

Pollen Viability and Autogamy Fitness in *Bauhinia forficata* Link (Fabaceae)

Luana Camila Capitani¹, Ana Paula Moreira Rovedder¹,
José Carlos Corrêa da Silva Júnior¹, Aline Peccatti¹

¹Universidade Federal de Santa Maria – UFSM, Santa Maria/RS, Brasil

ABSTRACT

Bauhinia forficata (Fabaceae) occurs in many phytophysiognomies of southern Brazil, however its ecological relevance is not well understood. The present study was developed in the Central Depression of Rio Grande do Sul and aimed to determine variations in pollen viability along flowering, ability to perform autogamy and dye efficiency for the viability test. Pollen viability was determined by colorimetry as well as the ability to perform autogamy by isolating floral buds, being evaluated in eleven matrices. Average pollen viability was 81.43%, with the highest average value obtained with the dye 2,3,5- Triphenyltetrazolium Chloride (TTC) (84.11%). Safranin was not a good indicator at the tested concentration. No correlation was found between pollen viability and flowering time. The species demonstrated an inability to perform autogamy.

Keywords: colorimetric method, pollen, TTC.

1. INTRODUCTION

Pollen is primarily the transport vehicle of the male gamete, in addition to being the most important source of proteins for floral visitors and pollinators (Agostini et al., 2014; Westerkamp, 2004). Its composition is mainly protein, however it also contains sugars, lipids, and phosphorus among other substances important for the survival of floral visitors (Willmer, 2011). In addition to the reproductive function of pollen, it is likely that it was evolutionarily the first floral resource to be used by animals as a source of food (Lunau, 2006).

Pollen quality expressed by viability is extremely important for the sexual reproductive success of plant species, since they cannot directly choose their reproductive partners (Oliveira & Maruyama, 2014), and the greater the viability, longevity and pollen germination, the greater the genetic variability of a species will be (Akoroda, 1983).

Maintaining genetic diversity is essential for the evolution of a species to occur and to reduce the chances of extinction in the ecosystem, even in the face of natural or induced changes. Therefore, the adoption of diversity maintenance strategies in conservation plans is essential (Zanella et al., 2012; Frankham, 2010). However, the reproductive success of plant species not only depends on pollen viability rates, but also on the efficiency in the pollen transfer to the feminine part of the flower, preferably belonging to an individual with genetic characteristics different from the plant that originated the male gamete.

Therefore, it is worth highlighting that autogamy can lead to a loss of genetic variability, making the species more susceptible to environmental changes and, sometimes resulting in the local extinction of a species. In this way, many plants developed mechanisms over time to prevent self-pollination from occurring, including the adoption of strategies to induce genic flow, as the offer of resources (Rech et al., 2014).

Among the species of Deciduous Seasonal Forest in Rio Grande do Sul, *Bauhinia forficata* stands out because it presents easy adaptation to different climates and regions. It can be found in diverse phytophysognomies such as the Mixed Ombrophilous Forest, both in Montaineous as well as Sub-Montaineous, Semideciduous Mesophyll Forest, Ciliary and Riparian Forest, Secondary Semideciduous

and Seasonal Semideciduous Forest, and Deciduous Forest (Pezzopane et al., 2003; Marangon et al., 2008; Longhi et al., 2005; Valério et al., 2008; Avila et al., 2011; Moraes & Almeida, 2009).

The species also presents medicinal potential (Engel et al., 2008; Silva & Cechinel, 2002; Marques et al., 2013), being frequently used in folk medicine and pharmacognosy. In addition to standing out due to the quantity and quality of the floral resources offered, it also contributes by attracting and maintaining the nectarivorous and pollinating fauna (Paulino, 2013), therefore acting as a key species in the maintenance of ecological interactions in ecosystems associated with it. For these reasons, it is also indicated for the restoration of degraded areas.

Therefore, the present study aimed to determine the pollen viability of *Bauhinia forficata* through colorimetric analysis as well the efficacy of Basic Fuchsin (1%), Safranin (1%), Methyl Red (1%) and 2, 3, 5 - Triphenyltetrazolium Chloride (TTC) (1%) dyes for determining the pollen viability of the species, and to test its ability to perform autogamy through isolating the action of pollen biotic vectors.

