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ARTICLE

Cytogenetic characterization of *Angelonia integerrima* Sprengel, a native species with ornamental potential

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Abstract: *Angelonia integerrima Sprengel is a native species of the state of Mato Grosso do Sul and the Southern region (Paraná, Santa Catarina and Rio Grande do Sul) of Brazil, with features such as an unusual appearance and color of the flowers, indicating an ornamental use. To optimize the use of this species and to fill in gaps regarding its cytogenetic characterization, this study determined the chromosome number, meiotic index and pollen viability of plant individuals of four A. integerrima populations. All plant individuals of the four populations had 2n = 20 chromosomes. Still, the meiotic index of most analyzed plant individuals exceeded 90%, while pollen viability of all plant individuals was higher than 80%. These data suggest considerable cytological stability of the analyzed A. integerrima plant individuals, which may favor the selection of future genotypes for commercial purposes or their use in conservation and breeding programs of the species.*

Keywords: *Caracol-do-campo, chromosome number, meiotic index, pollen viability, plant genetic resources.*

INTRODUCTION

Angelonia integerrima Sprengel, popularly known as Angelonia, caracol-do-campo or violeta-do-campo in Brazil, is a native species that occurs in stony fields and rocky outcrops in Mato Grosso do Sul and the Southern Region (Paraná, Santa Catarina and Rio Grande do Sul) of Brazil, and close to the Brazilian borders of Paraguay and Argentina (Souza and Giulietti 2009).

This species has a considerable ornamental potential in view of features such as herbaceous growth, the contrast caused by the color difference between stem and leaves, inflorescences with flowers at different maturation stages (increasing the duration of flowering), aside from the peculiar aspect of the flowers. It can be cultivated in flowerbeds, forming clumps, as well as in rock gardens, flower planters and even pots (Stumpf et al. 2009).

Although the Brazilian flora presents a great biodiversity, including species with ornamental characteristics such as *A. integerrima*, the number of native species cultivated commercially is still derisory (Heiden et al. 2006). However, a reduction or even replacement of exotic ornamental species by native ones is a trend that has been consolidating in the modern landscaping (Heiden et

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al. 2007), emerging as a new niche in the floriculture market, with great potential of production and commercialization (Oliveira Junior et al. 2013).

However, to introduce *A. integerrima* into cultivation, some aspects must be known. Among these, the cytogenetic characterization is essential, for providing a foundation for related studies that contribute both to the use of native material with economic potential and to the preservation of the species germplasm (Valls 2007). Moreover, the information generated may provide a basis underlying breeding, with a view to developing the commercially most promising material (Cruz et al. 1993).

The knowledge about the chromosome number of a species provides data that contribute to phylogeny and taxonomy, as well as helping to determine the most appropriate procedures to obtain hybrids in breeding programs (Heslop-Harrison 2000, Auler et al. 2006). While the analysis of the meiotic index and pollen viability allow obtaining information on genetic variability, sterility problems and possibilities of crosses (Souza et al. 2004).

The cytogenetic characterization of the genus *Angelonia* is doubtful, since only chromosome numbers are reported for some species such as *A. grandiflora* C. Morr. (Raghavan and Srinivasan 1940), *A. salicariaefolia* Humb. & Bonpl. (Chandran and Bhavanandan 1983), *A. cubensis* (var *alba*) (Subramanian and Pondmudi 1987), *A. gardneri* Hook. (Molero et al. 2006) and *Angelonia* spp. (hybrid) (Plaschil and Olbricht 2008). In these studies, the chromosome number of the species was n = 10 or 2n = 20, suggesting that x = 5 or x = 10 is the basic chromosome number of the genus. Only one study in the literature determined the chromosome number of *A. integerrima,* based on a single Paraguayan population, as 2n = 20 chromosomes (Molero et al. 2006).

Thus, to deepen the knowledge about the basic aspects of the species *A. integerrima*, which could contribute to enhance conservation strategies and underlie future breeding studies, this study addressed the description of the species in terms of chromosome number, meiotic index and pollen viability of different populations of the state of Rio Grande do Sul (RS), Brazil.

MATERIAL AND METHODS

The analyses were carried out at the Laboratory of Cytogenetics of the Department of Forage Plants and Agrometeorology (DPFA) and the Laboratory of Biotechnology of the Department of Horticulture and Forestry (DHS), at the Faculty of Agronomy of the Federal University of Rio Grande do Sul (UFRGS) in Porto Alegre, RS, Brazil.

