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CHROMOSOMIC STUDIES IN *ZEPHYRANTHES CITRINA* BAKER (AMARYLLIDACEAE), A POLYPLOID ORNAMENTAL

ESTUDIOS CROMOSÓMICOS EN *ZEPHYRANTHES CITRINA* BAKER (AMARYLLIDACEAE), UN POLIPLOIDE ORNAMENTAL

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ABSTRACT

Zephyranthes citrina is an ornamental American bulbous plant used as an ornamental garden crop for the aesthetic qualities of its yellow perigonium. The objective of this work was to characterize the species by classical chromosome staining and fluorochrome banding. A sporophytic chromosome number of $2n=8x=48$ chromosomes was observed, being the karyotypic formula 20 m + 26 sm + 2 st. Satellites were detected in the short arm of metacentric chromosomes 8, 9, 11 and 12, which colocalized with constitutive heterochromatin $CMA^+/DAPI^{-/0}$ bands. The karyotype comprised chromosome pairs with terminal constitutive heterochromatin bands that included satellites and heteromorphic clusters indicating that it is an allooctoploid. These results will be used as a tool for monitoring genetic improvement, in interspecific crosses and its progenies and in biotechnological procedures by *in vitro* culture.

Key words: constitutive heterochromatin, chromosome banding, bulbous, plant genetic resources, karyotype

RESUMEN

Zephyranhtes citrina es una planta bulbosa americana, ornamental, utilizada en jardines por las cualidades estéticas de su perigonio amarillo. El objetivo de este trabajo fue caracterizar citogenéticamente la especie con tinción clásica convencional y bandeo cromosómico. Se observó un número cromosómico esporofítico de $2n=8x=48$, siendo la fórmula cariotípica 20 m + 26 sm + 2st. Se detectaron satélites en el brazo corto de los cromosomas metacéntricos 8, 9, 11 y 12, que co-localizaron con bandas de heterocromatina constitutiva $CMA^+/DAPI^-$. El cariotipo comprendió pares de cromosomas con bandas de heterocromatina constitutivas terminales que incluyeron satélites y grupos heteromórficos que indican que es un alooctoploide. Estos resultados serán usados como herramientas en el monitoreo del mejoramiento genético, en análisis de cruzamientos interespecíficos y progenies y en procedimientos biotecnológicos de cultivo *in vitro*.

Palabras clave: heterocromatina constitutiva, bandeo cromosómico, bulbosa, recursos fitogenéticos, cariotipo

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INTRODUCTION

Zephyranthes Herb. is a genus of perennial bulbous plants belonging to the Amaryllidaceae family, which stands out for its high ornamental potential and, at the same time, as a producer of phytochemicals. The species of this genus are of American origin but have been cultured and naturalized as ornamentals in various countries (Meerow *et al.*, 1999; Tapia-Campos *et al.*, 2012; Katoch and Singh, 2015). Phytochemical research of the genus began around 1940 with the report of the presence of alkaloids, such as lycorine, and is currently one of the areas of greatest scientific interest in these bulbous plants due to the pharmacological, antimicrobial, antifungal, acetylcholinesterase and cytotoxic properties of their active principles (Greahouse, 1941; Katoch and Singh, 2015). Taxonomically, *Zephyranthes* belongs to the tribe Hippeastreae (Amaryllidaceae) and its species inhabit tropical and subtropical regions of America (Meerow *et al.*, 2000; Tapia-Campos *et al.*, 2012), and although several efforts have been made to understand its evolutionary complexity, it is still a controversial clade, due to interspecific cross-linked hybridization revealed by molecular data and phylogenetic analyzes (García *et al.*, 2014, 2019).

Cytogenetically, *Zephyranthes* exhibits a wide range of chromosome numbers ranging from $2n=2x=10$ to $2n=96$, diploid and polyploid species, polyploid complexes, and the presence of aneuploid-polyploid polymorphisms with varied karyotypic formulas (Raina and Khoshoo, 1971; Bhattacharyya, 1972; Greizerstein and Naranjo, 1987; Daviña and Fernandez, 1989; Daviña, 2001; Felix *et al.*, 2011a; Daviña and Honfi, 2018). Furthermore, there are at least three basic numbers $x=5$, 6 and 7 whose diploids are found in the subtropical zone of South America (Daviña *et al.*, 2019). Fluorescent chromosome banding techniques allow longitudinal differentiation of chromosomal regions (Honfi *et al.*, 2017). In plants, the specific identification of constitutive heterochromatin regions with a sequential triple staining with chromomycin, distamycin and 4'-6-diamidino-2-phenylindole (CMA/DA/DAPI) (Daviña, 2001) has been used infrequently in the clade Hippeastreae and there are some antecedents in the genera *Zephyranthes* (Daviña, 2001; Felix *et al.*, 2011b) and *Habranthus* Herb. (Barros e Silva and Guerra, 2010).

