Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: S.I.R. Conceição, A.S. Róis, A.D. Caperta (2019) Nonreduction via meiotic restitution and pollen heterogeneity may explain residual male fertility in triploid marine halophyte *Limonium algarvense* (Plumbaginaceae). *Caryologia* 72(3): 53-62. doi: 10.13128/caryologia-761

Published: December 13, 2019

Copyright: © 2019 S.I.R. Conceição, A.S. Róis, A.D. Caperta. This is an open access, peer-reviewed article published by Firenze University Press (http://www.fupress.com/caryologia) and distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Nonreduction via meiotic restitution and pollen heterogeneity may explain residual male fertility in triploid marine halophyte *Limonium algarvense* (Plumbaginaceae)

Sofia I. R. Conceição[1], Ana Sofia Róis[1,2], Ana D. Caperta[1,*]

[1]*Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal*
[2]*School of Psychology and Life Sciences, Universidade Lusófona de Humanidades e Tecnologias (ULHT), Campo Grande, 376, 1749-024 Lisboa, Portugal*
* Corresponding author; E-mail address: anadelaunay@isa.ulisboa.pt

Abstract. The cosmopolitan halophylic genus *Limonium* (Plumbaginaceae) presents high cytogenetic interest because of the natural occurrence of diploid and polyploid variants. Natural triploids are very rare in nature but common in this genus, including the widespread triploid *Limonium algarvense* found in the Iberian Peninsula and in Morocco. This study describes male sporogenesis and gametogenesis, pollen formation and germination, and seed production in triploid *L. algarvense* and diploid *Limonium ovalifolium* using various cytological approaches. The diploid species presented regular meiosis. The triploid species was defective in male meiosis due to unpaired chromosomes, trivalent and tetravalent pairing, unbalanced chromosome segregation in meiosis I, and meiotic restitution in both meiosis I and II. These results may be explained by indeterminate and broad first meiotic restitution. Dyads and restitution nuclei at meiosis I were the most frequent meiotic products in the triploid species. Cytomixis was observed in both species, and callose deposition did not differ among them. In the diploid species, regular, tricolpate pollen grains, which germinated in vitro were found. Contrastingly, the triploid species produced heterogeneous pollen in morphology and size, with moderate to no viability that poorly germinated in vitro. We conclude that even if most triploids male gametes are non-functional, they seem to generate small numbers of viable gametes via nonreduction of chromosomes. Flow cytometric seed screening demonstrated that the diploid species presented a diploid progeny whereas triploids only showed triploid progenies. In the triploids low pollen fertility coupled with viable seed production may assure their persistence in natural populations.

Keywords. Apomixis, *In vitro* pollen germination, *Limonium*, Male sporogenesis and gametogenesis, Meiotic restitution, Polyploidy.

INTRODUCTION

In flowering plants, polyploidy (i.e. the condition of having three or more copies of the basic set of chromosomes) has been considered to be one of the

main drivers of plant speciation (Ramsey and Schemske, 1998; Adams and Wendel, 2005). A major route of polyploidization rely on alterations of the meiotic cell cycle involving meiotic nuclear restitution during micro- and megasporogenesis, originating unreduced gametes (Bretagnolle and Thompson, 1995; De Storme and Mason, 2014). Several processes can lead to these gametes like cytokinetic defects, omission of a meiotic division (De Storme and Geelen, 2013) or alterations in spindle biogenesis and polarity. These processes can lead to spindle absence or malformation during metaphase I or II (MI or MII), spindle co-orientation (parallel spindles) in MI (Bretagnolle and Thompson, 1995), and tripolar and fused spindles in MII (Rim and Beuselinck, 1996). Meiotic restitution nuclei can be associated with First Division Restitution (FDR) or Second Division Restitution (SDR) due to the omission of the first or second meiotic division, respectively (Ramanna and Jacobsen, 2003).

