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REGULAR PAPER

The role of triploids in the origin and evolution of polyploids of *Turnera sidoides* complex (Passifloraceae, Turneroideae)

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Abstract Triploids can play an important role in polyploid evolution. However, their frequent sterility is an obstacle for the origin and establishment of neotetraploids. Here we analyzed the microsporogenesis of triploids (x = 7) and the crossability among cytotypes of *Turnera sidoides*, aiming to test the impact of triploids on the origin and demographic establishment of tetraploids in natural populations. Triploids of *T. sidoides* exhibit irregular meiotic behavior. The high frequency of monovalents and of trivalents with non-convergent orientations results in unbalanced and/or non-viable male gametes. In spite of abnormalities in chromosome pairing and unbalanced chromosome segregation, triploids are not completely sterile and yielded up to 67% of viable pollen. Triploids that originated by the fusion of $2n \times n$ gametes of the same taxon showed more regular meiotic behavior and higher fertility than triploids from the contact zone of diploids and tetraploids or triploids of hybrid origin. The reproductive isolation of *T. sidoides* cytotypes of different ploidy level is not strict and the 'triploid block' may be overcome occasionally. Triploids of *T. sidoides* produce diploid and triploid progeny suggesting that new generations of polyploids could originate from crosses between triploids or from backcrosses with diploids. The capability of *T. sidoides* to multiply asexually by rhizomes, would enhance the likelihood that a low frequency of neopolyploids can be originated and maintained in natural populations of *T. sidoides*.

Keywords Cytotype crossability · Microsporogenesis · Polyploidy · Triploids

Introduction

Polyploidy, or whole genome duplication (WGD), is a common phenomenon across numerous eukaryotic taxa and is believed to be an important factor of adaptation and speciation in plants and other eukaryotes (Mable 2003; Gregory and Mable 2005; Otto 2007; Soltis et al. 2014). In flowering plants, polyploidy is especially widespread and is considered the major mechanism of speciation (Grant 1981), since it raises an immediate reproductive isolation barrier, increasing biodiversity and providing new genetic material for evolution (Levin 1983, 2002).

Different mechanisms may induce polyploidy in plants (Karpechenko 1927; Harlan and De Wet 1975; Thompson and Lumaret 1992). However, the main pathway to polyploid formation is via sexual polyploidization (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Brownfield and Köhler 2011). Particularly, autotetraploids were suggested to be formed in a single step through fusion of two 2*n* gametes produced by diploids (bilateral polyploidization). Alternatively, tetraploids may arise in two steps (unilateral polyploidization) from matings involving viable triploids, which in turn resulted from fusion of *n* and 2*n* gametes produced by diploids (triploid bridge hypothesis). Because of the presumed low probability of fusion of two 2*n* gametes, it has been hypothesized that triploids might play an important role in the origin and evolution of tetraploids (Harlan and de

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Wet 1975). However, one of the main obstacles to the triploid bridge is that, in many species, newly formed tetraploids often have to overcome fertility bottlenecks, because mating with partners of lower ploidy causes incompatibilities in the endosperm leading to seed abortion ("triploid block", Marks 1966; Erilova et al. 2009). The establishment of tetraploids may also be limited by the inviability of triploids. Thus, triploid individuals are rarely found in natural populations. However, sometimes the triploid block is overcome, when triploid plants are not completely sterile, yield some viable gametes and, produce viable diploid, triploid or tetraploid progeny from self-crossings or from crossings with diploids or tetraploids (Burton and Husband 2000).

The *Turnera sidoides* L. autopolyploid complex offers a suitable model system to study the evolutionary dynamics of polyploids in natural populations. *Turnera sidoides* is a complex of perennial rhizomatous herbs that is widely distributed in mid-latitude South America, ranging from southern Bolivia, Paraguay and Brazil to Uruguay and Argentina, where it reaches 39°S. *Turnera sidoides* is an outbreeder due to dystily (Arbo 1985) and genetic self-incompatibility (Solís Neffa 2000). It includes five taxonomic subspecies that differ in their geographical distribution and exhibit a wide morphological variability (Arbo 1985; Solís Neffa 2010). Different ploidy levels with $x = 7$, from diploid to octoploid, were found in *T. sidoides* (Fernández 1987, 2001; Solís Neffa et al. 2004; Speranza et al. 2007; Roggero Luque 2010; Elías et al. 2011; Kovalsky and Solís Neffa 2012). Diploid populations are rare and occupy restricted areas, except in *T. sidoides* subsp. *pinnatifida* (Juss. ex Poir.) Arbo. Most of the subspecies distribution area is covered by tetraploid cytotypes. Hexaploids, octoploids and odd-polyploids are rare and usually located at marginal areas (Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Speranza et al. 2007; Roggero Luque 2010; Elías et al. 2011).

Since all polyploids of *T. sidoides* analyzed so far have an even ploidy level (Fernández 1987; Solís Neffa and Fernández 2001; Solís Neffa et al. 2004), it was firstly hypothesized that bilateral polyploidization would be the most important mechanism of polyploid origin in this species (Panseri et al. 2008). However, findings of triploid plants in some diploid populations (Kovalsky and Solís Neffa 2012) as well as in diploid-tetraploid contact zones of *T. sidoides* (Elías et al. 2011; Moreno et al. 2015), suggested that unilateral polyploidization by a triploid bridge may be an alternative mechanism of polyploid formation in the complex. The last hypothesis was supported by preliminary studies suggesting that triploids of *T. sidoides* are not completely sterile (Elías and Solís Neffa 2008; Moreno et al. 2015). However, to date not detailed studies on the meiotic behavior and microsporogenesis were performed in such triploids. Moreover, the crossability among triploid hybrids with their diploid and tetraploid subspecies parents was verified in a hybrid zone of *T. sidoides* (Moreno et al. 2015), but the study reported just seed sets without information on the ploidy level of the progeny.

In this context, aiming to analyze the type of gametes produced by triploids and to account for the role of triploids in the origin and demographic establishment of tetraploids in natural populations, in the present study the microsporogenesis of triploids as well as the crossability among diploid, triploid and tetraploid plants of *T. sidoides* complex were analyzed.

