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POLLEN VIABILITY AND MEIOTIC IRREGULARITIES IN A COLLECTION OF *Stevia rebaudiana* (BERTONI) BERTONI CULTIVATED IN TUCUMÁN, ARGENTINA

VIABILIDAD DE POLEN E IRREGULARIDADES MEIÓTICAS EN UNA COLECCIÓN DE *Stevia rebaudiana* (BERTONI) BERTONI CULTIVADA EN TUCUMÁN, ARGENTINA

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ABSTRACT

Stevia rebaudiana (Bertoni) Bertoni (2n=2x=22) (Asteraceae family) is a species of economic value due to the presence of steviol glycosides in leaves -mainly stevioside and rebaudioside A- which are non-caloric sugars. In 2013, a collection of plants (genotypes) from four contrasting environments was established in Tucumán, Argentina, for evaluation under local conditions and, eventually, breeding purposes. As a first step, pollen viability and meiosis was studied in a sample of 56 plants. The percentage of pollen viability varied from medium (69.4%) to high (99.6%) in 52 of them, but from low (36.5%) to relatively low (51.5%) in the remaining four. The latter four plants also presented pollen grains of heterogeneous size, which were classified as n (normal, the most frequent size in the sample), <n and >n. Abnormalities were observed in meiosis and at the tetrad stage. Pollen viability appears not to be a problem for planning crossing experiments within the collection. Moreover, the observation of parallel spindles at Anaphase II and dyads and triads at the tetrad stage entails the possibility of eventually exploring ploidy manipulations in breeding.

Key words: *Stevia*, pollen viability, meiosis, sporads

RESUMEN

Stevia rebaudiana (Bertoni) Bertoni (2n=2x=22) (familia Asteraceae) es una especie de valor económico debido a la presencia de glucósidos de esteviol en sus hojas, principalmente esteviósido y rebaudiósido A, que son azúcares no calóricos. En 2013, se estableció una colección de plantas (genotipos) provenientes de cuatro ambientes contrastantes en Tucumán, Argentina, para su evaluación en condiciones locales y, eventualmente, con fines de mejoramiento genético. Como primer paso, se estudió la viabilidad del polen y la meiosis en una muestra de 56 plantas. El porcentaje de viabilidad del polen fue de medio (69,4%) a alto (99,6%) en 52 de ellas, pero de bajo (36,5%) a relativamente bajo (51,2%) en las cuatro restantes. Las últimas cuatro plantas también presentaron granos de polen de tamaño heterogéneo, que se clasificaron como n (normal, el tamaño más frecuente en la muestra), <n y >n. Se observaron anomalías en la meiosis y en el estadio de tétrada. La viabilidad del polen no parece ser un problema para planificar cruzamientos experimentales en la colección. Además, la observación de husos paralelos en Anafase II de díadas y tríadas en la etapa de tétrada conlleva la posibilidad de explorar, eventualmente, manipulaciones de ploidía en el mejoramiento de esta especie.

Palabras clave: *Stevia*, viabilidad de polen, meiosis, espórada

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INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni (2n=2x=22) belongs to the Asteraceae family. This species, native to the Amambay region of Northeastern Paraguay (Soejarto, 2002; Brandle and Telmer, 2007), was introduced for cultivation in Argentina by the end of the XXth century. Its leaves accumulate steviol glycosides, mainly stevioside and rebaudioside A, which are non-caloric natural sugars widely used in food products and as dietary supplements in many countries (Kinghorn and Soejarto, 1985; Brandle and Rosa, 1992; Brandle and Telmer, 2007). Several of the widely cultivated lines of *S. rebaudiana* were experimentally obtained at the University of New Mexico, USA, in a program designed to develop polyploids richer in stevioside than their diploid counterparts (Valois, 1992). In Brazil, triploid and tetraploid lines from those experiments were initially cultivated at the CENARGEN/EMBRAPA germplasm bank before the transference of part of the collection to CPQBA/UNICAMP (Oliveira *et al.*, 2004). In Argentina studies on glycoside content, plant morphology and reproductive biology in different lines or varieties were carried out by Kolb *et al.* (2001), Kryvenki *et al.* (2007), Taiariol and Molina (2010), Guerrero *et al.* (2015) and Caponio *et al.* (2016).