The cosmopolitan species-rich genus *Limonium* Mill. (Plumbaginaceae) contains complex aggregates of diploids and polyploids (Róis et al., 2016; Caperta et al., 2017; Róis et al., 2018). These species are of interest because of the occurrence of diploid (2*n* = 2x = 16, 18 chromosomes), triploid (2*n* = 3x = 24, 25, 27), tetraploid (2*n* = 4x = 32, 35, 36), pentaploid (2*n* = 5x = 43), and hexaploid (2*n* = 6x = 51, 54, 56) variants (e.g., Erben 1978, 1993; Brullo and Pavone 1981; Arrigoni and Diana 1993; Castro and Rosselló 2007). Meiotic studies in the genus are scarce (D'Amato, 1940a, 1940b, 1949; Róis et al. 2012), although essential to assess fully the nature of cytological variation of polyploid species. Studies on male and female sporogenesis and gametogenesis revealed that diploid *Limonium ovalifolium* (Poir.) Kuntze (2*n* = 2x =16) presented regular meiosis, whereas tetraploid *Limonium multiflorum* Erben (2*n* = 4x =35, 36) showed unbalanced and irregular meiosis (Róis et al., 2012; Róis et al., 2016). Triploid cytotypes appear to be the predominant *Limonium* cytotypes in the Iberian Peninsula and in the Balearic Islands (Erben, 1978, 1979; Cowan et al., 1998; Castro and Rosselló, 2007). However, meiotic studies in triploids are limited to a few works on female development in *Statice oleaefolia* Scop. var. *confusa* Godr. (synonym *Limonium virgatum* (Willd.) Fourr.; 2*n* = 27 chromosomes; Erben, 1993) (D'Amato, 1940a, 1940b, 1949).

In the present study, our goal was to compare male sporogenesis and gametogenesis in triploid *Limonium algarvense* Erben with diploid *L. ovalifolium* (Poir.) Kuntze. Both the diploid and triploid species are perennial and capable of vegetative reproduction, and have a widespread occurrence in the Iberian Peninsula and in Morocco (Erben, 1993; Fennane et al., 2014; Caperta et al., 2017). Flowers of the triploid species have potential agro-food industry applications due to antioxidant and anti-inflammatory properties (Rodrigues et al., 2015, 2016).

MATERIAL AND METHODS

Cytological analysis of microsporogenesis and gametogenesis

Five *L. algarvense* (2*n* = 25 chromosomes, 5.69 ± 0.15 pg/2C; Caperta et al., 2017) and one *L. ovalifolium* (2*n* = 16, 3.58 ± 0.04 pg/2C; Róis et al., 2012) genotypes from *ex situ* collections established in a greenhouse at Instituto Superior de Agronomia (Lisbon, Portugal) were used.

Microsporogenesis and gametogenesis were analysed in floral buds in distinct developmental stages, as described in Róis et al. (2012). Buds selection was based on size: bud with 0.1 – 0.3 cm (from pre-meiotic interphase to metaphase I - stage I); and bud > 0.3 – 0.5 cm (from anaphase I to pollen grain -stage II). In brief, staged buds were fixed in a fresh absolute ethanol : glacial acetic acid (3:1) solution overnight and stored in 70 % ethanol solution at –20 º C until used. Then, buds were digested in a pectolytic enzyme mixture [2 % cellulase (Sigma), 2 % cellulase "Onozuka R-10" (Serva), and 2 % pectinase enzyme (Sigma)] in 1xEB in a humid chamber for 2 h at 37 °C. Meiocytes, chromosomes and pollen grains spreads were prepared from anthers. Preparations were stained with 4', 6-diamino-2-phenylindole hydrochloride (DAPI) (1 mg ml $^{-1}$) in Vectashield (Vector Laboratories).

The percentage (%) of meiotic products from cell fusion in the first division was calculated as the number of fused dyads/telophase I cells; and the percentage of meiotic products from cell fusion in second division was calculated as the number of fused triads or tetrads/ telophase II cells, respectively.

For tetrad analysis, staged flower buds previously fixed in ethanol : glacial acetic acid (3:1) solution were used. The buds were further placed in an aceto-carmine solution for 1 h, and dissected in a drop of aceto-carmine solution.

For histochemical callose staining, flower buds at stages I and II were collected and stained through a modified procedure by Musiał et al. (2015). Buds were kept in 80 % ethanol for 30 min in agitation and transferred to 1 M NaOH solution for 3 h at 37 ºC. Then, the buds were washed twice in distilled water for 2 min with agitation, and placed in 0.1 M KPO_4 for 2 min at room

temperature (rt), and subsequently in 0.1 % aniline blue (Merck) in 0.1M KPO_4 for 48 h, at rt. Finally, anthers were dissected in multiwall-slides in a drop of 0.1M KPO_4: glycerol (1:1).

Pollen size, viability and germination

Anthers from mature flowers stored in 70 % ethanol were stained using Alexander's stain (Alexander, 1969) under a coverslip, and observed under light microscopy. Total pollen viability estimates were performed by one person using three to five flowers per plant and counted with a 63x objective. About 300 pollen grains per flower were recorded. Pollen grains dimensions were estimated as described in Róis et al. (2012) by calculating mean, standard deviation, and standard error.