Materials and methods

Plant material

Information about the taxa studied the provenance of the materials, collectors and collection numbers as well as ploidy levels are listed in Table 1. The voucher specimens are deposited at the Herbarium of the Instituto de Botánica del Nordeste, Corrientes, Argentina (CTES). Some plants of each site were also transported to Corrientes city where they were grown under homogeneous greenhouse conditions in the same Institute. Ploidy levels of all individuals analyzed were determined previously (Elías et al. 2011; Kovalsky 2012; Kovalsky and Solís Neffa 2012).

Three types of triploids were analyzed: triploids resulted from intrasubspecific and intersubspecific crossings among diploids that produce unreduced gametes ($2n + n$), and triploids from diploid–tetraploid contact zones (Table 1).

Microsporogenesis analysis

Meiotic chromosomes of S_{431}, S_{215} ($2n \times n$) and, S_{250}-$n \times S_{215}$-$2n$ triploids were examined in pollen mother cells (PMCs) of young floral buds of suitable sizes, fixed in 5:1 absolute ethanol: lactic acid (Fernández 1973) for 12 h at 4 °C and stored in 70% ethanol at 4 °C. Anthers from the developing floral buds were squashed in a drop of 2% aceto-orcein. PMCs were studied at different meiotic stages. Chromosomes were viewed and photographed with a Zeiss Axioplan HBO 100W/2 microscope equipped with a computer-assisted Cannon Powershot A-640 digital camera system, at a magnification of 1000×.

Microspores at tetrad stage were also examined in the same triploids. For this analysis, young floral buds were fixed as above and the anthers were stained with carmine:glycerin. At least 300 sporads in each of the five anthers per bud were counted to record the number of tetrads, triads, dyads, monads and other types of sporads. The meiotic index was determined as ratio of abnormal sporads to total sporads × 100. Furthermore, since the number of monads, dyads, triads and tetrads during meiosis allows evaluating the theoretical

Table 1 Populations of *T. sidoides* analyzed in this study

T. sidoides	Code	Locality (voucher references)	Ploidy level
subsp. *carnea* (Cambess.) Arbo	S_{215}	Argentina, Corrientes, Dpt. Mercedes. 29°33′44″S, 57°30′40″W, 66 m a.s.l. (Solís Neffa and Seijo 960)	2x
	S_{216}	Argentina, Corrientes, Dpt. Mercedes. 29°33′2″S, 57°30′14.2″W, 41 m a.s.l. (Solís Neffa 981)	2x
	S_{429}	Argentina, Corrientes, Dpt. Mercedes. 29°31′52.8″S, 57°32′53.7″W, 68 m a.s.l. (Solís Neffa et al. 2221)	4x
	S_{430}	Argentina, Corrientes, Dpt. Mercedes. 29°33′00″S, 57°32′15.5″W, 69 m a.s.l. (Solís Neffa et al. 2222)	4x
	S_{431}	Argentina, Corrientes, Dpt. Mercedes, 29°33′45.7″S, 57°31′07.1″W, 57 a.s.l. (Solís Neffa et al. 2223)	2x, 3x
	$S_{215\,(2n\times n)}$	Cultivated in Argentina, Corrientes, Dpt. Capital	3x
subsp. *pinnatifida* (Juss. ex Poir.) Arbo	S_{235}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′57.8″S, 64°29′29.3″W, 1082 m a.s.l. (Solís Neffa and Seijo 967)	2x
	S_{319}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′47.7″S, 64°29′51.3″W, 1070 m a.s.l. (Elías s/n)	2x
	S_{318}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte, camino al C° Uritorco. (Elías s/n.)	4x
	S_{326}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°53′12.30″S, 64°29′, 52.10″W, 1249 m a.s.l. (Elías 7)	2x, 3x
	S_{237}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′44.96″S, 64°31′,49.76″W, 977 m a.s.l. (Solís Neffa and Seijo 969)	4x
	S_{331}	Argentina, Córdoba, Dpt. Punilla, La Falda. 31°04′60,00″S, 64°30′00,00″W, 952 m a.s.l. (Elías 14)	4x
	S_{234}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′44.96″S, 64°30′40.66″W, 1007 m a.s.l. (Solís Neffa and Seijo 966)	4x
	S_{236}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′41.55″S, 64°30′21.43″W, 1027 m a.s.l. (Solís Neffa and Seijo 968)	4x
	S_{322}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′40.14″S, 64°30′29.00″W, 1116 m a.s.l. (Elías s/n)	4x
	S_{332}	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51′42.49″S, 64°30′18.78″W, 1116 m a.s.l. (Elías s/n)	4x
pinnatifida (2x)×*carnea* (2x)	S_{250}-$n\times S_{215}$-$2n$	Cultivated in Argentina, Corrientes, Dpt. Capital	3x

frequency of 2*n* pollen (Xue et al. 2011), the frequency of the expected *n*, 2*n* and 4*n* pollen was estimated based on the assumption that each monad produced one jumbo pollen, each dyad two 2*n* pollen grains, each triad one 2*n* pollen and two *n* pollen grains, and each tetrad four *n* pollen grains. Since the frequency of the production of the different types of sporads did not differ statistically between long (L) and short-styled (S) plants of *T. sidoides* (Kovalsky and Solís Neffa 2012), the effect of floral morph type was not considered in this analysis.

The frequency of unreduced microspores in S_{431} and S_{215} ($2n\times n$) triploid plants was analyzed based on the pollen size distribution. Pollen grains from freshly dehisced anthers (three flowers per plant) were tapped into a drop of 1:1 solution of carmine-glycerin and stained for at least 2 h before scoring. Pollen size was estimated for each grain by the Em index, i.e. the distance between the aperture angle and the middle point of the opposite side (Van Campo 1957). Pollen grains showing a size 1.25 times larger than the population mean were considered as giant, while deeply stained grains showing a size 1.5 times larger than the population mean were considered as jumbo pollen. Moreover, since pollen size variation is associated with distylous dimorphism in *T. sidoides* (Solís Neffa 2000), measurements for short-styled and long-styled flowers were estimated independently. A χ^2 test was performed to compare the expected and observed giant pollen frequencies.