Plant material

The plant material used included flower buds and seeds of four natural populations, resulting in a total of 44 plant individuals collected in 2016 and 2017, in localities of the state of Rio Grande do Sul (RS).

The populations with the respective geographic coordinates are listed below: Morro do Osso (MO), Porto Alegre, RS (lat 30º 07’ 77” S, long 51º 14’ 87” W, alt 143 m asl); Morro Santana (MS), Porto Alegre, RS (lat 30º 02’ 14” S, long 51º 06’ 33” W, alt 311 m asl); Barão do Triunfo (BT), Barão do Triunfo, RS (lat 30º 21’ 36” S, long 51º 43’ 55” W, alt 401 m asl) and Parque Saint’Hilaire (PSH), Viamão, RS (lat 30º 5’ 42.72” S, long 51º 5’ 9.36” W, alt 114 m asl). The regional climate is Cfa, i.e., humid subtropical (Pessoa 2017). The sites where the species was found were composed of native grassland areas characteristic of the Pampa biome.

Chromosome number

To determine the chromosome number, seeds of 29 *Angelonia integerrima* plant individuals, previously refrigerated (4 to 6 ºC), were sterilized in 70% alcohol and 1% sodium hypochlorite followed by triple washing with distilled and autoclaved water. Thereafter, they were placed to germinate in Petri dishes double-lined with pH-neutral filter (Germitest®) paper moistened with distilled and autoclaved water. Then, the dishes were placed in a growth chamber, at a mean temperature of 25 °C ± 2 and photoperiod of 16 h light. When the radicles reached an approximate length of 0.5 cm (seven days after sowing), they were pre-treated with 0.002M 8-hydroxyquinoline for 23 h 30 min at 4 ºC in a refrigerator. To determine the adequate pretreatment, preliminary tests were performed. After this step, the roots were fixed in Carnoy 3:1 (absolute ethanol/glacial acetic acid v/v) for a period of 24 h at room temperature and stored

in 70% ethanol under refrigeration until further analysis. To analyze the mitotic metaphases, the radicles were washed in distilled water, hydrolyzed in 1N hydrochloric acid at 60 °C in a water bath for 10 min and rinsed again in distilled water. Thereafter, the rootlets were stained with Feulgen for about 12 h. For the slides, root tips (meristematic region) were ground in 2% propionic carmine, covered with coverslips and sealed with nail polish.

For each plant individual, 10 - 20 cells were analyzed in mitotic metaphases, with good chromosome distribution and degree of contraction. The analyses were performed with a ZEISS microscope, coupled with an AxioCam ERc5s camera. The best metaphases were photographed and the images edited in the Adobe Photoshop program.

Meiotic index and pollen viability

Due to the development stage of the bud at collection, only the pollen grain viability could be analyzed in some plant individuals (44 plants), while in others (19) the final microsporogenesis products were also analyzed.

After collection, the flower buds at different development stages were immediately fixed in Carnoy 3:1 (absolute ethanol/glacial acetic acid v/v) for 24 h at room temperature. Subsequently, they were transferred to 70% ethanol and refrigerated until use. For the slides, the floral buds were dissected and the anthers removed, stained with 2% propionic carmine and crushed with a glass stick, by the methodology of Guerra and Souza (2002).

To determine the meiotic index, a mean of 310 tetrads per plant individual were analyzed. Tetrads with four cells (quartet) were considered normal, while the abnormal had other numbers (two, three or more). The meiotic index (MI) was calculated by the formula: MI = [number of normal tetrads/total number of tetrads (dyads, triads, tetrads, polyads] observed] x 100 (Love 1949).

To estimate the pollen viability, more mature flower buds were used, and the slides were also prepared by the methodology of anther squashing. Two dyes were used for pollen grain staining: 2% propionic carmine and Alexander staining. When using 2% propionic carmine dye, the fully stained pollen grains were considered viable and the unstained or weakly stained unviable. When using Alexander staining, purple pollen grains were considered viable and blue-green grains unviable, and the slides were analyzed 24 h after preparation for a better reaction of the dye. Two slides per plant individual and dye were prepared, resulting in a total of 2000 pollen grains per plant individual (500 per slide). Pollen viability was estimated by the percentage of viable grains, dividing the number of grains considered viable by the total number of grains multiplied by 100.

For each population, the longitudinal and transverse axes of 10 plant individuals (10 grains per plant individual) were measured, except for the populations of Barão do Triunfo and Parque Saint-Hilaire, in which the pollen grains of three plant individuals (all plant individuals of both populations) were measured. The measurements were performed with an ocular micrometer coupled to an optical microscope.