For pollen tube growth analysis, five flowers (five anthers each flower) per plant were used following the procedure described in Róis et al. (2012). The pollen grains were collected from plants soon after anther dehiscence and cultured in a media containing 20 mM boric acid, 6 mM calcium nitrate, 0.1 % casein hydrolysate and 7 % sucrose (Zhang et al., 1997). A dialysis tubing and filter paper support combined with 23 % polyethylene glycol –20,000 as an osmoticum in the medium, provided appropriate physical conditions for pollen germination. Pollen grains were incubated at 37 °C during 48 h or 72 h in the dark. The grains were considered germinated when they present a tube length that was equal or greater than the diameter of the pollen grain. For measurement of pollen tube length, 10 pollen tubes were selected randomly from each treatment, and measured on micrographs recorded with a 63x objective using Axiovision 4.0 (Zeiss) software.

Optical microscopy analysis and imaging

Slides of cell preparations, meiocytes, pollen grains, and pollen tubes were observed using a Zeiss Axioskop 2 fluorescence microscope and photographed with an AxioCam MRc5 digital camera (Zeiss).

Flow cytometric screening of seeds

Flow cytometric seed screening (Matzk et al., 2000) was used to estimate the genome size of seeds derived from each plant progenies in at least 40 seeds, which were analyzed in pooled groups of 20 seeds. Nuclei were isolated following the procedure of Galbraith et al. (1983), in which 0.5 cm^2 of fresh leaf tissue of each sample was chopped with a razor blade, simultaneously with 0.5 cm^2 of fresh leaf tissue of the internal reference standard, in a Petri dish containing 1 ml of WPB buffer (Loureiro et al., 2007). As internal standard *Secale cereale* 'Dankovske' (2C = 16.19 pg) (Doležel et al., 1998) was utilized. The suspension was filtered through a 50 µm mesh nylon filter, and propidium iodide (50 µg/ml) was added to stain the DNA. A Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a green solid state laser (Cobolt Samba 532 nm, operating at 30 mW; Cobolt, Stockholm, Sweden) was used to measure the relative fluorescence of stained nuclei. Results were obtained using PARTEC FLOMAX software (v. 2.9). About 1300 nuclei per sample were analyzed.

The DNA-ploidy level was inferred as a relative position of the sample G1 peak to that of the internal standard. The exact chromosome numbers and DNA ploidy level of the progenitor plants were determined by chromosome counting (please see Caperta et al., 2017). The value of genome size in mass units (2C in pg; *sensu* Greilhuber et al., 2005) was obtained for each individual analysed using the following equation: *Limonium* 2C nuclear DNA content (pg) = (*Limonium* G_1 peak mean / reference standard G_1 peak mean) * genome size of the reference standard.

Statistics analysis

An analysis of variance (ANOVA) was applied to assess the significance of differences among the studied individuals in relation to meiotic products and pollen types ($p < 0.05$ and $p < 0.001$) (Khan and Rayner, 2003). Percentages were logit-transformed before the statistical analysis to ensure homogeneity of variance.

RESULTS

The diploid *L. ovalifolium* presented a regular meiosis whereas triploid *L. algarvense* showed several meiotic abnormalities (Fig.1). At pre-meiotic interphase, the triploid species exhibited the maximum of three nucleoli while the diploid species showed two nucleoli (data not shown). In the beginning of first meiotic division at the pachytene stage, abnormal meiocytes were observed in *L. algarvense*, with unpaired chromosomes (Fig.1b), although *L. ovalifolium* showed full pairing of all chromosomes (Fig. 1a). In *L. ovalifolium* eight bivalents were found at diakinesis (Fig. 1c) whereas in *L. algarvense*, chromosome abnormalities proceeded as meiosis advanced to the next prophase stages. In this lat-