Finally, in order to estimate pollen viability of triploids, the same pollen samples were used. At least 300 grains per flower were scored. Well-filled pollen grains with stained nuclei were scored as fertile, while pollen grains with unstained or poorly stained cytoplasm were counted as sterile.

All statistical analyses were performed using the software Infostat version 2014 (Di Rienzo et al. 2014).

Progeny test

Twelve diploid plants from natural populations S_{215}, S_{216}, S_{235}, S_{319}, and S_{326}, two triploid plants (S_{215}-$2n \times n$, and S_{326}), as well as 14 tetraploid plants from natural populations S_{429}, S_{430}, S_{237}, S_{331}, S_{234}, S_{236}, S_{237}, S_{322}, S_{332}, and S_{318} were used in experimental crosses (Fig. 1; Table 1). Since, in diploid populations, some individuals produce higher frequencies of unreduced gametes (Kovalsky and Solís Neffa 2012, 2016), non-producer individuals were selected for crossing experiments. Pollen viability of diploid (≈95%) and tetraploid plants (≈85%) was determined previously (Elías 2010; Kovalsky and Solís Neffa 2016). Diploid, triploid and tetraploid plants were crossed in all possible combinations. All crosses were between L and S plants. Crosses were done in greenhouse to exclude unwanted pollination according to Fernández and Arbo (1989). Open flowers used as females were emasculated before pollinating them with pollen from anthers of plants selected as males. The number of crosses for each parental combination varied according to the availability of plants and simultaneous flowering. Maturing seeds capsules were wrapped in small tulle bags to prevent loss of seeds during dehiscence. Capsules were fully developed after approximately 20 days, then mature seeds were collected. Viable seeds of each cross were sown in individual pots, and the resulting individuals were transplanted after having developed the first pair of leaves.

Crossability between cytotypes was estimated from the following parameters: fruit set (number of fruits obtained/number of pollinated flowers), seed set (mean number of viable seeds per fruit), and the ploidy level of the resulting progeny. The ploidy level was measured by estimating the relative DNA content by flow cytometry using leaf tissue and following the recommendations with the Partec kit CyStain UV Precise P (05-5002) used to prepare the samples. For each individual, 0.5 cm^2 of leaf was placed in a petri dish with a similar amount of tissue from an internal standard (*T. sidoides* subsp. *carnea*, individual S_{215-48} and *T. sidoides* subsp. *pinnatifida*, individual S_{235-3}); the ploidy level of which was inferred previously from chromosome counts during meiosis (Elías 2010; Kovalsky and Solís Neffa 2012). After adding 0.5 ml of extraction buffer, the leaf tissue was chopped with a razor blade. Following a 2-min incubation, samples were filtered through a 50 µm nylon mesh into the sample tube with 1.5 ml of DAPI (4,6-diamidino-2-phenylindole) staining solution. The mixture was incubated for 2 min at room temperature. The fluorescence intensity of DAPI-stained nuclei was determined using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) with the wavelength used for excitation operating at 355 nm. About 3,000 nuclei were measured for each sample. Ploidy levels were estimated by comparing the DNA peak of the samples to the internal standard. For this purpose 124 individuals of the progeny of all crosses were analyzed. The data was evaluated using PA II's Partec FloMax software.

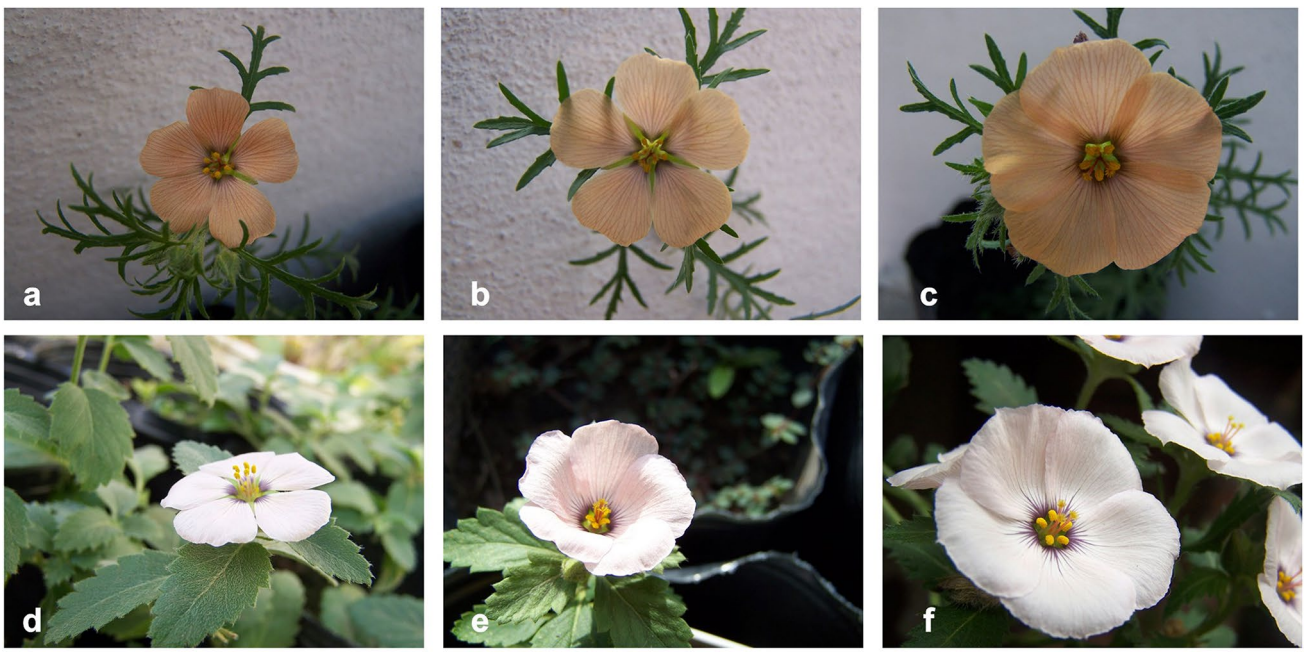

Fig. 1 Plants of *Turnera sidoides* used as progenitors in intercytotype crosses. **a–c** *Turnera sidoides* subsp. *pinnatifida*, **d–f** *Turnera sidoides* subsp. *carnea*, **a, d**, diploid plants ($2n = 2x = 14$), **b, e** triploid plants ($2n = 3x = 21$), **c, f** tetraploid plants ($2n = 4x = 28$)