Zephyranthes citrina Baker is a species native to the Gulf of Mexico, described for the first time in 1882, when it was spread to South America and is currently used ornamentally for the aesthetic qualities of its perigonium, particularly for the intense yellow coloration of its tepals (Hume, 1935; Tapia-Campos *et al.*, 2012). In a genus where white and pink shades are the most widespread, the intense yellow tepals are of great interest and value in breeding. Likewise, various phytochemicals have been found in this species, some of them of pharmacological importance (Boit *et al.*, 1957; Herrera *et al.*, 2001; Kohelova *et al.*, 2021). Recently, 27 different alkaloids have been detected in this species, among them, seven were unknown to science. Some of these alkaloids have shown biological activity associated with Alzheimer's disease and cytotoxic activity linked to oncological diseases (Prakash and Vedanayaki, 2019; Kohelova, 2021; Kohelova *et al.*, 2021). Within the framework of the characterization of the phytogenetic resources of the Amaryllidaceae family of ornamental and phytochemical interest, the objective of this work was to describe the species chromosomally and to detect karyotypic markers that easily identify it.

MATERIALS AND METHODS

Within the framework of scientific cooperation between CIATEJ (Mexico) and UNaM (Argentina), we studied individuals from a population of *Zephyranthes citrina* (Daviña 681) cultivated in Posadas, Misiones, Argentina, whose control specimen is deposited in the herbarium of the Universidad Nacional de Misiones (MNES) (Figure 1).

Figure 1. *Zephyranthes citrina* (D 681) in bloom visited by pollinators.

Standard cytological techniques

The protocols used by Daviña (2001) were applied and the number of chromosomes in mitotic cells was determined using the meristems of the root tips pretreated with a 0.002M saturated solution of 8-hydroxyquinoline for 8 h at room temperature. They were fixed in absolute ethanol:glacial acetic acid in a 3:1 ratio and stored in the same fixative at about 4° C. The conventional Feulgen

staining was then performed, which consists of an acid hydrolysis of the rootlets in 1N HCl for 10 min at 60° C and a subsequent staining with basic fuchsin (Schiff's reagent) in a dark chamber for at least 20 min. The meristematic zones were macerated in 2% acetic orcein and subsequently squashed.

Molecular chromosome techniques

Pretreated and fixed roots were used as described above in standard staining techniques and also following the protocol suggested by Schwarzacher *et al.* (1980), which consists of macerating the root tips in an enzymatic solution (2% cellulase, 1% pectinase, in 0.01 M citrate buffer, pH 4.8) and squashing in 45% acetic acid. The coverslip was removed with liquid nitrogen and air dried for 1 d at room temperature before use.

A triple sequential CMA/DA/DAPI staining was performed. For the CMA (chromomycin A3) bands, the procedure developed by Schweizer (1976) was followed. The slides were incubated in CMA staining solution (McIlvaine buffer pH 7, 10 mM Cl_2Mg, 0.12 mg/ml chromomycin A3) for 2 h in the dark at room temperature, washed and air dried, and mounted in a solution 1:1 glycerol:McIlvaine buffer with 5 mM Cl_2Mg. Next, they were stained with distamycin A (DA) drops dissolved in McIlvaine buffer pH 7. They were incubated in DA solution at room temperature in the dark in a humid chamber for 15 to 30 min. Subsequently, they were washed and dried. Finally, for the DAPI (4'-6-diamidino-2-phenylindole) bands, the method suggested by Schweizer (1976) was used. The slides were incubated in DAPI staining solution (McIlvaine buffer pH 7, 1-2 µg/ml DAPI) for 30-45 min in the dark at room temperature, washed and air dried, and mounted in the same solution as above.