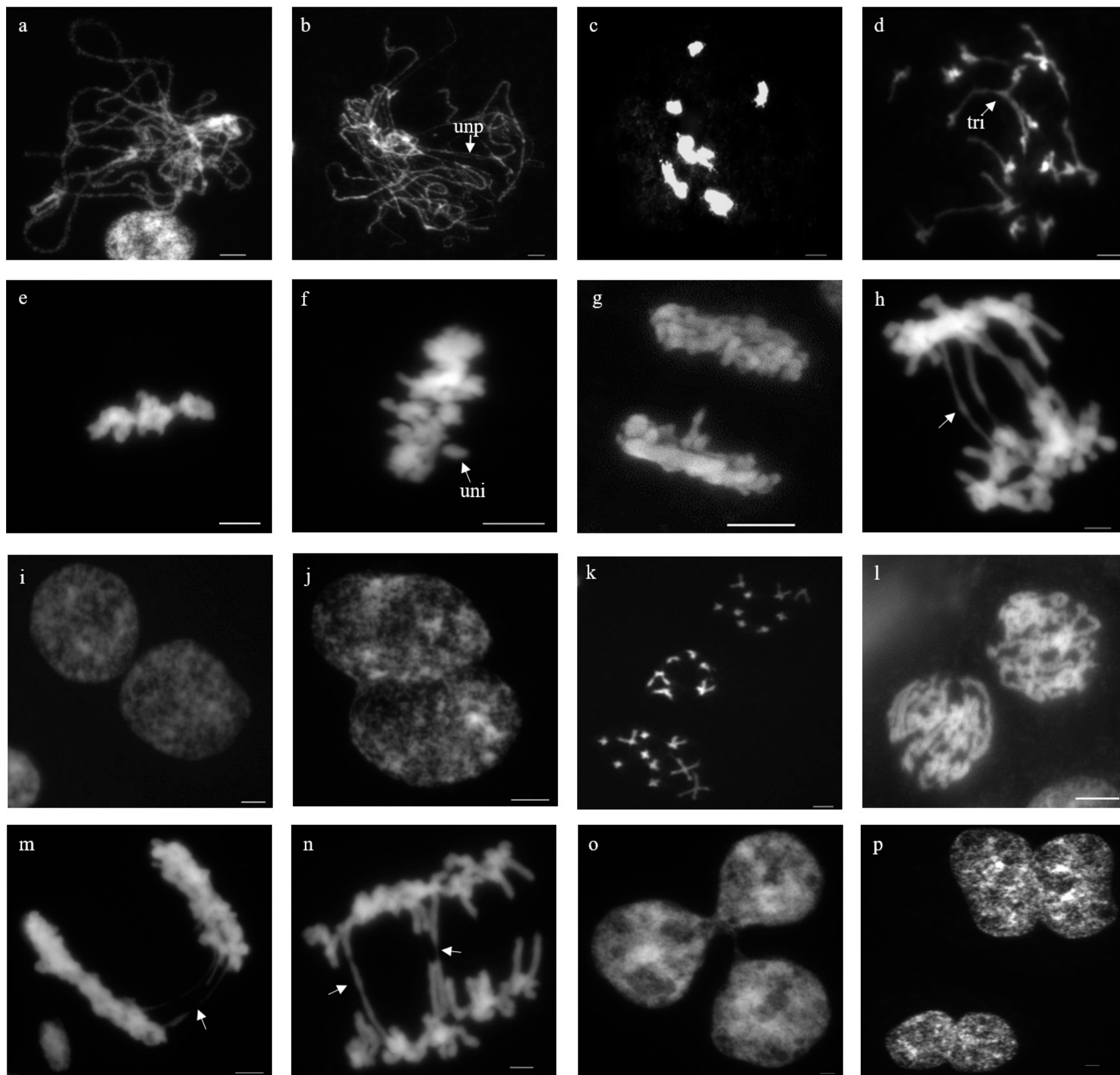

Fig. 1. Chromosome pairing and segregation in DAPI-stained male sporocytes in diploid *Limonium ovalifolium* and triploid *Limonium algarvense***.** **a** Full pairing of chromosomes at pachytene in *L. ovalifolium*; **b** Pachytene with unpaired (unp, arrowed) chromosomes in *L. algarvense*; **c** Diakinesis showing eight bivalents in *L. ovalifolium*; **d** Diplotene with trivalents (tri) and different chromosome associations in *L. algarvense*; **e** Metaphase I in *L. ovalifolium*; **f** *L. algarvense* metaphase I showing an univalent (uni, arrowed); **g** Anaphase I in *L. ovalifolium*; **h** Abnormal anaphase I with chromosome bridges in *L. algarvense* (arrowed); **i** Dyad and fused dyads in *L. algarvense* (**j**); **k** Chromosome arrangement after a tripolar spindle in *L. algarvense* showing three groups of chromosomes (10, 6 and 9, respectively totalizing $2n$ = 25 chromosomes); **l** Prophase II in *L. ovalifolium*; **m** Metaphase II and abnormal anaphase II (**n**) showing chromosome bridges in *L. algarvense* (arrowed); **o** Triad fusion in *L. algarvense*; **p** Co-existence of a fused dyad and a fused tetrad in *L. algarvense*. Bars = 5 μm.