Results

Microsporogenesis analysis of triploids

The analysis of 1583 PMCs showed that triploids generally exhibit irregular meiotic behavior. Most of the PMCs showed the presence of trivalents (0–7) and univalents (0–6) at diakinesis and metaphase I (Fig. 2a, b; Table 2). At these phases, the most frequent configuration were 7 III and 1I + 1II + 6III, recorded in S_{431} and, S_{215} ($2n \times n$) triploids; while in the triploid hybrid, S_{250}-$n \times S_{215}$-$2n$, the most frequent configurations were 4I + 4II + 3III, 3I + 3II + 4III and, 2I + 2II + 5III (Table 3). Three types of trivalent configurations were observed: lineal (51.22%), convergent (26.82%), and indifferent (21.95%).

The further meiotic course was highly abnormal. The most frequent meiotic abnormalities found during metaphase I were out of plate univalents, bivalents or trivalents, particularly in S_{215} ($2n \times n$); while in anaphase I laggard chromosomes were mostly detected in the triploid hybrid, S_{250}-$n \times S_{215}$-$2n$ (Table 4). Irregular distribution of chromosomes between the poles was also observed. In S_{215} ($2n \times n$), 12 + 9 and, 11 + 10 were the most frequent distributions, although 9 + 10 and 3 chromosomes outside

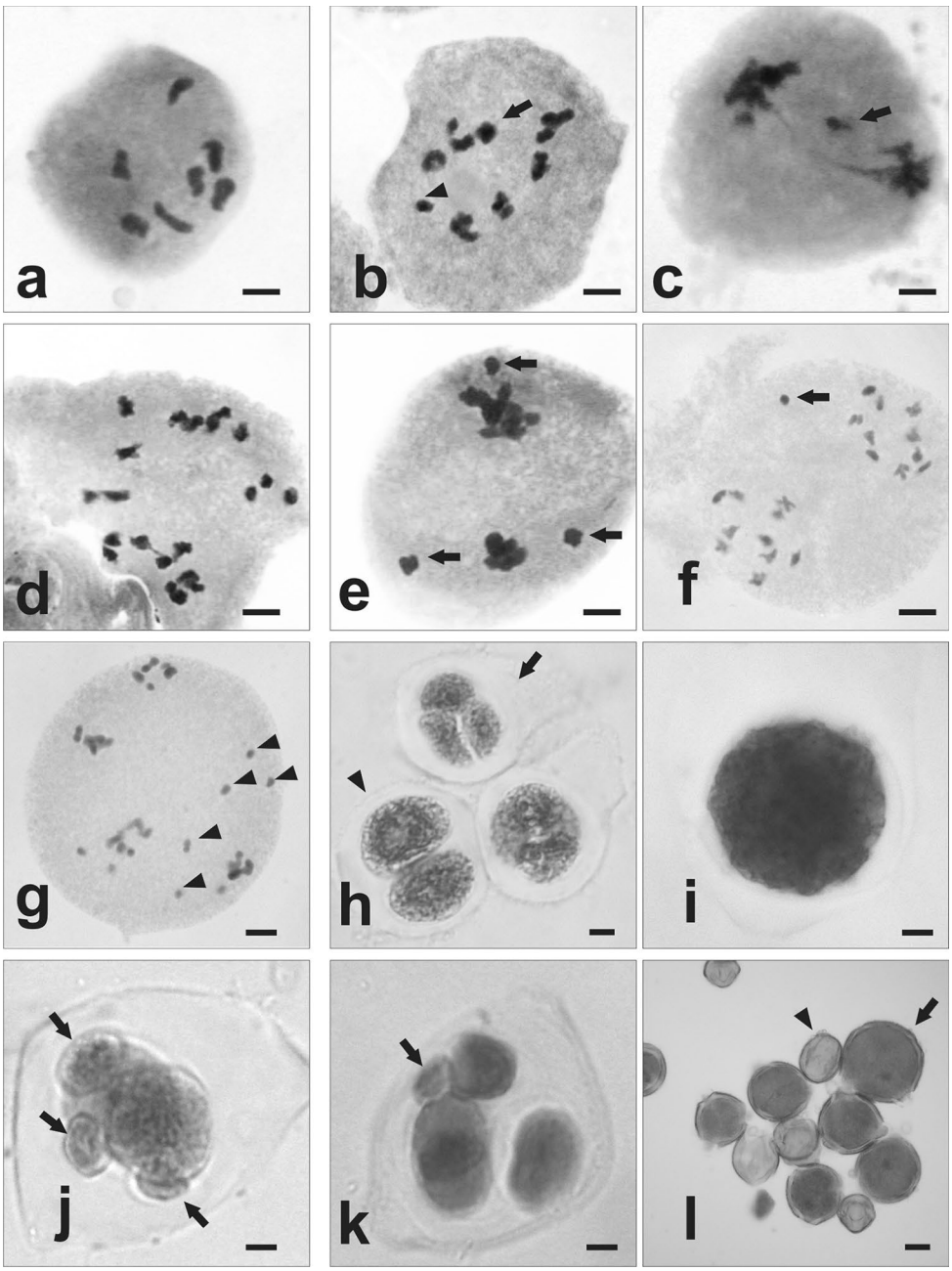

Fig. 2 Meiotic chromosomes, sporads and pollen of *Turnera sidoides* triploids. **a, b** Diakinesis. **a** 7III, **b** 6 III + 1 II (*arrow*) + 1I (*arrowhead*), **c** AI, bridge and laggard chromosome (*arrow*). **d** PII, **e** MII chromosomes out of plate (*arrows*), **f** PII with ten chromosomes in each nuclei and a missintegrated chromosome (*arrow*), **g** AII four poles with different number of chromosomes each and, chromosomes out of poles (*arrowheads*), **h–k** sporads, **h** Tetrad, tryad (*arrow*) and dyad (*arrowhead*), **i** monad, **j** dyad with three microspores (*arrows*). **k** tetrad with a micromicrospore (*arrow*), **l** Pollen grains, viable pollen grain (2*n*), jumbo pollen (4*n*) (*arrow*), inviable pollen (*arrowhead*). *Bar* 5 µm