Meiosis and pollen viability were also analyzed under a ZEISS microscope, coupled with an AxioCam ERc5s camera, and the photomicrographs edited, using Adobe Photoshop.

Statistical analysis

Data were analyzed by descriptive statistics (mean and standard deviation). For pollen viability means between populations, plant individuals and dyes, the data did not meet the assumptions of analysis of variance, even after transformations, and were therefore subjected to non-parametric analysis by the Kruskal-Wallis test, followed by means comparison by Dunn's test, using software SigmaPlot 11.0.

RESULTS AND DISCUSSION

Chromosome number

This is the first study to determine the somatic chromosome number of a large number of plant individuals of four *Angelonia integerrima* populations (Figure 1a). A chromosomal number of 2n = 20 was found for all plant individuals of the four populations (Table 1). This result agrees with reports of Molero et al. (2006) for a population of *A. integerrima* in Paraguay. However, it is emphasized that the photomicrographs of the chromosomes of the species presented in this

Table 1. Populations of *Angelonia integerrima* Spreng., number of plants and cells analyzed and respective chromosome number.

Population (acronym)	**Number of plant individuals** (number of cells analyzed)	**Chromosome number** (2n)
Morro do Osso (MO)	12 (160)	2n = 20
Morro Santana (MS)	13 (130)	2n = 20
Barão do Triunfo (BT)	01 (20)	2n = 20
Parque Saint'Hilaire (PSH)	03 (40)	2n = 20

study are new and unpublished (Figures 1b, 1c and 1d). As the number of chromosomes found in the four analyzed *A. integerrima* populations is the same (2n = 20), analogous to that of the Paraguayan population (Molero et al. 2006), it is assumed that there is probably no intraspecific variability.

Studies with other *Angelonia* species indicate that there is no intrageneric variation affecting the chromosome number either, at least with regard to the cultivated species: *A. grandiflora* C. Morr. (Raghavan and Srinivasan 1940), *A. salicariaefolia* Humb. & Bonpl. (Chandran and Bhavanandan 1983), *A. cubensis* (var *alba*) (Subramanian and Pondmudi 1987), *A. gardneri* Hook. (Molero et al. 2006), and *Angelonia* spp. (Hybrid) (Plaschil and Olbricht 2008). In these studies, the species had n = 10 or 2n = 20 chromosomes, suggesting that x = 5 or x = 10 is the basic chromosome number of the genus.

With regard to the small sample size of the population of Barão do Triunfo (one plant individual with seeds), it is emphasized that this population is known to have comprised several *A. integerrima* plant individuals a few years ago, however, with the intensive conversion of native field areas into pastures and monocultures, the population was drastically reduced. Thus, this study reinforces the importance of describing this species, as a contribution to the conservation of the still existing germplasm.

Due to the small chromosome size of *A. integerrima* (< 2 µm), as also reported for other species of the genus (Raghavan and Srinivasan 1940), the centromere could not be accurately identified, nor the arm length measured for karyotype assembly.

According to Guerra (1990), the analysis of several populations of a same species can provide information about the cytological stability of the species, as observed in *A. integerrima,* and on the existence of intraspecific variability or even subspecies. It is worth remembering that the chromosome number of a species must be determined in several plant individuals and populations, and voucher specimen of the analyzed material should be deposited in a herbarium, underlying the identification of the species under study (Castro et al. 2006).

Meiotic index and pollen viability

Data resulting from the analysis of microsporogenesis end products indicated a high percentage of normal tetrads, with meiotic index average of 92.9% (Table 2). In this study, some irregularities such as dyads, triads and polyads (Figure 2a, 2b, and 2c) were observed, although the number of normal tetrads (2d) was the highest.

There are no literature reports on the chromosome behavior at the end of meiosis and/or on the pollen viability of *A. integerrima*. For the genus *Angelonia*, Molero et al.