Stevia rebaudiana has a complex reproductive system, which is not completely understood. Its flowers are hermaphrodite, and also protandry has been reported, thus, cross-pollination appears to be the prevalent mode of sexual reproduction (Yadav *et al.*, 2014; Caponio *et al.*, 2016; Benelli *et al.*, 2017). In addition, a sporophytic homomorphic autoincompatibility system would further promote cross-pollination (Monteiro, 1980; Brandle *et al.*, 1998; Yadav *et al.*, 2014; Caponio *et al.*, 2016). For commercial production, however, it is reproduced agamically via stem cuttings (Kryvenki *et al.*, 2007; Tamura *et al.*, 1984a; b) or micropropagation (Miyagawa *et al.*, 1986; Swanson *et al.*, 1992; Akita *et al.*, 1994; Constantinovici and Cachita, 1997) due the poor and highly variable percentage of viable seed production (Monteiro, 1980; Duke, 1993; Carneiro *et al.*, 1997).

Besides the described sexual and agamic reproduction, in embryological studies Monteiro (1980) observed that *S. rebaudiana* can also reproduce apomictically. Along with agamospermy, this author reported the production of viable pollen in sexual plants and sterile pollen in the apomictics, as well as relatively low (65%) pollen viability in three batches of 10 plants each, introduced in Brazil from Paraguay in the 1960 and 1970 decades. Furthermore, Oliveira *et al.* (2004) only observed sterile pollen in diploid, triploid and tetraploid lines obtained from CENARGEN/EMBRAPA, in Brasilia. Caponio *et al.* (2016), however, reported pollen viability values between 94.8 and 97.9% in six plants from two geographic provinces of Argentina: Misiones (four ecotypes) and Entre Ríos (two ecotypes).

Pollen viability can be used as an indicator of regularities/irregularities in the nuclear division processes during male sporogenesis and/or gametogenesis. Low pollen viability in higher plants might have a genetic and/or an environmental base (Larrosa *et al.*, 2012; Farco and Dematteis, 2014). Thus, meiotic analyses can provide relevant information for both basic and applied studies (De Souza *et al.*, 2003), particularly for parental selection in breeding. In *S. rebaudiana*, Raina *et al.* (2013) observed mostly normal chromosome behavior in two morphotypes with low pollen viability, but they also observed a few univalents at diplotene and diakinesis in some meiocytes, which revealed synaptic problems underlying the sterility.

In 2013, a collection of *Stevia rebaudiana* was established in Tucumán, Argentina, to evaluate germplasm from four contrasting environments for adaptation to local conditions and, eventually, breeding purposes. The aim of this communication is to report the results of pollen viability and meiotic studies carried out in a sample of plants of this collection.

MATERIALS AND METHODS

In November 2013, 120 plants from four origins (30 plants/origin) were established in the experimental field at *Estación Experimental Agropecuaria Famaillá, INTA* (27° 00´ 49" S - 65° 22´ 32" W), in Tucumán province, Argentina. This locality has an average annual temperature of 20° C and an average annual precipitation of 965 mm. From this collection, 56 plants from the "criolla" landrace were included in the study, which were assigned the codes T (Tucumán), J (Jujuy), M (Misiones) and F (Formosa), according to their provenance, followed by consecutive numbers (Table 1). Plants were placed in the field, in furrows at a distance of 1 m between furrows and 0.4 m between plants; irrigation was provided by dripping as needed. The field grown plants were vegetatively propagated in September-October 2014 by stem cuttings, and the derived plantlets were cultivated in 2 l pots in the open, also with irrigation by dripping. Pollen viability was determined in 56 well-grown plants. Pollen samples were obtained from mature anthers prior to anthesis, from florets of 14 of the pot-grown plants per origin (56 plants in total) in February 2015. From each plant, four florets were analyzed, and significant differences between means were detected by Fisher´s Least Significant Difference (LSD) test at the 5% level with Infostat 2018 (Di Rienzo *et al.*, 2018).

Fresh pollen from five anthers per floret were placed on glass slides and stained with a 0.5% acetocarmine solution (0.5 g carmine, 45 ml glacial acetic acid and 55 ml distilled water, 50 ml glycerol) (Marks, 1954), for observation under an optical microscope at 125x. On average, 300 pollen grains per plant were observed. Pollen grains that were fully stained with well-defined contours were considered viable, whereas those that were poorly (or not) stained were considered sterile. In plants with pollen of heterogeneous size, 200 pollen grains per plant were measured with a micrometric eyepiece and, according to diameter, were classified as n (normal, most frequent diameter in the sample), <n and >n. Following Larrosa *et al.* (2012) and based on the volume of a sphere, pollen grains with diameters 1.26 times larger than the average pollen diameter of each sample were considered to be chromosomically unreduced (2n), and those with diameters 1.59 times larger were considered to be chromosomically double unreduced (4n).