Table 2 Average chromosome associations at diakinesis and metaphase I in triploids of *T. sidoides*

	I	II	III	IV	CMP
S_{431}	22.62 ± 21.89 (0–2)	22.62 ± 21.89 (0–2)	33.33 ± 24.16 (5–7)	–	42
S_{215} (*2n* × *n*)	9.78 ± 11.,89 (0–3)	14.84 ± 13.58 (0–3)	15.09 ± 16.79 (4–7)	–	137
S_{326}	–	–	–	–	–
$S_{250} \times S_{215}$-*2n*	5.82 ± 3.95 (0–6)	5.25 ± 3.84 (0–7)	5.46 ± 3.74 (0–7)	1.82 ± 0 (0–1)	55

Mean ± SD

Table 3 Meiotic configuration of triploids of *T. sidoides* at Metaphase I

Configuration	S_{431} (%)	S_{215} (*2n* × *n*) (%)	S_{250}-*n* × S_{215}-*2n* (%)
7III	54.76	41.61	5.45
3I + 6III	0	3.65	0
1I + 1II + 6III	38.10	29.20	3.64
3II + 5III	0	0	3.64
2I + 2II + 5III	7.14	13.14	10.91
2I + 5III + 1VI	0	0.73	0
1II + 5III + 1IV	0	0	1.82
1I + 4II + 4III	0	0	7.27
3I + 3II + 4III	0	2.19	10.91
5I + 2II + 4III	0	0	1.82
2I + 5II + 3III	0	0	5.45
4I + 4II + 3III	0	0	14.55
6I + 3II + 3III	0	0	5.45
3I + 6II + 2III	0	0	1.82
5I + 5II + 2III	0	0	3.64
6I + 6II + 1III	0	0	1.82
3I + 7II + 1IV	0	0	1.82
PMC	42	137	55

PMC pollen mother cells

the metaphase I plate were also observed in some PMC. S_{250}-*n* × S_{215}-*2n* progenie exhibited a wide range of irregular distribution of chromosomes, 9 + 10 + 2 outside plate being most frequent. Bridges were also observed in some PMC of S_{215} (*2n* × *n*) (Fig. 2c; Table 4).

At metaphase II 'out of plate' chromosomes were observed in all triploids, with the highest frequency in S_{431} and S_{250}-*n* × S_{215}-*2n* (Fig. 2e). At anaphase II laggard chromatids were detected mostly in S_{250}-*n* × S_{215}-*2n* (Fig. 2g). At anaphase II–telophase II, both balanced and unbalanced segregations were detected. In S_{215} (*2n* × *n*) 7 + 6 + 4 + 4 and, 6 + 5 + 5 + 5 distribution patterns were observed; while, as in AI, S_{250}-*n* × S_{215}-*2n* showed a range of irregular chromosomes segregations (Table 4).

The meiotic products yielded a variety of sporad types (Fig. 2h–k). Beside the four expected normal sized microspores, triads, dyads and monads, were found in all triploids, with triads being the most frequent type, particularly in the S_{431} triploid. Moreover, sporads with 1–3 minute microspores and polyads were also observed. The major frequency of minute microspores were observed in S_{215}-*2n* × *n*, and in the triploid hybrid S_{250}-*n* × S_{215}-*2n* (Table 5).

The average pollen size was 46.68 µm (±6.16 µm), varying between 37.24 and 76.44 µm. The percentages of observed *n*, 2*n* and 4*n* pollen were 0.90, 0.07 and, 0.02%, respectively. The χ^2 test showed that there were no significant differences between the percentages of expected *n*, 2*n* and 4*n* pollen (0.99, 0.01 and 0.02%, respectively), and the percentage of unreduced pollen observed (*2n:* $\chi^2 = 0.316$; *4n:* $\chi^2 = 0.162$) (Fig. 2l).

Pollen viability varied between 21% in S_{326} to 67% in S_{215}-*2n* × *n* (Table 5).

Progeny test

Results are summarized in Table 6 and in Figs. 2 and 3. Of the 259 crosses performed to validate crossability between cytotypes, 56% of the progeny produced fruits. The 2*x* × 2*x*, 4*x* × 4*x*, 2*x* × 4*x* and 2*x* × 3*x* reciprocal crosses, were successful, whereas no fruits were obtained from 3*x* × 3*x* and 4*x* × 3*x* crosses (Table 6).

Fruit set was highest in 2*x* × 2*x*, 4x × 4*x* and 2*x* × 4*x* crosses, intermediate in 2*x* × 3*x* and 3*x* × 4*x* and lowest in 3*x* × 2*x* crosses. 85% of seeds were well-developed. Seed set per fruit was highest in 4x × 4*x* and 4x × 2*x* crosses (69%), intermediate in 2*x* × 2*x* and 2*x* × 4*x* crosses and lowest in 2*x* × 3*x* and 3*x* × 2*x* (Fig. 3; Table 6). Some aborted eggs were observed in 2*x* × 3*x*, 3*x* × 2*x*, 4*x* × 2*x* and 2*x* × 4*x* crosses.