ter species, at the diplotene stage trivalents were detected besides bivalent formation (Fig. 1d), and univalents were also visible in metaphase I (Fig. 1f). Conversely, in *L. ovalifolium* metaphase I cells were regular (Fig. 1e). At anaphase I, the majority of *L. ovalifolium* meiocytes were generally normal, although occasionally lagged chromosomes were found (Fig. 1g). By contrast, in *L. algarvense* most anaphase I cells presented chromosome laggards (data not shown) and chromosome bridges (Fig. 1h). At the end of telophase I, some dyads and fused dyads

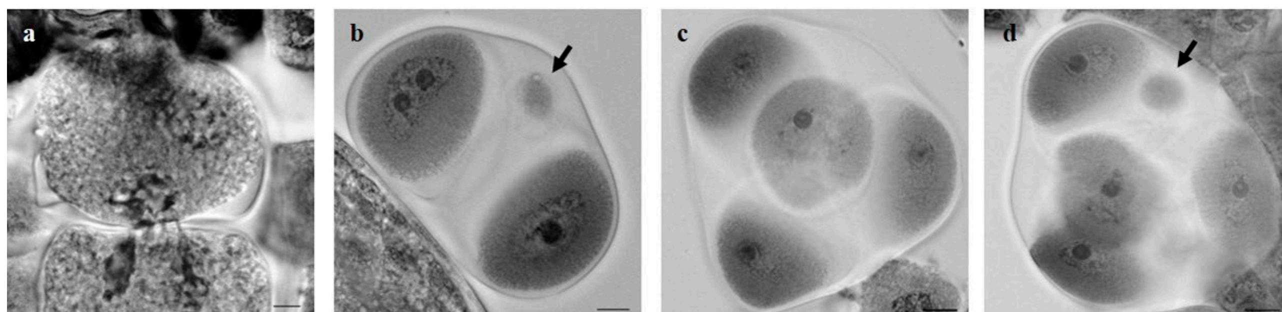

Fig. 2. Tetrad analysis of *L. algarvense***. a** Cytoplasmatic bridges with chromosome passing (cytomixis); **b** Unbalanced triad (a micronucleus is arrowed); **c** Tetrad; **d** Polyad (a micronucleus is arrowed). Bars = 5 μm.

Table 1. Percentage of meiotic products from *ex-situ* collection *Limonium* plants used.

Species	Accession number	Meiotic Products					1st Division restitution nuclei	2nd Division restitution nuclei	Total of cells analysed
		Monads	Dyads	Triads	Tetrads	Polyads			
L. ovalifolium	2009I4SR	0	10.9	0	83.6	5.5	0	0	65
L. algarvense	2009I1AL	0	23.3	18.5	7.2	0	45.8	5.2	249
	2009I2AL	0	59.6	6.1	0.5	0	28.8	5.1	198
	2009I7AL	10	69.5	5	2.1	0.2	9.4	4.0	479
	2009I18AL	21.9	20.6	1.3	0	0	57.6	2.6	155
	2010I15PA	0	36.9	0	0.2	0.2	57.6	5.2	465

resulting in first division nuclei in the triploid species were found (Fig. 1i-j). However, nuclei fusion was seldom seen in the diploid species. After telophase I in *L. algarvense*, tripolar spindles originate a particular chromosome arrangement where it was possible to detect $2n$ = 25 chromosomes arranged in three groups, respectively ten, six and nine chromosomes (Fig. 1k). In this chromosome arrangement, associations of chromosomes with different sizes and shapes was moreover detected. In meiosis II, regular prophase II cells were observed in *L. ovalifolium* (Fig. 1l). In *L. algarvense* chromosome bridges were still visible in metaphase II and in anaphase II (Fig. 1m, n). At the end of meiosis, the diploid species showed mostly tetrads, while dyads were the more common meiotic product in the triploid species (Table 1). In addition to monads and dyads, the triploid species showed triads and tetrads. The coexistence of fused dyads (Fig. 1p) and tetrads (Fig. 1p) as well as fused triads (Fig. 1o) (second division restitution nuclei) and dyads with micronuclei (Fig.2). Although, the frequency of polyads formation is low in both the diploid and triploid species, its occurrence was rarer in triploid (0.2 %) than in diploid (5.5 %) species (Table 1; Fig. 2d).