2. MATERIAL AND METHODS

The study area is located in the district of Arroio Grande, part of the Santa Maria municipality - RS (29°41'25" S, 53°48'42" W). The region is located in the Atlantic Forest Biome, with a predominantly subtropical humid climate (ST UM) (Maluf, 2000) and a Deciduous Seasonal Forest of subtropical climate.

Identification of the species was performed by the Herbarium of the Forestry Sciences Department- Santa Maria Federal University (HDCF). The choice of *Bauhinia forficata* matrices was based on phenotypic characteristics associated with the viability of the collection and experimental execution such as the size and accessibility of the treetop, characteristics of physiological vigor such as health and the appearance of the treetop and leaves, absence of symptoms of nutritional scarcity in leaves and branches, the presence, quantity and maturation of flower buds and flowers, and on the analysis of the individuals' phytosanitation, according to the visual classification proposed by Nicodemo et al. (2009).

Only those individuals that presented the best combination of the phenotypic and physiological vigor factors, and that were included in Grade 1 (Light: less than 25% of the damaged plant) in the phytosanitary analysis were selected as matrices. Additionally, given that the species presents the ability to sprout from the roots, individuals at least 50 m apart from one another were characterized as distinct matrices, or at a lower distance when the existence of distinct individuals could be verified.

Flower collection was performed in 11 matrices during January 2015, in seven non-consecutive evaluations. Anthers samples of three flowers per evaluation day were collected and stored in solution (Ethanol - Acetic acid (3: 1)) for each matrix, and maintained in a refrigerated environment ($\approx -18^\circ\text{C}$) until the time of evaluation (9 months after collection).

Pollen viability was determined by colorimetric methods with the aid of Basic Fuchsin (1% solution) dye, which reacts with pollen protoplasm blushing pink; Safranin dye (1%), related to cell wall integrity and also blushing pink; Red Methyl dye (1%), which reacts with the viable pollen by blushing an intense red; and finally 2,3,5-Triphenyltetrazolium Chloride (TTC-1%), which indicates the presence of active dehydrogenases enzymes involved in the process of cellular respiration, turning the pollen a reddish-pink (Santos et al., 2006; Munhoz et al., 2008).

The anthers were macerated and the pollen transferred to microscopy slides containing one drop of dye and semi-permanent blades were subsequently assembled, of which the total number of pollen grains and the number of viable pollen grains per sample was determined (adapted of Munhoz et al., 2008 and Vida et al., 2011). Larger pollen grains with more intense staining were considered viable. Pollen viability was calculated by the following Formula 1 (Santos et al., 2006):

$$\text{Viability (\%)} = \frac{\text{number of stained pollen grains}}{\text{total number of pollen grains}} \times 100 \quad (1)$$

For the autogamy aptitude test (with the purpose of preventing the interaction of pollen biotic vectors with the flower), three floral buds were isolated per matrix with the aid of Voil bags, totaling 33 floral buds, which were thus protected until the fruit ripening period (adapted from Aguiar & Gaglianone, 2008 and

Mendes et al., 2011). This test determined whether anthesis of the isolated buds and the formation of fruit occurred or not.

A comparison of significant differences between pollen viability averages was carried out with the aid of Software Assisat (Santos-e-Silva, 2015), through the Kruskal-Wallis test (Cargnelutti et al., 2001). The correlation between pollen viability and flowering time was tested by regression analysis, and the significance of correlations was determined through the estimation of the "t" statistic, comparing to Student's t-values for n-2 DF and a significance level of 5%.

3. RESULTS AND DISCUSSION

Bauhinia forficata pollen presented structures named pollenkitt, which serve to aid in adhesion of the pollen to the floral visitors, as well allowing for the identification of the species, since the conformation of pollenkitt is unique for each species (Lin et al., 2013) (Figure 1).

Average pollen viability was 81.43%. Regarding the dyes, the highest viability average was obtained using the TTC dye (84.11%) (Figure 2). The average viability obtained with the Basic Fuchsin dye was 81.77% (Figure 3), and with the Methyl Red dye it was 78.4% (Figure 4), all considered high according to Souza et al. (2002). Germinated pollen grains could also be observed throughout the evaluations, presenting a mean germination percentage of 5.52% (Figure 3A).

In studying the pollen of two species of the genus *Tabebuia*, Arenas-de-Souza et al. (2014) found an average viability value of approximately 75%. Nascimento et al. (2014) found pollen viability from 56.60-81.10%, for *Delonix regia* and Hister & Tedesco (2016) found an average viability of 80.3% when studying *Psidium cattleianum*; values similar to those obtained in this research.