There were differences in reciprocal crossability, since certain combinations yielded higher seed set in one cross-direction than in the other. In crosses between diploids and tetraploids, more fruits and seeds were produced when tetraploids were used as a maternal parent. Crosses between diploids and triploids were effective in both directions, although

Table 4 Meiotic analysis of frequencies of abnormal configurations for each meiotic stage in triploids of *T. sidoides*

Configurations	S_{431} (%)	S_{215} ($2n \times n$) (%)	S_{250}-$n \times S_{215}$ $2n$ (%)
Metaphase I			
1 out of plate univalent	1.10	14.62	0
2 out of plate univalent	1.10	7.15	0
3 out of plate univalent	1.10	6.84	0
4 out of plate univalent	0	2.02	0
1 out of plate bivalent	0	1.56	0
2 out of plate bivalents	0	0.62	0
3 out of plate bivalents	0	0.31	0
1 bivalent + 2 univalent out of plate	0	0.31	0
1 out of plate trivalent	0	0.16	0
2 out of plate trivalent	0	0.16	0
3 out of plate trivalent	0	0.16	0
Anaphase I			
Laggard chromosomes	0	0	45.26
Laggard chromosomes + bridge	0	0.62	0
Telophase I			
Laggard chromosomes	0	0.31	2.11
Prophase II			
Micronucleous	0	0	1.05
Metaphase II			
1 outside plate chromosome	13.19	1.71	13.16
2 outside plate chromosomes	5.49	2.64	3.68
3 outside plate chromosomes	3.30	1.71	6.32
4 outside plate chromosomes	4.40	1.09	0.53
5 outside plate chromosomes	3.30	0	0
7 outside plate chromosomes	1.10	0	0.53
Anaphase II			
Laggard chromatids	0	0	0
1 laggard chromatid	0	0	1.05
2 laggard chromatids	0	0.47	2.11
3 laggard chromatids	0	0	0.53
Laggard chromatids + bridge	0	0.31	0
1 laggard chromosome	0	0	2.63
2 laggard chromatids	0	0	3.16
3 laggard chromatids	0	0	1.05
4 laggard chromatids	0	0	1.05
5 laggard chromatids	0	0	0.53
6 laggard chromatids	0	0	0.53
7 laggard chromatids	0	0	0.53
Telophase II			
1 laggard chromatid	5.49	0	0
2 laggard chromatids	2.20	0.16	0
3 laggard chromatids	1.10	0	0
1 micronucleus	1.10	0	7.37
3 nuclei	1.10	1.09	1.58
4 nuclei + 1 laggard chromatid	0	3.11	0
4 nuclei + 2 laggards chromatids	0	1.56	1.05
3 nuclei + laggard chromatid	0	0.31	0.53
3 nuclei + 4 laggards chromatids	0	0.16	0
2 nuclei	0	0.31	0

Table 4 (continued)

Configurations	S_{431} (%)	S_{215} ($2n \times n$) (%)	S_{250}-$n \times S_{215}$ $2n$ (%)
2 nuclei + 1 laggard chromatid	0	0.31	0
2 nuclei + 3 laggards chromatid	0	0.16	0
2 nuclei + 2 micronuclei	0	0.16	0
3 nuclei + 2 micronuclei	0	0.31	0
4 nuclei and +micronuclei	0	0.31	1.58
5 nuclei	0	0.62	0
6 nuclei	0	0	0.53
6 nuclei + 1 micronuclei	0	0	0.53
Bridge	0	0	1.05
Total number of analysed cells	124	643	816

Table 5 Sporads analysis and pollen viability in triploids of *T. sidoides*

Triploid	Tetrads (%)	Triads (%)	Dyads (%)	Monads (%)	Minute microspores (%)	Polyads (%)	Total sporads analysed	Pollen viability (%)
S_{431}	92.79	3.88	1.33	0.4	1.47	0.13	747	54
S_{215} ($2n \times n$)	92.38	1.28	0.41	0.12	5.77	0.04	1680	67
S_{326}	0	0	0	0	0	0	0	21
S_{250}-$n \times S_{215}$-$2n$	88.37	1.22	0.55	1.66	5.65	0.78	903	0

Table 6 Results of the experimental intercytotype crosses in *Turnera sidoides*

Type of cross	Number of crosses	Fruit set	Seed set	Ploidy level of progeny	%
subsp. *carnea*					
2x × 2x	29	100	16	2x	100
2x × 3x	30	48	4	2x	91.31
				3x	8.69
2x × 4x	29	72	12	3x	97.56[a]
				4x	2.44[a]
3x × 2x	13	23	4	2x	100
3x × 3x	10	0	0	–	0
3x × 4x	5	60	4	3x	100
4x × 2x	16	81	14	3x	100
4x × 3x	11	0	0	–	0
4x × 4x	12	100	17	4x	100
subsp. *pinnatifida*					
2x × 2x	10	46.16	19	2x	100
2x × 3x	17	5.55	26	2x	66.66
				3x	33.33
2x × 4x	39	25.7	17	3x	100
3x × 3x	2	0	0	–	0
4x × 3x	11	0	0	–	0
4x × 4x	25	26	16	4x	100

[a]From Kovalsky and Solís Neffa (2016)

more fruits and seeds were produced when diploids were used as maternal parent; while crosses between tetraploids and triploids were effective only when a triploid was the maternal parent (Table 5).

Offspring (124 individuals) was 100% diploid, triploid and tetraploid in $2x \times 2x$, $3x \times 3x$ and $4x \times 4x$ crosses, respectively; diploid (66.66%) or triploid (33.33%) in $2x \times 3x$ crosses; while that of $2x \times 4x$ crosses was mostly triploid (97.62%) (Table 6).

Discussion

Triploids are often sterile because of meiotic irregularities and a high frequency of aneuploid gametes (Lange and Wagenvoort 1973; Costa and Forni-Martins 2004; Zhang et al. 2004; Comai 2005). To ensure full fertility, all chromosomes of triploids must form trivalents that should be orientated in a convergent way and should segregate two chromosomes to one pole and one to the other, to produce two diploid gametes and two haploid gametes. However, this occurs rarely and triploids show different configurations at metaphase I (Clark and Wall 1996). Moreover, the occurrence of monovalents may cause sterility, owing to their variable and unpredictable a segregation at anaphase I