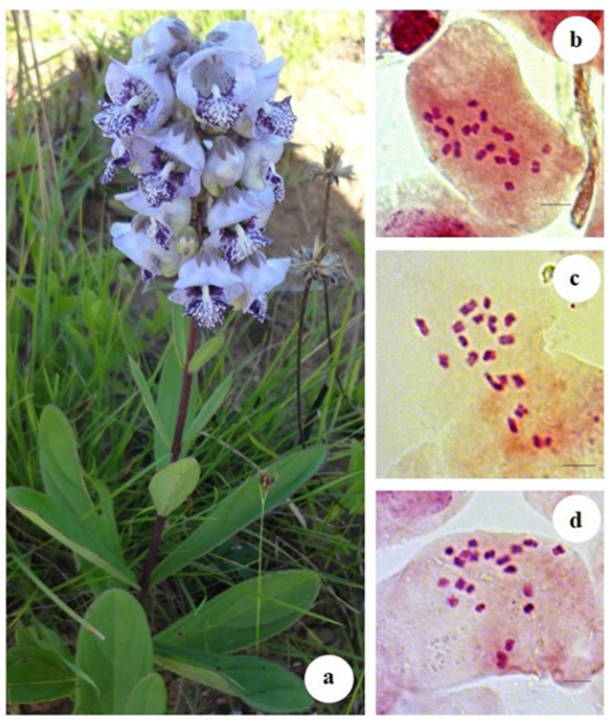

Figure 1. (a) General aspect of *Angelonia integerrima* Spreng. in natural environment (Parque Saint-Hilaire); (b - d): Mitotic metaphases with 2n = 20 chromosomes of the populations from Morro do Osso, Morro Santana and Parque Saint-Hilaire, respectively.

(2006) studied the meiotic behavior of *A. gardneri*, identifying normal chromosome segregation, with few irregularities. Ten bivalents (n = 10) in diacinesis/metaphase I were found.

A single plant individual presented had a low meiotic index (64.15% - data not shown), indicating that a series of irregularities may have occurred during meiosis, resulting in gamete imbalance. The same plant individual also had the lowest percentage of pollen viability (82.3% - data not shown). As pollen viability is directly related to gamete formation during meiosis (Diegues et al. 2015), it is expected that a plant with a low meiotic index will also have a lower pollen grain viability.

In addition, in relation to the meiotic index, the values of the other individuals analyzed were on average greater than 90%. (Table 2). According to Love (1949), plants with a meiotic index of more than 90% are considered to be cytologically stable and can therefore be included in breeding programs and used for species preservation.

Despite the simplicity of the parameter, the meiotic index is a commonly used indicator of regularity in studies on the meiotic behavior of plants (Love 1949). Consequently, this index is also widely used to identify the best genotypes for inclusion in breeding and conservation programs, particularly with regard to native species, which have been increasingly addressed by specific studies in the last two decades.

The high meiotic indices found here, indicating the regularity in the meiotic behavior of *A. integerrima*, corroborate the statement of Füller et al. (2015) that high-frequency meiotic irregularities in natural populations are not a common phenomenon. This was also observed in studies with natural populations of *Elionurus muticus* (Spreng.) Kuntze (Füller et al. 2015), *Bidens pilosa* L. (Fachinetto et al. 2008), *Baccharis trimera* (Less.) DC. (Auler et al. 2006), and *Eugenia involucrata* DC. (Guerra et al. 2016), which are also native species of the South-Brazilian flora.

Pollen viability was analyzed in 44 plant individuals of four *A. integerrima* populations. The mean value of pollen viability was used as the carmine propionic dye was of 96.1 and with the alexander staining was of 96.5 (Table 2). The statistical analysis showed no significant difference when comparing pollen viability between populations and between dyes (p-value for populations: 0.644; p-value for dyes: 0.378). However, a significant difference (p-value: <0.001) was observed among individuals within the populations, which indicates that the species may present a reasonable genetic variability for the characteristic pollen viability under the conditions of this study. This difference can also be explained by environmental factors such as temperature, humidity and pollen stage (Kelly et al. 2002), since the samplings were carried out on days with different climatic conditions.

Information on pollen viability and morphology are scarce for both the genus *Angelonia* and the family Plantaginaceae. Thus, our results constitute important information for the characterization of this taxon. The pollen grains of *A. integerrima* are predominantly rounded, with a mean polar axis (P) length between 18.33 and 20.86 µm and equatorial axis (E) between 18.67 and 20.86 µm. Compared to the pollen grains of other herbaceous species, such as *Sisyrinchium micranthum* Cav. (Tacuatiá et al. 2012), with an equatorial axis length of grains between 29.71 and 36.35 µm and polar axis between 25.19 and 30.34 µm, the *A. integerrima* pollen grains are considered small.

The pollen viability was tested with two dyes, propionic carmine and Alexander staining, since some authors claimed that their efficiency is different, for staining the pollen grains differently (Auler et al. 2006, Coelho et al. 2012, Hister and Tedesco 2016). While propionic carmine reddens viable grains and leaves unviable grains unstained (Figure 2e),

Table 2. Final meiotic products, meiotic index (%) and mean of pollen viability of four populations of *Angelonia integerrima* Spreng.