Karyotype analysis

Chromosomes were observed and photographed with a Leica DML binocular epifluorescence microscope equipped with a DF C310 FX video equipment. 10 optimal metaphases were analyzed, and the nomenclature proposed by Levan *et al.* (1964) was used to classify chromosomes according to the centromeric index (i=s*100/c, where s=length of the short arm and c=total length of the chromosome). In addition, the total length of the chromosome complement (TCL) and the arm ratio (r=l/s) were calculated. In the idiograms, the chromosomes were grouped according to their morphology and within each group they were ordered by decreasing size. As it is a polyploid species, the idiogram was made considering all the chromosomes. The satellites were classified according to the nomenclature suggested by Battaglia (1955, 1999) with which microsatellites were distinguished from macrosatellites, since the former have diameters less than half the diameter of the chromosome. The value of the length of the satellites was included within the total length of the arm to which they were associated.

RESULTS

Mitotic metaphases revealed the octoploid condition of *Z. citrina* with $2n=8x=48$ chromosomes and a basic number of $x=6$ (Figure 2A). The karyotypic formula was 20 m + 26 sm + 2 st, (Figure 3) and the total complement length was 271.31 µm (Table 1). Satellites were observed in the short arm of metacentric chromosomes 8, 9, 11 and 12, all located terminally. In the case of chromosomes 8 and 9 they were macrosatellites, while those of chromosomes 11 and 12 were microsatellites. The mean centromeric index (i) was 36.04 and the mean chromosome length was 5.65 *µm*. The CMA/DA/DAPI triple fluorescent staining pattern showed constitutive GC (guanine-cytosine)-rich heterochromatin bands (Figure 2B, C). The terminal bands located on the short arm of chromosomes 8 and 9 (m) revealed the presence of a type of constitutive heterochromatin $CMA^+/DAPI^0$, whose size includes the satellite and was 1.6 *µm*. On chromosomes 11 and 12 (m), a $CMA^+/DAPI^-$ fluorescent band, rich in GC, 0.3 *µm* long, was identified on the short arm. The amount of constitutive GC-rich heterochromatin corresponded to 0.7% of the polyploid genome. The karyotype comprised chromosome pairs with terminal constitutive heterochromatin bands that included satellites and heteromorphic clusters indicating that it was an allooctoploid (Figure 3).

DISCUSSION

Zephyranhtes citrina is an octoploid species that belongs to the group of species with a basic number $x=6$, which is the most frequent of the genus; this group contains, in addition to diploids, the largest number of polyploid and aneuploid species?. The detected number agrees with those reported by Soontornchainaksang and Chaiyasut (1996), Bobby *et al.* (2003) and Raina and Khoshoo (1972a) (as *Z. sulphurea*) and differs from $2n=47$ registered by Gonzalez *et al.* (1980) in provenances from Cuba. This is the first description of constitutive heterochromatin for the species. So far, there are only two antecedents in the Hippeastreae clade on the presence of $DAPI^+$ bands, which correspond to *Habranthus robustus* Herb., a diploid with $2n=2x=12$ (= *Zephyranthes robustus, sensu* García *et al.*, 2019) and *Habranthus brachyandrus* (Baker) Seally, a tetraploid with $2n=4x=24$ (=*Zephyranhtes brachyandra, sensu* García *et al.*, 2019) (Barros e Silva and Guerra, 2010; Felix *et al.*, 2011b). The other species of *Habranthus* and