In both species, the presence of cytoplasmatic bridges with passage of nuclear content from one cell to another (cytomixis, Fig. 2a) was observed, although with very rare incidence in diploid *L. ovalifolium*. No significant differences were detected between *L. algarvense* individuals in relation to the frequency of the meiotic products types for dyads (P =0.36), triads (P = 0.0692) and tetrads (P = 0.29). No significant differences were found between fused meiotic products for dyads (P =0.49), triads (P = 0.869) and tetrads (P = 0.29).

To substantiate possible causes of nuclei fusion, callose deposition was verified using aniline blue labelling during different stages of microsporogenesis. At tetrad stage both in diploid and triploid plants, bright fluorescence callose labelling was visible around the meiotic products (Fig. 3), without differences between species. The diploid species formed regular tricellular pollen grains with one vegetative nucleus and two sperm nuclei (Fig. 4a) whereas in the triploid species bicellular pollen grains having only one or two vegetative nuclei and one sperm nucleus were observed (Fig. 4b).

In diploid (100%) and triploid species (*L. algarvense* ~ 70%) most pollen grains showed three *colpi* (Fig. 5c, Table 2). Nevertheless, in the triploid species pollen grains with one *colpus*, two, four and five *colpi* were also found (Fig. 5, Table 2). In general, *L. ovalifolium* pollen grains measured 53.52 ± 5.6 μm (n = 41). By contrast, pollen grain size

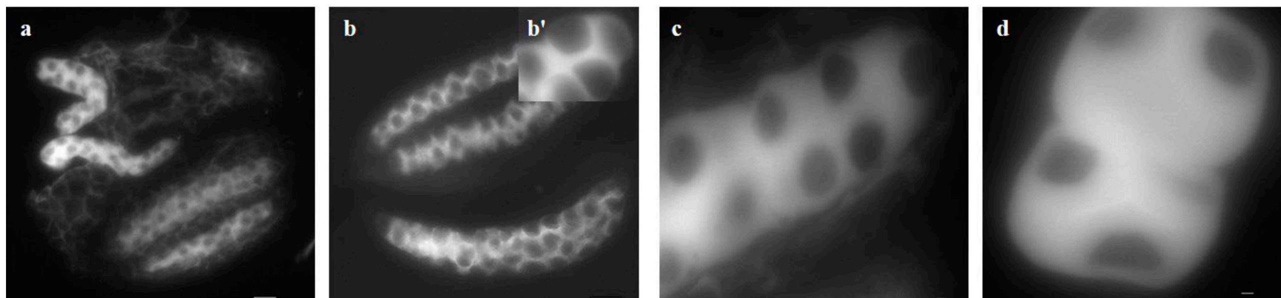

Fig. 3. Callose deposition in *Limonium ovalifolium* and *L. algarvense*. a Anther from a flower bud at stage II with callose labelling in *L. ovalifolium* (bar = 50 μm); **b** Anther from a flower bud at stage II exhibiting bright fluorescence in *L. algarvense* (details shown in the inset – **b**') (bar = 50 μm)**;** Tetrads with strong labelling in *L. ovalifolium* (**c**) and in *L. algarvense* (**d**) (bar = 5 μm).

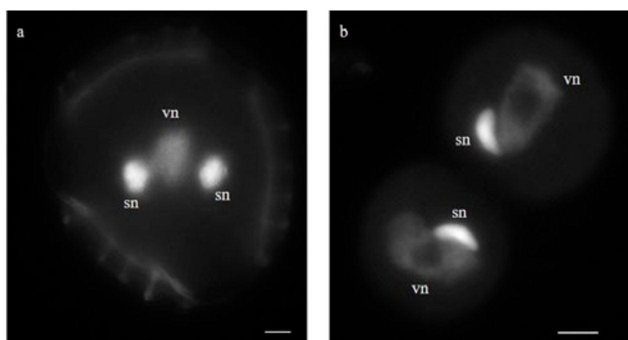

Fig. 4. Dapi staining of pollen grains. **a** Regular tricellular pollen grain in *L. ovalifolium* with one vegetative nucleus and two sperms nuclei; **b** Bicellular polar grain showing one vegetative nucleus (vn) and one sperm nucleus (sn). Bars = 5 μm.

differences were detected in the triploid species: one *colpus* (17.5 ± 2.0 μm, *n* = 2), two (37.8 ± 4.4 μm, *n* = 1), three (56.2 ± 6.3 μm, *n* = 65), four (68.8 ± 3.0 μm, *n* = 51) and five (78.4 ± 1.3 μm, *n* = 9) *colpi*. The pollen grain types did not have significant differences among the studied triploid individuals for 1 *colpus* (*P* = 0.177), 3 *colpi* (*P* = 0.836), 4 *colpi* (*P* = 0.224), 5 *colpi* (*P* = 0.587), and for a significance level between 0.05 and 0.1 for 2 *colpi* (*P* = 0.0545).