Population (acronym)	**Number of individuals**	**Microsporogenesis products** (total)		**Meiotic index** (MI %)	**Pollen viability** (%)	
		Regular (tetrad)	**Irregular (dyads, triads, polyads)**		**Propionic carmine**	**Alexander staining**
Morro do Osso (MO)	20	3174	197	94.1^{ns}	95.4^{ns}	95.9^{ns}
Morro Santana (MS)	18	2184	166	92.9	96.2	96.7
Barão do Triunfo (BT)	03	471	34	93.2	95.7	96.1
Parque Saint-Hilaire (PSH)	03	66	6	91.7	97.2	97.3
Mean	-	1473.5	100.7	92.9	96.1	96.5

$^{ns:}$ no significant differences in average (5% probability) by Dunn's test.

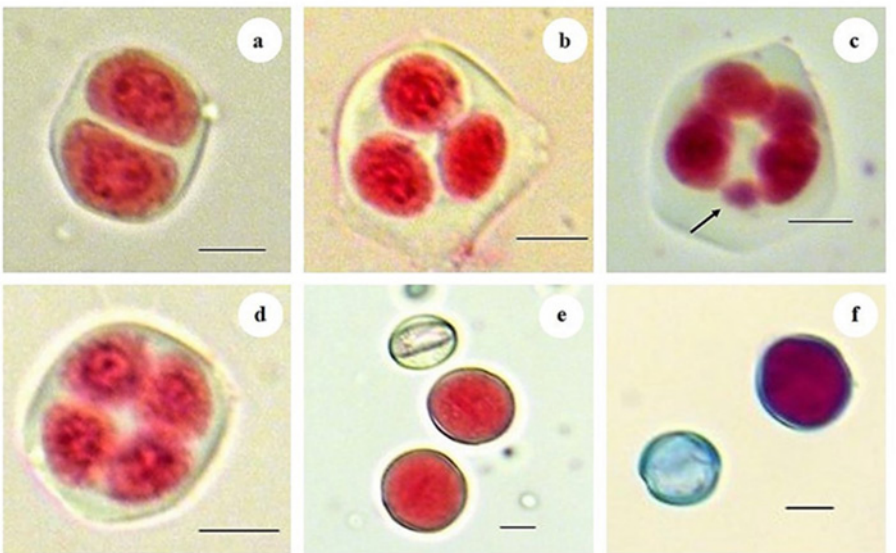

Figure 2. Pollen grains and microsporogenesis end products of *Angelonia integerrima* Spreng. (a) dyad; (b) triad; (c) polyad with microcyst (arrow); (d) normal tetrad; (e) pollen grains stained with propionic carmine: viable (stained red) and unviable (unstained); (f) pollen grains stained with Alexander staining: viable (stained purple) and unviable (stained blue). Scale: 10 µm.

the Alexander staining, which contains the dyes fuchsin and malachite green, produces a differential staining between viable and unviable pollen grains. The cellulose in the pollen cell wall reacts with malachite green to produce a blue-green color, while the protoplasm reacts with acid fuchsin to produce a purple color (Alexander 1980). Thus, as unviable grains have no protoplasm, they have a blue-green color, while viable grains have a blue-green cellulose wall and purple protoplasm (Figure 2f).

Nevertheless, the means of pollen viability, determined with propionic carmine and Alexander staining, were not significantly different in this study (Table 2), indicating that both are efficient to estimate the viability of *A. integerrima* pollen grains.

In a study with *Citrus* species, Moreira and Gurgel (1940) defined that pollen viability above 70% can be considered high. Based on this definition, high pollen viability was detected in all plants analyzed of both populations. Parallel to the meiotic indices found, a high percentage of viable pollen grains is expected in view of the high percentage of normal tetrads, which are a direct reflection of a regular meiotic process (Corrêa et al. 2005), as pointed out in this study. If the process of megasporogenesis also occurs regularly, the seed production of these plants will be problem-free and the viability good, recommending their use in breeding programs.

In addition, it is emphasized that several authors, such as Krycki et al. (2016), Costa et al. (2018), Fachinetto et al. (2018), Tolomeotti et al. (2018) and Silva et al. (2018), have carried out studies involving native species of economic importance, such as the present study.

CONCLUSION

The somatic chromosome number of all plant individuals of the four analyzed populations of *A. integerrima* is 2n = 20. The meiotic index is superior to 90% in most of the analyzed plant individuals, whereas pollen viability exceeded 80% for all plant individuals. These data suggest a regular microsporogenesis process of the analyzed plant individuals.

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