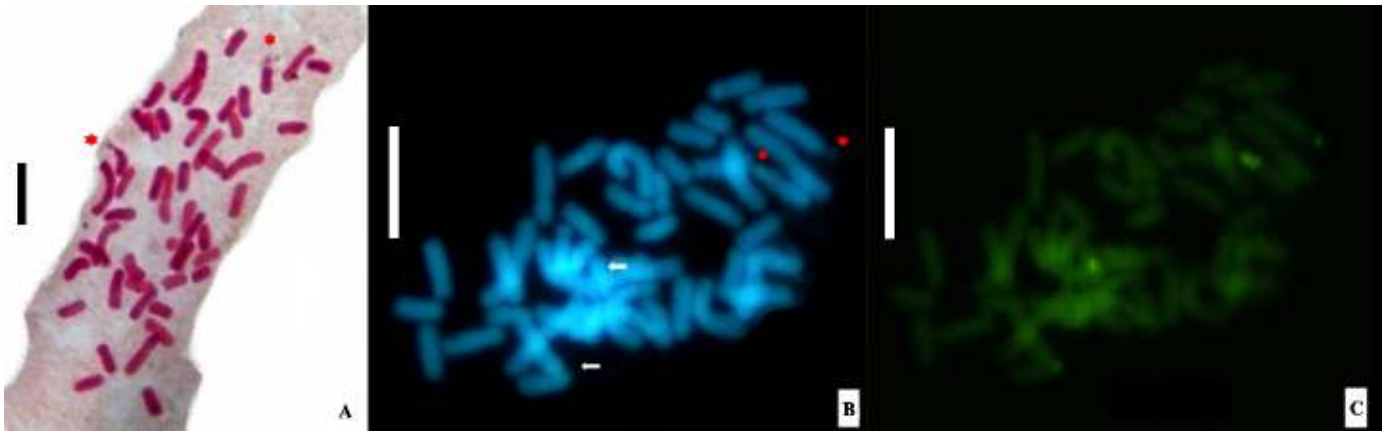

Figure 2. Mitotic metaphase of *Z. citrina*: **A-** conventional staining, 2*n*=8x=48, asterisks indicate the satellites of chromosomes 8 and 9 (m). **B-C-** Sequential banding CMA/DA/DAPI; arrows indicate sites DAPI – (bands CMA+/DAPIo) on chromosomes 8 and 9 (m); asterisk bands indicate CMA+/DAPI- on metacentric chromosomes 11 and 12 (m). Bars =10 µm.

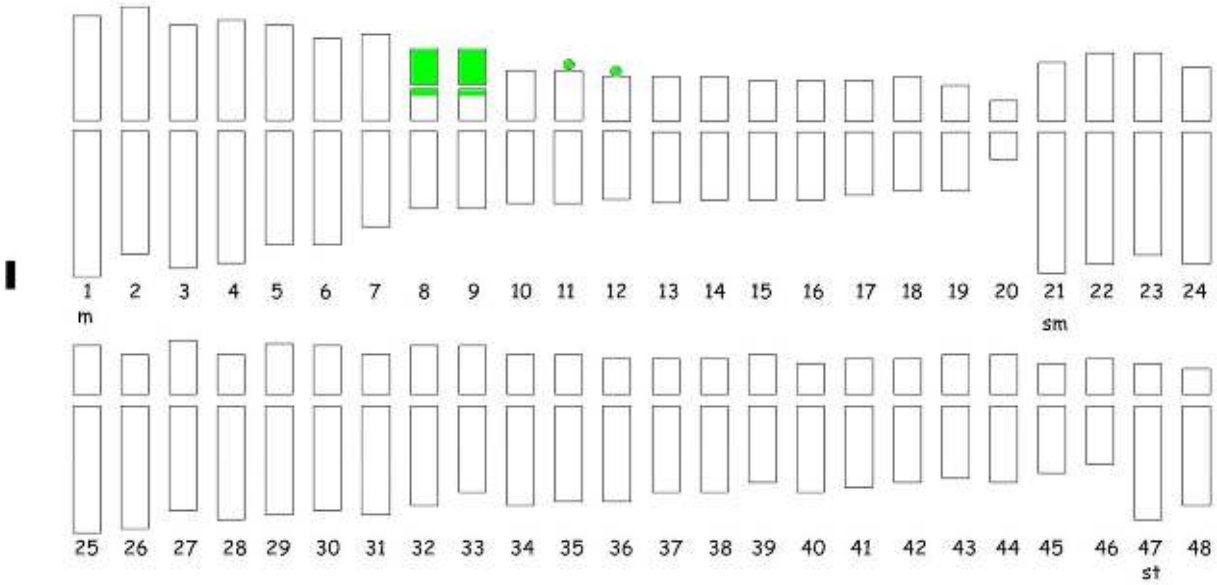

Figure 3. Idiogram of the complete chromosome set of *Z. citrina* 2*n*=48 (20 m + 26 sm + 2 st). Heterochromatic bands (in green) CMA+/DAPIo on chromosomes 8, 9, CMA+/DAPI- on chromosomes 11 and 12 (m), in the short arm. Bars =1 µm.