Still, pollen grains viability and germination revealed marked differences between species. Comparing to the diploid species, which had 84.8 % (*n* = 1006) of viable grains, the triploid species ranged from low (genotype 2009I1AL, 14.8 %; *n* = 824) to moderate (genotype 2010I15PA, 41.2 %; *n* = 900) viable pollen grains. The diploid species showed the highest frequency (60.5 %, *n* = 885) of germinated grains, while in the triploid species pollen germination frequencies varied among accessions, from 0.8 % (*n* = 900, in 2009I1AL) to 8.2 % (*n* = 883, in 20109I15PA).

The triploid species was able to produce seeds (*c.* 150/per scape) with a moderate seed germination frequency 65 % (*n* = 587). The estimation of embryo and residual endosperm nuclear DNA contents by flow cytometry showed that in *L. ovalifolium* only histograms with a single 2C DNA peak was found representing diploid seeds whereas in *L. algarvense* histograms with an unique 3C DNA peak was detected (Fig. 6).

DISCUSSION

Polyploid plants can arise by the fusion of unreduced gametes or through a mechanism that employs an intermediate step generating triploids (triploid bridge hypothesis) (Ramsey and Schemske, 1998). Triploids are considered to be meiotically unstable, resulting in frequent chromosome loss and fragmentation (McClintock

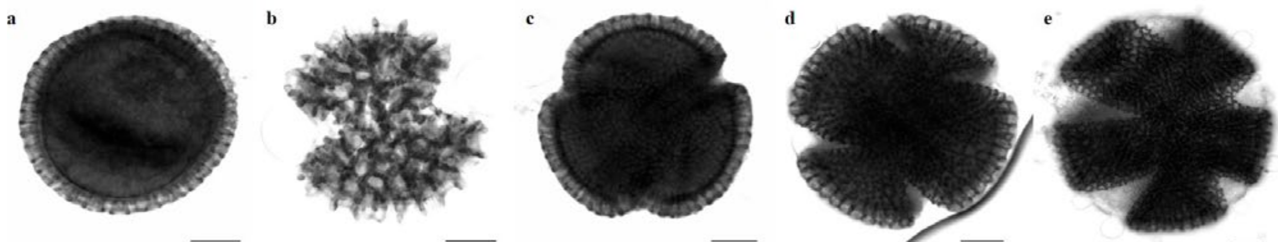

Fig. 5. Pollen grains types in *L. algarvense***.** Pollen grains with one *colpus* (**a**), two (**b**), three (**c**), four (**d**) and five (**e**) *colpi.* Bars = 5 μm.

Table 2. Percentage of pollen grains morphotypes in *L. algarvense*. The percentage of each of the different pollen types and standard deviation is indicated.

Accession	Pollen grains				
	1 *colpus*	2 *colpi*	3 *colpi*	4 *colpi*	5 *colpi*
2009I1AL	0.24 ± 0.2 (2)	1.09 ± 0.5 (9)	74.15 ± 1.8 (611)	23.18 ± 1.8 (191)	1.33 ± 0.7 (11)
2009I7AL	0.61 ±0..3(5)	3.03 ± 1.5 (25)	75.30 ± 3.7 (622)	20.10 ± 2.4 (166)	0.97 ± 0.7(8)
2010I15PA	1.11 ± 0.5 (10)	4.33 ± 2.2(39)	74.11 ±1.5 (667)	19.78 ±3.3(178)	0.67 ± 0.6 (6)
2010I16PA	1.11 ± 1.0 (10)	4.89 ± 1.6 (44)	78.89 ± 5.3 (710)	14.56 ± 7.1 (131)	0.56 ± 0.7 (5)
2009I18AL	0.35 ± 0.3 (3)	1.76 ±1.5 (15)	69.65 ± 6.3 (592)	25.65 ± 3.3 (218)	2.59 ± 2.7 (22)