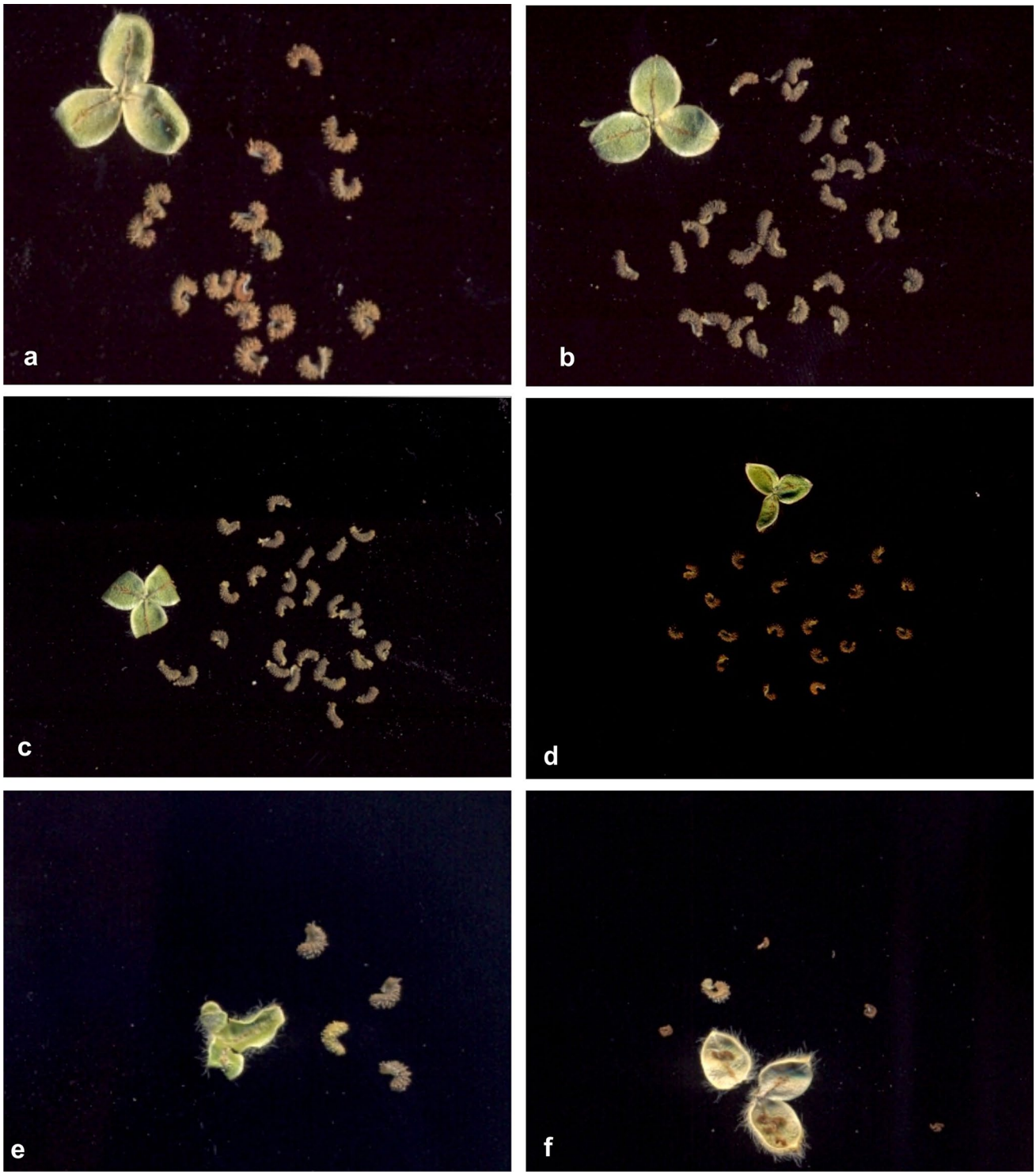

Fig. 3 Fruit and seeds obtained from intercytotype crosses in *T. sidoides*. **a** 2*x*×2*x*, **b** 4*x*×4*x*, **c** 2*x*×4*x*, **d** 2*x*×3*x*, **e** 3x×4*x*, **f** 3*x*×2*x*

(Sybenga 1975). Since monovalents are often not positioned at the equator when bivalents separate instead they lag at the cell periphery form of micronuclei and eventually get lost. Consequently, gametes do not receive full sets of chromosomes, thus getting unbalanced and non-viable, and cause reduced fertility (Clark and Wall 1996). The high frequency of trivalents with non-convergent orientations (mostly lineal) and of monovalents is apparently the cause of unbalanced and/or non-viable gamete formation and of the reduction of pollen viability in triploids of *T. sidoides*.

Even though, our results prove that, in spite of frequent abnormalities in chromosome pairing and of unbalanced chromosome segregation during the first or second meiotic division, triploids of *T. sidoides* can form some viable male and female gametes that possess euploid or mainly aneuploid chromosome numbers (Zhou et al. 2008). Monovalents may randomly become incorporated into the telophase nuclei or may remain in the cytoplasm and not incorporated into the telophase I nuclei. Even bivalents out of the metaphase plate may persist as laggards apart from the poles (Clark and Wall 1996). Therefore, additional minute microspores may be formed by such laggards. For minute microspore in a sporad, an aneuploid microspore with n − 1 chromosomes is to be expected (Seijo and Solís Neffa 2006). Fortuitous misintegration of monovalents into the main nuclei may lead to fertile aneuploid gametes in triploids of *T. sidoides*. However, our findings of 7 + 6 + 4 + 4 segregation and of some normal sporads prove that triploids can also form fertile euploid gametes.

Pollen viability of up to 67% substantiated that triploid *T. sidoides* plants are not completely sterile, although they are less fertile than diploids and tetraploids. These results are in agreement with those obtained in natural (Moreno et al. 2015) and artificial triploid hybrids of *Turnera* (Arbo and Fernández 1983; Fernandez et al. 2017), with up to 50% viable pollen.

The cytogenetic analyses in *T. sidoides* also revealed that the meiotic behavior, percentage of normal and irregular sporads as well as pollen viability vary among triploids. Triploid originating by the fusion of $2n \times n$ gametes of the same taxon display a more regular meiotic behavior and higher fertility than triploids from the contact zone of diploids and tetraploids and triploids of hybrid origin.