Zephyranthes, with known constitutive heterochromatin patterns, present banding patterns with regions rich in GC (Daviña, 2001; Barros e Silva and Guerra, 2010; Felix *et al.*, 2011b), as well as the type of pattern detected for *Z. citrina* in this work.

The origin of the polyploids in the Hippeastreae clade remains uncertain in many cases. The main reason is due to the few registered meiotic studies, since both microsporogenesis and megasporogenesis are processes that occur when the flower bud is still inside the bulb without showing external signs of such events and therefore, numerous bulbs must be sacrificed with no guarantees of finding the coveted meiotic stages. Reported male meiosis in both cultivars and natural species have revealed high percentages of bivalents and regular meiosis; multivalent and irregular meiosis to meiotic aberrations such as bridges, lagging chromosomes, micronuclei, among others (Coe, 1953; Sharma and Ghosh, 1954; Tandom and Mathur, 1965; Yokouchi, 1965; Raina and Khoshoo, 1972b; Daviña and Fernandez, 1989; Thoibi Devi and Borua, 1997; Daviña, 2001). For these reasons and based on the described characteristics of the karyotype, *Z. citrina* is considered to be allopolyploid, which may be clarified with future meiotic studies.

Natural and synthetic hybrids of *Zephyranthes* have been reported, resulting from intra- and inter-specific crosses, designed to expand options for growers (Raina and Khoshoo, 1972a; Chowdhury and Hubstenberger, 2006; David, 2011). Chowdhury and Hubstenberger

Table 1. Average of the morphometric parameters of the chromosomal set of *Z. citrina*.

Cromosoma	Long Total	Brazo Corto	Brazo Largo	Indice	Tipo	r
1	9,5	4	5,5	42,1052631579	m	1,375
2	9	4,3333333333	4,6666666667	48,1481481481	m	1,0769230769
3	8,8333333333	3,6666666667	5,1666666667	41,5094339623	m	1,4090909091
4	8,8333333333	3,8333333333	5	43,3962264151	m	1,3043478261
5	8	3,6666666667	4,3333333333	45,8333333333	m	1,1818181818
6	7,5	3,1666666667	4,3333333333	42,2222222222	m	1,3684210526
7	7	3,3333333333	3,6666666667	47,619047619	m	1,1
8	5,6666666667	2,6666666667	3	47,0588235294	m	1,125
9	4,8333333333	2	2,8333333333	41,3793103448	m	1,4166666667
10	4,8333333333	2	2,8333333333	41,3793103448	m	1,4166666667
11	4,8333333333	2,1666666667	2,6666666667	44,8275862069	m	1,2307692308
12	4,5	1,8333333333	2,6666666667	40,7407407407	m	1,4545454545
13	4,5	1,8333333333	2,6666666667	40,7407407407	m	1,4545454545
14	4,3333333333	1,6666666667	2,6666666667	38,4615384615	m	1,6
15	4,3333333333	1,6666666667	2,6666666667	38,4615384615	m	1,6
16	4,1666666667	1,6666666667	2,5	40	m	1,5
17	4,1666666667	1,8333333333	2,3333333333	44	m	1,2727272727
18	3,8333333333	1,5	2,3333333333	39,1304347826	m	1,5555555556
19	2,15	0,95	1,2	44,1860465116	m	1,2631578947
20	7,6666666667	2,3333333333	5,3333333333	30,4347826087	sm	2,2857142857
21	7,6666666667	2,6666666667	5	34,7826086957	sm	1,875
22	7,3333333333	2,6666666667	4,6666666667	36,3636363636	sm	1,75
23	7,1666666667	2,1666666667	5	30,2325581395	sm	2,3076923077
24	6,8333333333	2	4,8333333333	29,2682926829	sm	2,4166666667
25	6,3333333333	1,6666666667	4,6666666667	26,3157894737	sm	2,8
26	6,1666666667	2,1666666667	4	35,1351351351	sm	1,8461538462
27	6	1,6666666667	4,3333333333	27,7777777778	sm	2,6
28	6	1,8333333333	4,1666666667	30,5555555556	sm	2,2727272727
29	6	2	4	33,3333333333	sm	2
30	5,8333333333	1,6666666667	4,1666666667	28,5714285714	sm	2,5
31	5,8333333333	2	3,8333333333	34,2857142857	sm	1,9166666667
32	5,5	2	3,3333333333	36,3636363636	sm	1,6666666667
33	5,5	1,6666666667	3,8333333333	30,303030303	sm	2,3
34	5,3333333333	1,6666666667	3,6666666667	31,25	sm	2,2
35	5,1666666667	1,5	3,6666666667	29,0322580645	sm	2,4444444444
36	4,8333333333	1,5	3,3333333333	31,0344827586	sm	2,2222222222
37	4,8333333333	1,5	3,3333333333	31,0344827586	sm	2,2222222222
38	4,6666666667	1,6666666667	3	35,7142857143	sm	1,8
39	4,6666666667	1,3333333333	3,3333333333	28,5714285714	sm	2,5
40	4,6666666667	1,5	3,1666666667	32,1428571429	sm	2,1111111111
41	4,5	1,5	3	33,3333333333	sm	2
42	4,5	1,6666666667	2,8333333333	37,037037037	sm	1,7
43	4,6666666667	1,6666666667	3	35,7142857143	sm	1,8
44	4,33333333	1,33333333	3	30,769230716	sm	2,2500000056
45	4	1,3333333333	2,6666666667	33,3333333333	sm	2
46	3,8333333333	1,5	2,3333333333	39,1304347826	sm	1,5555555556
47	5,6666666667	1,3333333333	4,3333333333	23,5294117647	st	3,25
48	5	1,1666666667	3,8333333333	23,3333333333	st	3,2857142857