Meiosis was studied in plants with low pollen viability (<52%) and heterogeneity in pollen size. To that end, immature flower buds at various developmental stages were fixed in absolute ethanol: glacial acetic acid (3:1, v/v) for 24 hs, and stored in 70% ethanol until use (Mok and Peloquin, 1975). Meiocytes were removed from anthers placed on a drop of 0.5% acetocarmine (0.5 g carmine, 45 ml glacial acetic acid and 55 ml distilled water) for staining, and observed with an optical microscope, starting from the most advanced stages and working backwards.

Table 1. Geographical provenance of the *Stevia rebaudiana* plants collected in Argentina.

Plant code	Locality, province	Geographic coordinates
T1.1, T2.12, T1.6, T1.2, T2.4	Las Mesadas, Tucumán	27º 06’ S 65º 30’ W
J3.16, J3.14, J3.2, J3.12, J3.15, J6.5, J6.16, J6.20, J8.9	Perico, Jujuy	24º 22’ S 65º 08’ W
M9.18, M11.7, M11.11, M11.6, M11.2	Cerro Azul, Misiones	27º 39’ S 55º 26’ W
F12.1, F12.3, F12.4, F12.11	Colonia Laishí, Formosa	26º 28’ S 58º 38’ W

RESULTS

Fifty-two out of the 56 analyzed plants had medium to high pollen viability (69.4 to 99.6%), whereas the remaining plants had low viability (36.5 to 51.5%) (Table 2). Plants with low to relatively low pollen viability (M10.8, M11.7, M11.11 and F12.1) also produced pollen grains of heterogeneous size (Table 3, Figure 1A, B).

Pollen grains were classified as n (mean diameter $\bar{x}$ =20.37 μ, the most common in the sample), >n ($\bar{x}$=33.83 μ) and <n ($\bar{x}$=11.55 μ) (Table 4). The mean diameter of the largest-sized pollen was 1.66 times that of the n pollen, and the mean diameter of the smallest-sized pollen was 0.57 times the diameter of the n pollen. Plant M10.8 had the highest percentage of <n pollen (24.0%), whereas M11.11 and M11.7 had the highest percentages of >n pollen (12.0% and 11.9%, respectively).

All plants produced normal tetrads; nevertheless, abnormalities were observed in the four plants with low pollen viability (Table 3). These abnormalities included monads in M10.8 (2.6%) and M11.7 (1%) (Figure 2A); dyads in M11.7 (20.8%), M11.11 (2%), and F12.1 (1.4%) (Figure 2B); and triads in M11.7 (0.9%), M11.11 (16%), and F12.1 (12.7%) (Figure 2C). Moreover, pentads were observed in M11.7 (0.9%) and M10.8 (0.5%).

The number of cells of the previously mentioned four plants (M10.8, M11.7, M11.11 and F12.1) analyzed at various meiotic stages and the irregularities observed are presented in Table 5. In Metaphase I, out of plate chromosomes (Figure 3A) were observed in 18.5% of the cells of M10.8, whereas in the other three plants, this percentage varied from 2.4 to 6.2%. In Anaphase I, laggards (Figure 3B) were observed in percentages varying from 2.7 to 7.9. Metaphases II (not included in the table) were normal, except for the presence of parallel spindles (Figure 3C) in F12.1. In Telophase II, 2.8 to 6.7% of the cells presented chromosomes grouped in three poles instead of four (Figure 3D).

Table 2. Percentage of pollen viability in individual plants of *Stevia rebaudiana.*

Plant	%	Plant	%	Plant	%	Plant	%
T1.1	98.0a	J3.12	98.2a	M9.1	99.1a	F12.1	51.5b
T1.2	89.8a	J3.14	97.4a	M9.4	88.5a	F12.2	97.1a
T1.5	94.0a	J3.15	83.8a	M9.8	95.6a	F12.3	98.5a
T1.6	98.6a	J3.16	97.7a	M9.12	97.8a	F12.4	97.1a
T1.8	87.3a	J3.2	99.4a	M9.18	98.3a	F12.5	98.7a
T2.2	97.0a	J4.3	98.8a	M10.2	78.5a	F12.6	87.8a
T2.4	95.6a	J5.5	98.8a	M10.4	98.9a	F12.7	99.3a
T2.5	99.5a	J6.16	99.0a	M10.6	89.7a	F12.8	99.3a
T2.10	92.4a	J6.20	96.8a	M10.8	36.5b	F12.10	98.4a
T2.12	99.2a	J6.5	98.7a	M10.10	95.5a	F12.11	99.6a
T2.15	95.5a	J7.5	94.3a	M11.2	98.5a	F12.12	98.3a
T2.16	97.3a	J8.12	69.4a	M11.6	98.0a	F12.13	95.5a
T2.18	79.4a	J8.7	98.4a	M11.7	38.4b	F12.14	79.6a
T2.4	95.6a	J8.9	98.5a	M11.11	38.1b	F12.15	92.4a

*Different letters indicate significant differences based on Fisher´s LSD (α=5%).