Safranin dye was not a good indicator of *Bauhinia forficata* pollen viability. The reaction intensity was low in all analyses, showing weak pollen coloration, which made it difficult to differentiate between viable and unviable pollen. On the other hand, Santos et al. (2006) and Bolat & Pirlak (1999) obtained satisfactory results using Safranin for other plant species, even if they did not indicate it as the most effective dye.

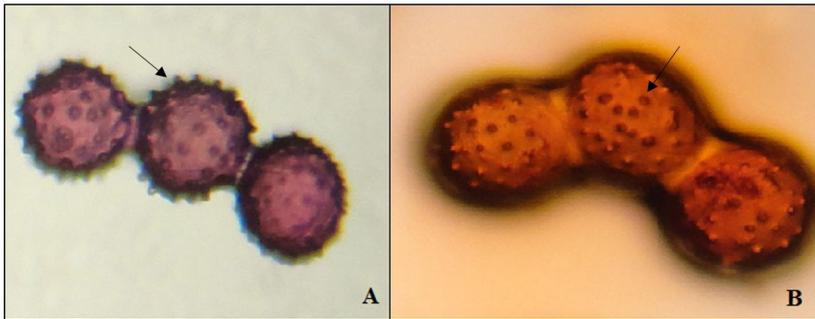


Figure 1. Viable pollen grains stained with Basic Fuchsin (1%) (A) highlight the structure of pollenkitt - and pollen grains stained with Methyl Red; (B) highlight the structure of pollenkitt.

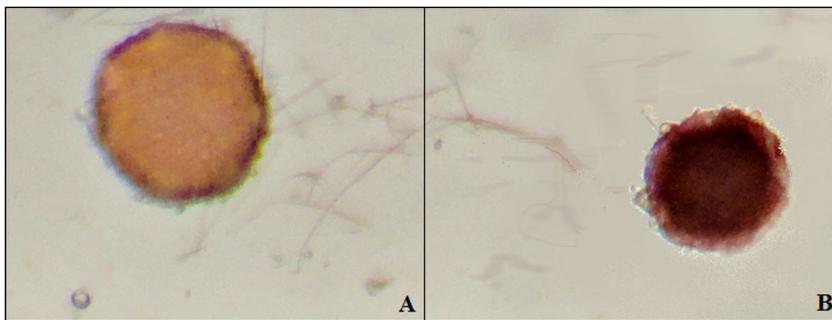


Figure 2. *Bauhinia forficata* pollen grain stained with 2,3,5- Triphenyltetrazolium Chloride (TTC): viable pollen (A) and unviable pollen (B).

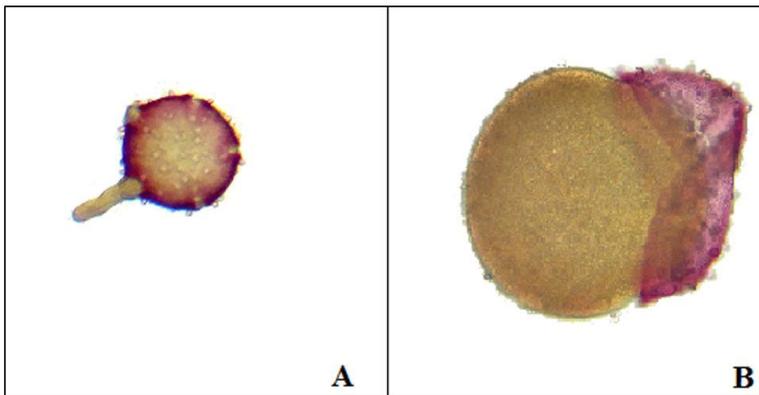


Figure 3. *Bauhinia forficata* pollen grain stained with Basic Fuchsin (1%): germinated Pollen (A); ruptured cell wall (B).