Abbreviations: **Nº**=chromosome number; **c**=total length of the chromosome in *µm* (c=s+l); **l**=long arm length in µm; **s**=short arm length in *µm*; i=centromeric index (i=sx100/c); **type**=chromosomal morphology according to Levan *et al.*, 1964: **m**=metacentric, **sm**=submetacentric, **st**=subtelocentric; **r**=arms ratio (r=l/s).

(2006) highlight seven barriers to the formation of hybrids in *Zephyranthes*, among which chromosome number and ploidy level are preponderant due to the existing chromosome variety in the genus. Another crucial aspect is the existence of reproduction by apomixis and pseudogamy in species of the genus, reproductive events that constitute barriers to obtain simple hybrids (Raina and Khoshoo, 1972a; Chowdhury and Hubstenberger, 2006; Crane, 2019). Obtaining hybrids implies the identification of possible progenitors suitable for crossbreeding plans, both to increase aesthetic and ornamental varieties and to obtain new phytochemical combinations.

At least two successful hybrid lineages are known from crosses with *Z. citrina*. One of them is of interspecific origin and the other is intergeneric. Among the interspecific hybrids with fertile progeny, the tri-hybrid "Best Pink Trihybrid" stands out, product of the cross [(*Z. candida* x *Z. citrina*) x *Z. macrosiphon*] (Chowdhury and Hubstenberger, 2006), where *Z. citrina* was used as a pollen donor because it is an apomictic species (Howard, 1996). On the other hand, *Zephyranthes ajax* is a commercial hybrid with pale yellow tepals resulting from a cross between *Z. citrina* x *Z. candida*, a somatic chromosome number of 2*n*=42 and variable ploidy in the endosperm of its progeny (Tandon and Kapoor, 1962). These endosperm characteristics with mitotic aberrations and variable ploidy was also observed in *Z. citrina* (Bobby *et al.*, 2003). In the lineage of hybrids of intergeneric origin, there is a hybrid known as *Cooperanthes* "*Percy*" (also *Zephyranthes x Percyi*) that was introduced by Traub in 1954 by crossing *Z. citrina* and *Cooperia drummondii* Herb. (David, 2011).

Having cytogenetic markers provides a useful tool to detect in early stages if the hybridization was successful, before the first flowering period of the obtained progeny. It is evident that *Z. citrina* is a species of high value as a parent, of interest in crossbreeding plans due to the qualities of its corolla and the fact that cytological markers for the species have been detected in this work. These results characterize *Z. citrina* as an octoploid and contribute to the knowledge of its cytogenomic structure. Future crosses using this species as a male parent will allow the initiation of new hybrid lineages of ornamental and/or phytochemical interest, which will be able to multiply massively. Protocols for mass multiplication of bulbs (Rodriguez Mata *et al.*, 2018) and *in vitro* culture protocols, adjusted to obtain plants without ploidy alteration and with an efficiency of 85% in the acclimatization stage, are available (Syeed *et al.*, 2021).

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