Table 3. Percentage of pollen according to size and sporad types in plants of *Stevia rebaudiana* with low pollen viability.

Plant	Pollen			Sporad Type (%)			
	Viability (%)	Size (%) < n	Size (%) > n	Monad	Dyad	Triad	Pentad
M11.7	38.4	2.59	11.94	1.0	20.8	21.3	0.9
M10.8	36.5	24.04	1.24	2.6	0.0	0.0	0.5
M11.11	38.1	0.0	12.0	0.0	2.0	16.0	0.0
F12.1	51.5	0.0	9.6	0.0	1.4	12.7	0.0

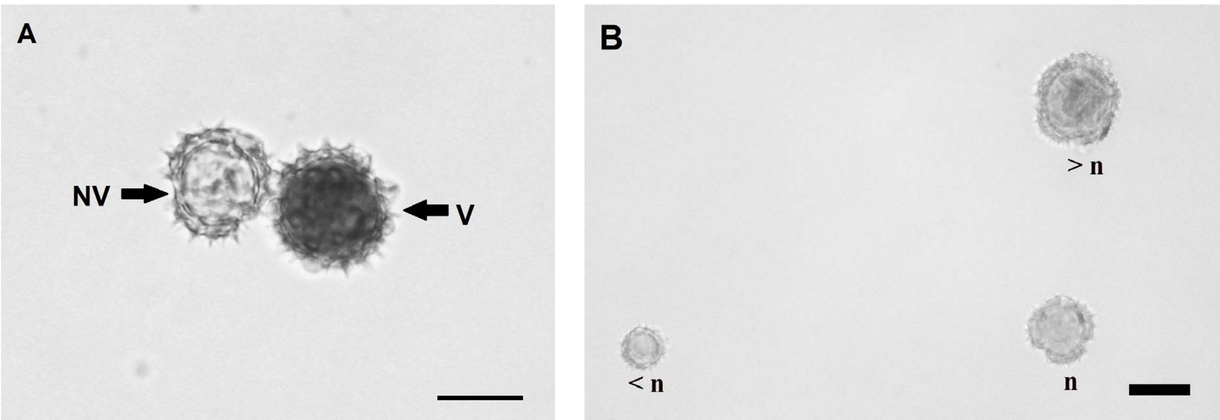

Figure 1. Variability in pollen grain viability and size: viable (stained), inviable (non-stained) (A); n, <n, >n, (B). Bar=20μ.

Table 4. Mean, minimum and maximum pollen diameter (μ) and standard deviations in *Stevia rebaudiana*.

Statistics	Pollen diameter (μ)		
	n	< n	> n
Mean	20.37	11.55	33.83
Minimun	18.80	8.48	30.78
Maximum	22.37	15.06	36.00
SD	1.02	2.52	1.32

SD: standard deviation.

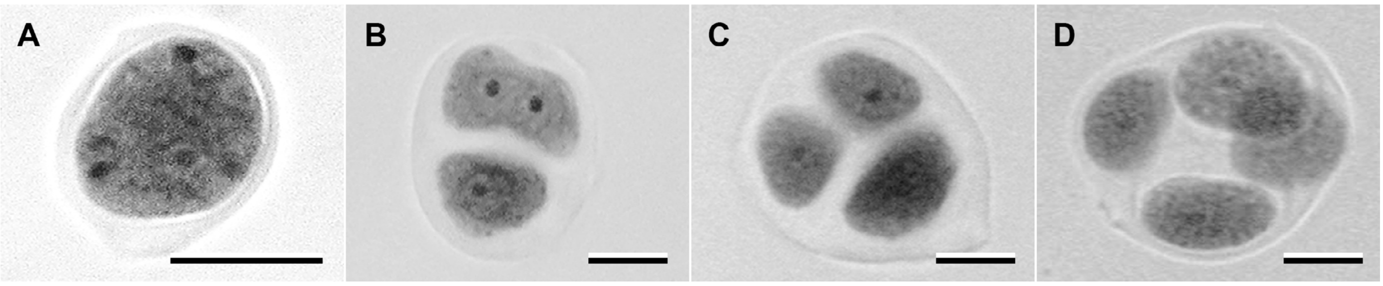

Figure 2. Normal and abnormal sporads in *Stevia rebaudiana*: monad (A), dyad (B), triad (C), normal tetrad (D). Bar=20μ.