Likewise, our results show that reproductive isolation among cytotypes of *T. sidoides* is not too strict. In some polyploid complexes, tetraploids are reproductively isolated from their diploid progenitors by postzygotic barriers (Futuyma 1998; Ramsey and Schemske 1998; Petit et al. 1999; Schuter 2001). The 'triploid block' for instance varies among taxa (Marks 1966) and may be strong as in *Solanum* L. (Werner and Peloquín 1991), *Trifolium pratense* L. (Taylor and Wieseman 1988) or *Lotus tenuis* Wald. et Kit. (Negri and Veronessi 1989), but not absolute (Felber and Bever 1997). In such species, the progeny of 2x × 4x crosses is characterized by a vast absence of triploids (Lumaret et al. 1987; van; Dijk et al. 1992) due to seed abortion caused by an unbalanced maternal–paternal genome ratio in the endosperm (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Köhler et al. 2010). However, the presence of triploids in contact zones between diploids and tetraploids and in the progeny of 2x × 4x experimental crosses in other species (Zohary and Nur 1959; Felber and Bever 1997; Lazzaroff et al. 2015), is considered as evidence that the 'triploid block' can be overcome (Husband and Schemske 1998; Husband 2004). In *T. sidoides* most populations are represented by a single cytotype, although some mixed diploid triploid and tetraploid populations were detected (Elías et al. 2011; Kovalsky and Solís Neffa 2012; Moreno et al. 2015). This fact together with our finding of triploids in the progeny of $2x \times 4x$ experimental crosses, suggest that the triploid block may be overcome in this species complex as well in natural populations of *T. sidoides*.

The fact that the progeny of 2x × 4x experimental crosses yielded more fruits and seeds when the maternal parent was tetraploid is in agreement with the results of interploidy crosses involving other *Turnera* species (Shore and Barret 1985; Arbo and Fernández 1987; Fernández and Solís Neffa 2004; Fernández et al. 2010) and species of other genera (Stebbins 1958; Woodell and Valentine 1961; Ockendon 1968; Levin 1971). It has been suggested that ploidy ratios between embryo, endosperm, and maternal tissue affect the development and viability of seeds generated by interploidy crosses (Ramsey and Schemske 1998). Two hypotheses were proposed to explain how changes in the expression level of imprinted genes in the endosperm could cause altered expression of target genes in interploidy crosses (Khöler and Kradolfer 2011). One proposed that a maternally expressed gene encodes a transcriptional activator that is inactivated by the binding of a paternally expressed gene product; while the other suggest that a maternally expressed gene encodes a transcription factor that acts as a repressor when it binds the target sequence as a homodimer. Consequently, in a $4n \times 2n$ interploidy cross, increased levels of activator/repressor will not cause changes in the expression of the target genes. However, in a $2n \times 4n$ interploidy cross, the relative levels of the activator/repressor in relation to the increased number of target sites are reduced, resulting in repression or activation of the target gene. Thus, in *T. sidoides*, the embryo collapse due to unbalances of the embryo to endosperm genome ratio might be more frequently overcome by higher ploidy level of maternal parent. Another interesting feature was the finding of only diploid and triploid progenies in $2x \times 3x$ crosses of *T. sidoides*, unlike the results observed in other species where tetraploids were also found (Ramsey and Schemske 1998). Since gametes produced by the diploid plants selected for crossings are reduced ($n = x$), the frequency of diploid and triploid progeny resulting from crosses $2x \times 3x$ is an indirect measure of the frequency of n and $2n$ gametes produced by the triploid parent. That triploids of *T. sidoides* produce most frequently haploid gametes is supported by the fact that the progeny of $3x \times 4x$ crosses was 100% triploid, resulting from fusion of n gametes of triploid and 2n gametes of tetraploid parents.

In Lily cultivars, progenies of triploid female parents were mostly aneuploid, while triploid male parents led to more diploid offspring because of a higher transmission

rate of aneuploid gametes by the female parent (Zhou et al. 2008), as also observed for other plant taxa (Brandham 1982). In *T. sidoides* when the female parent triploid was it gave rise to diploid progeny, but when the male parent was triploid diploid and triploid offspring was observed. The fact that, fruit set and seed set were lower when triploids were used as female parent, suggests that triploids more often form a higher frequency of aneuploid eggs yielding inviable embryos that get aborted at early developmental stages.

Although no tetraploids were found among the progeny of $2x \times 3x$ crosses and in diploid-tetraploid contact zones the facts that triploids can produce n and $2n$ gametes and unreduced gametes were detected in natural diploid populations of *T. sidoides* (Panseri et al. 2008; Kovalsky and Solís Neffa 2012, 2016) suggest that unilateral sexual polyploidization by a triploid bridge could be involved in the origin of neotetraploids of this species. In *T. sidoides*, the possible origination of tetraploidy via a triploid bridge and the fact that triploids are not completely sterile suggest that triploids contribute to the dynamics of ploidy levels in the diploid–tetrapoid contact zone of this species. In such contact zones, entirely sterile triploids resulting from crosses between diploid and tetraploid individuals, would favor the evolution of reproductive barriers between both cytotypes, contributing to their spatial segregation (Harlan and de Wet 1975; de Wet 1980; Felber and Bever 1997; Ramsey and Schemske 1998; Burton and Husband 2000). On the other hand, partially fertile triploids may allow gene flow between diploids and tetraploids (Levin 1971; Stebbins 1971; Savidan and Pernès 1981; Felber and Bever 1997; Lenormand 2002; Pannell et al. 2004; Stift et al. 2010). Model simulations have demonstrated that the evolution of tetraploids in a diploid population depends on the reproductive efficiency of triploids and the ploidy level of functional gametes ($n = x$, $n = 2x$ and, $n = 3x$) they produce, contributing to *the novo* the formation of polyploids in each generation (Husband 2004). Because triploid of *T. sidoides* plants are not sterile, and produce $n = x$ and $n = 2x$ gametes, as well as diploid and, triploid progeny in experimental crosses suggesting that new generations of triploids and tetraploids can originate by crossings between triploids or by backcrosses with diploid progenitors that produce some $2n$ pollen and $2n$ eggs. Therefore such triploids may contribute to the origin of new tetraploids and to gene flow among diploids and tetraploids in the contact zones. However, considering that triploids originated by the fusion of reduced and unreduced gametes of diploids are more fertile than triploids originating from $2x \times 4x$ crosses, the former have a higher probability of getting established in natural populations. These facts together with the capability of *T. sidoides* to multiply asexually by rhizomes (Solís Neffa 2000), enhances the likelihood that a low frequency of neopolyploids can be originated and maintained within natural populations of *T. sidoides*.

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