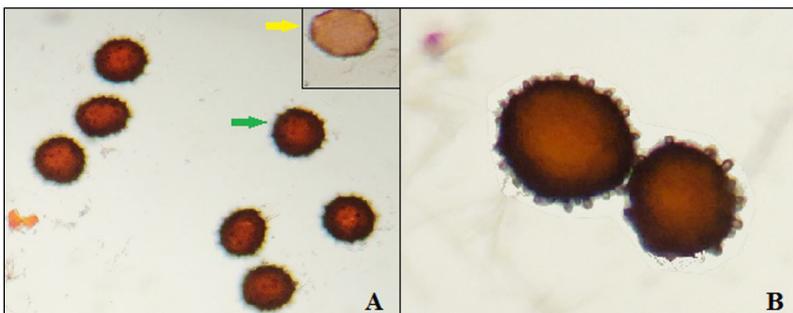


Figure 4. *Bauhinia forficata* pollen grains stained with Methyl Red (A) (green arrow highlighting viable pollen grain and yellow arrow highlighting unviable pollen grain); Highlighted for the pollenkitt structure (B).

A significant difference in pollen viability between the matrices was observed through applying the Kruskal-Wallis test ($p = 0.05$) (Table 1); however, the origin of this variation is difficult to determine, since genetic, biotic and/or abiotic factors may be associated.

No correlation ($r_{\text{pearson}} = 0.3001$), or causal relationship ($r^2_{\text{ajus}} = 0.05$) was found between pollen viability and flowering time (Figure 5). In addition to genetic factors of the species, high viability rates may have been obtained due to the form of storage (in a solution with a storage temperature of approximately $-18\text{ }^\circ\text{C}$), since there are reports of reduced pollen viability from desiccation processes and maintenance of viability with storage at low temperatures (Pio et al., 2007).

The time and form of storage can significantly reduce pollen viability. For example, in analyzing the storage

and pollen viability of Citrus cultivars for 9 weeks, Pio et al. (2007) found that pollen grain viability diminished with increased storage time. However, the data obtained for *Bauhinia forficata* show that the pollen of the species remains viable even after long storage periods (9 months in this case) and that the viability rate is high for all matrices, even throughout the flowering period.

There was a significant difference (Kruskal-Wallis, $p = 0.05$) between the basic Fuchsin and Methyl Red dyes; however, they did not differ statistically from the TTC dye, which even presented the highest viability rates amongst the results. Therefore, it is not possible to state that some of the dyes used overestimated the pollen viability of the species. Nevertheless, many authors cite the tendency of colorimetric tests to overestimate

Table 1. Pollen viability in *Bauhinia forficata* in the central region of Rio Grande do Sul.

MATRIX	POLLEN VIABILITY (%)	STATISTICAL RANKING*
M1	81.14	abc
M2	83.26	abc
M3	71.42	bc
M4	87.76	a
M5	86.94	abc
M6	82.98	abc
M7	83.77	abc
M8	83.48	abc
M9	87.63	ab
M10	78.68	abc
M11	64.31	c

*Values followed by the same letter do not differ statistically from one another by the application of Kruskal-Wallis test, at 5% level of significance.

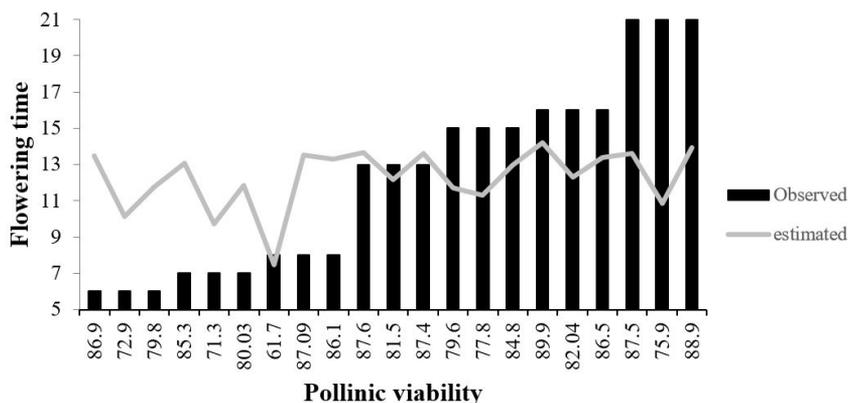


Figure 5. Correlation (cause and effect) between pollen viability and flowering time in *Bauhinia forficata*, demonstrating the viability observed throughout flowering (bars) and the viability expected by regression analysis (row) ($r^2_{\text{ajus}} = 0.05$).

the pollen viability of the species, suggesting that they be combined with other determination methods such as *in vitro* germination tests, tests of percentage and meiotic stability, amongst others, aiming to eliminate the intrinsic limitations of each method chosen (Munhoz et al., 2008; Arenas-de-Souza et al., 2014; Nascimento et al., 2014; Davide et al., 2009).