Table 5. Percentage of meiotic abnormalities in plants of *Stevia rebaudiana* with low pollen viability.

Phase/Type	Plant			
	M10.8	M11.7	M11.11	F12.1
Metaphase I				
regular	81.54	93.8	97.65	92.7
out-of-plate chromosomes	18.46	6.2	2.35	3.3
Total # analyzed cells	65	130	170	60
Anaphase I				
regular	95	97.3	92.12	92.4
laggards	5	2.7	7.88	7.6
Total # analyzed cells	80	150	203	104
Telophase II				
regular	95.8	94.4	97.2	93.3
irregular	4.2	5.6	2.8	6.7
Total # analyzed cells	110	187	170	165

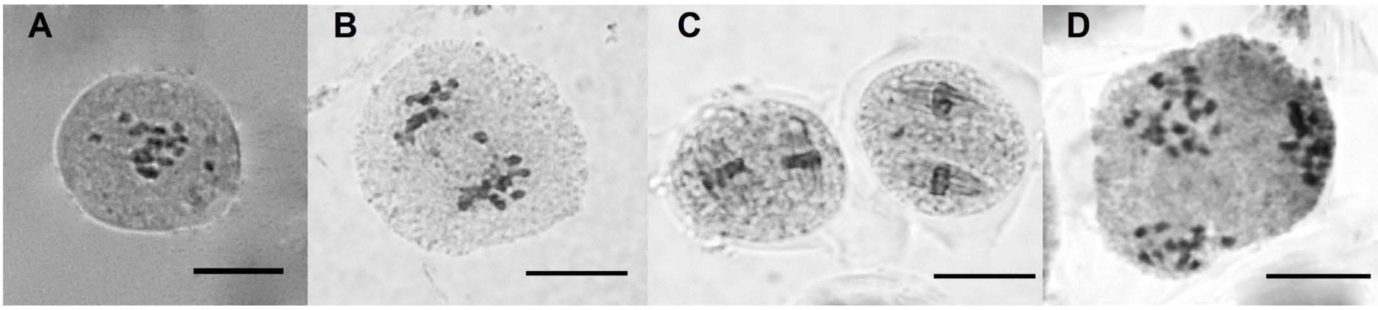

Figure 3. Meiotic abnormalities in *S. rebaudiana*: out of plate chromosomes at Metaphase I (A), laggards at Anaphase I (B), parallel spindles (der.) at Metaphase II (C), chromosomes grouped in three poles in Telophase II (D). Bar=20μ.

DISCUSSION

Most plants analyzed in this study (92.9%) had more than 85.0% pollen viability, thus, this factor is not expected to be a problem in planning crossing experiments with the *Stevia rebaudiana* collection established in Tucumán. However, abnormalities were detected in meiosis and at the tetrad stage in the other 8.0%, which could account for their low pollen viability.

Following Larrosa *et al.* (2012), 2n and 4n pollen grains were identified, along with n, <n and other >n pollen grains. The observation of monads, dyads and triads at the tetrad stage gave further support to this classification. Although there are reports on 2n pollen production in the Asteraceae family (*i.e.*, Noyes and Allison, 2005; Echeverría and Camadro, 2017), this is the first report on 2n and 4n pollen production in *S. rebaudiana*.

In Dicotyledons, the first meiotic division is not followed by cytokinesis; rather, two spindles are formed in the second division, which are oriented such that their poles define a tetrahedron; finally, simultaneous cytokinesis occurs yielding a tetrad of four n microspores. Abnormalities in spindle orientation and/or cytokinesis due to the action of meiotic mutants can lead to the formation of meiocytes with different numbers of microspores at the tetrad stage (Mok and Peloquin, 1975). In the present study, the presence of monads, dyads, triads, and parallel spindles in plants M10.8, M11.7, M11.11 and F12.1 allows the presumption of the presence of meiotic mutants. Similarly, the production of <n and other > n pollen grains could be due to the action of other types of mutants.

In summary, pollen viability was high in most of plants of the *S. rebaudiana* collection sample grown in Tucumán, but low pollen viability was also detected, which was underlined by meiotic irregularities. Some of these irregularities leading to 2n and 4n pollen formation are, presumably, under control of meiotic mutants. Thus, for breeding purposes, it is advisable to previously screen the collection for selecting fertile male parents. Moreover, it is feasible to explore heterotic responses for the traits of interest at higher ploidy levels by sexual polyploidization via 2n pollen.

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