The difference in germination performance between annatto seeds from domesticated and wild populations observed in this study (Table 1; Figure 1) may be related to the morphophysiological changes that occurred in the species during the domestication process (MEYER et al., 2012). These changes, known as the domestication syndrome, can include changes in various characteristics depending upon the crop, including rachis that do not shatter, increased fruit and seed size, changes in branching and height, among others (MEYER et al., 2012), as well as in seed dormancy (SUGIMOTO et al., 2010; SMÝKAL et al., 2018). Among the characteristics observed in the annatto related to domestication, changes were found in the shape of the fruit, an increase in the size of the fruit (1 - 2 cm to 2.5 - 4.5 cm), an increase in the number of seeds (2 - 10 to 30 - 60 seeds per fruit), aryl with pigment, pigment color and fruit dehiscence (MOREIRA et al., 2015). As far as we know, no study has been carried out comparing dormancy of wild and domesticated annatto seeds.

It is worth remembering that in cultivated species that have their wild type, such as annatto, seed dormancy has its advantages and disadvantages (SUGIMOTO et al., 2010). Low dormancy leads to uniform germination, which is advantageous for farmers, while deep dormancy provides germination for a long period, allowing germination to occur only when conditions are favorable for seedling survival

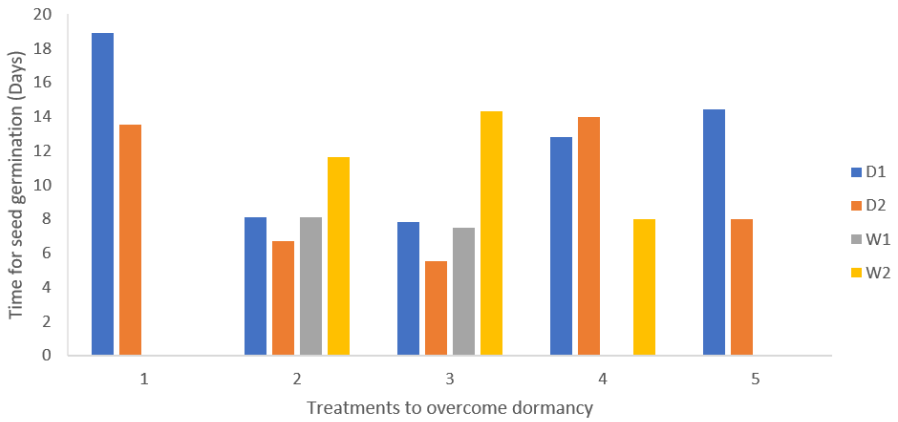

Figure 2 - Time for seed germination (days) for domesticated (D1 and D2) and wild (W1 and W2) annatto, considering the five treatments to overcome dormancy (T1 - Control, T2 - Mechanical scarification (with sandpaper), T3 - mechanical scarification (with sandpaper) + immersion in water at 36 ºC overnight, T4 – immersion in water at room temperature for 24 h, T5 - immersion in concentrated sulfuric acid (95 - 98%) for 15 min + running water for 3 min).

that represents an advantage for the survival of wild plants in natural conditions (DE WET & HARLAN, 1975; BASKIN & BASKIN, 2014).

The results found in this study are in line with what would be expected when comparing wild and domesticated populations and represent yet another character of the annatto domestication syndrome. However, it is interesting to observe, from the data reported in this and previous studies (PICOLOTTO et al., 2013; DAS et al., 2017), that seed dormancy was not totally lost in domesticated annatto, considering that only 10% germination, on average, was observed in this study for the domesticated populations in the control treatment (Table 1), probably because this characteristic does not affect its cultivation in agroecosystems.

We concluded that both mechanical scarification with sandpaper alone (T2), as well as mechanical scarification with sandpaper + immersion of the scarified seeds in water at 36 ºC overnight (T3) proved to be good alternatives for overcoming dormancy of both domesticated and wild annatto seeds. The germination percentage of domesticated types was higher when compared to the wild type for all treatments, corroborating the initial hypothesis that the seeds from domesticated populations present a lower degree of dormancy, which thus represents another character from annatto domestication syndrome.

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DECLARATION OF CONFLICT OF INTEREST

The authors declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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