It should also be noted that the TTC dye, among those used in this study, is the only one that presented positive staining for metabolic activities of the pollen grains (active dehydrogenases enzymes) and not only for the constituent's integrity.

None of the floral buds isolated from the action of biotic vectors presented fruit formation, having flowered at the same time and with the same duration as the other floral buds of the treetop, demonstrating the inability of the species to perform autogamy. This characteristic is preferable in plant species because it decreases the chances of genetic drift and consequently local extinction risks (Rech et al., 2014). However, autogamy, apomixis, vegetative or asexual reproduction may be advantageous in species adapted to extreme environmental conditions or in species colonizing new areas. Even in these cases, where the degree of inbreeding is also high, events such as crossing over or somatic changes occur at random, thus ensuring both the genetic stability of the population and consequent local perpetuation as a degree of genetic variability that allows reproductive flexibility (Rech et al., 2014).

The extreme behavior in the induction of genic flow in reproductive systems would in theory be dioecious, where the pollination process always results in genic flow, since inbreeding is virtually impossible (Oliveira & Maruyama, 2014). Nevertheless, any type of separation of sexual functions in plants, whether functional, structural, temporal, etc., would result in greater genic flow in the population and could characterize adaptive advantages in the species (Oliveira & Maruyama, 2014).

Despite all this evidence, hermaphrodite flowers such as *Bauhinia forficata* are common in the plant kingdom and characteristic of most angiosperms on the planet. Studies related to the evolution of floral systems indicate that unlike in the most common hypothesis where the existence of hermaphrodite flowers results in higher self-pollination rates, hermaphroditism in plant species emerged as a strategy to increase gene flow (Allen & Hiscock, 2008).

The Allen & Hiscock (2008) statement can be evidenced in this experiment, because the impediment of pollen biotic vectors through isolation with Voil bags does not preclude the wind transporting pollen grains from nearby flowers in the same individual to the stigma of the flower (a process denominated geitonogamy). However, *Bauhinia forficata* did not present fruit formation in the isolated floral buds, indicating that in addition to the inability to perform autogamy, the species also presents some mechanism of genetic incompatibility that prevents reproduction by geitonogamy, and consequently pollination between individuals with a high degree of kinship, thereby reducing the inbreeding of the populations.

Inability to perform geitonogamy is especially important to ensure genetic variability in species that have vegetative propagation such as *Bauhinia forficata*, which emits shoots from the root, and therefore forms genetically homogenous groups or with a high degree of kinship. Moreover, if confirmed, this would represent an important ecological adaptive mechanism of the species and would consolidate it as a key species for biodiversity reintroduction processes, being necessary with further studies on the sexual system(s) of the species with the objective of testing the above mentioned hypothesis.

4. CONCLUSION

The species presented an inability to perform autogamy. The pollen viability of *Bauhinia forficata* was considered high, independent of the dye used for the cytochemical tests.

The dyes used were able to clearly distinguish the viable pollen grains from the unviable ones, with the exception of the Safranin dye, with its use not being recommended to determine pollen viability for this species.

Due to the ability to detect enzymatic activity involved in the respiration process, the use of TTC dye to determine pollen viability of the species is recommended, however, it was noted that Methyl Red and Basic Fuchsin dyes also presented satisfactory results.

Optimization of strategies to encourage cross-breeding is evident, even with the existence of hermaphrodite flowers in *Bauhinia forficata*, such as: characteristics related to attraction of visitors, supply of floral resources, large amounts of pollen, high pollen viability and the

inability to perform autogamy, show that the species developed mechanisms to control and induce genetic flow, and thereby increase pollination quality.

ACKNOWLEDGEMENTS

FAPERGS for granting the scholarship to the first author of this article and to Mr. Oswaldo Carlesso and his wife for making the study area available.

SUBMISSION STATUS

Received: 11 June, 2017

Accepted: 26 July, 2017

CORRESPONDENCE TO

Luana Camila Capitani

Departamento de Ciências Florestais, Centro de Ciências Rurais, Universidade Federal de Santa Maria – UFSM, Rua João Costa, 490, Bairro Camobi, CEP 97105-200, Santa Maria, RS, Brasil e-mail: lumilacapitan@gmail.com

FINANCIAL SUPPORT

Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul – FAPERGS.

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