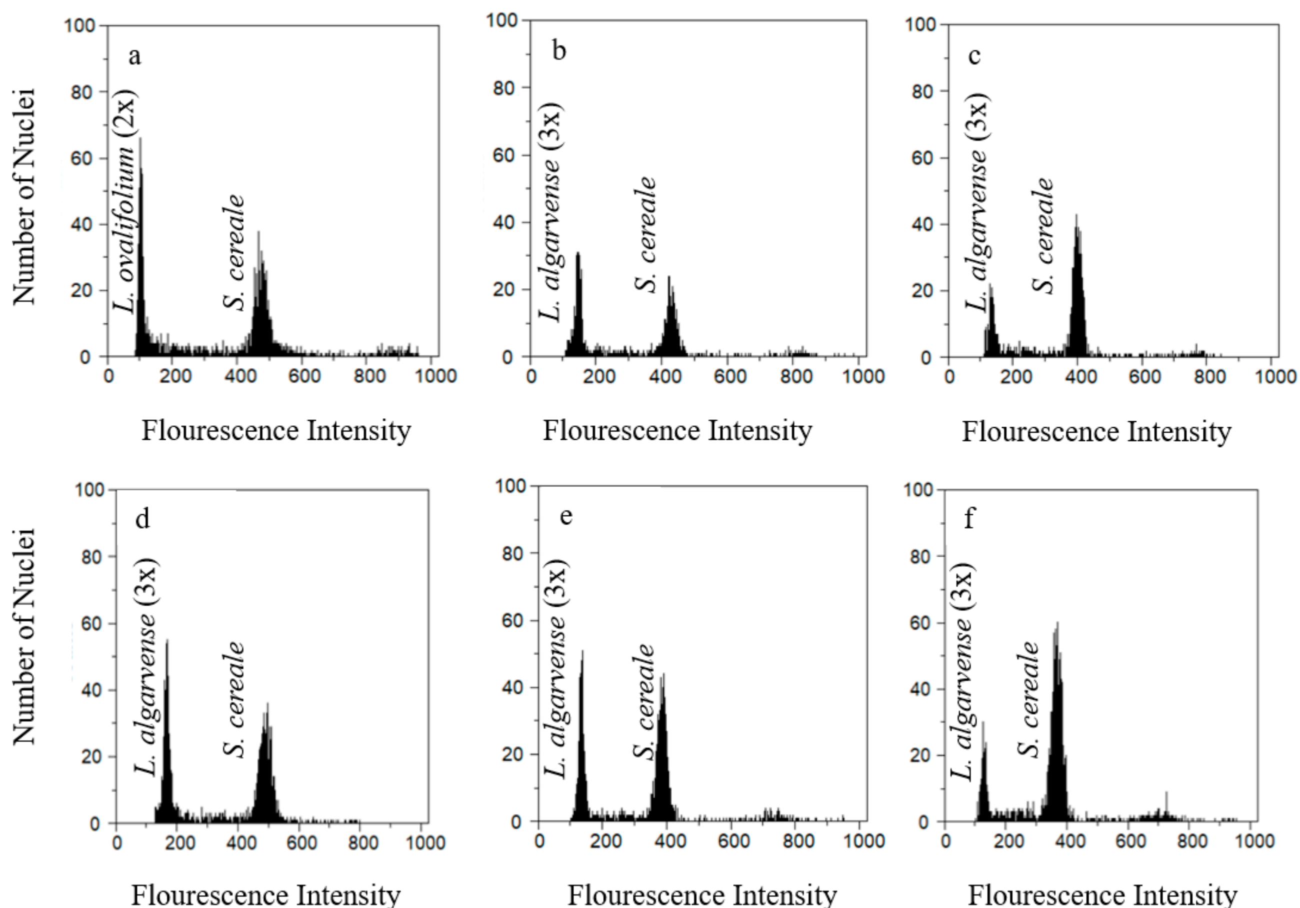

Figure 6. Flow cytometric histograms of seeds of *L. ovalifolium* **(a)** and *L. algarvense* (**b-f**).

1929). These plants can produce diploid, triploid and tetraploid progeny as well as populations of aneuploid individuals with diverse karyotypes (Henry et al., 2005).

During microspore formation, triploid *L. algarvense* showed diverse division anomalies related to chromosome pairing and segregation. In prophase I at pachytene, *L. algarvense* presented unpaired regions, probably as a result of a lack of chromosome homology in some of these regions and reduced recombination. At diplotene and diakinesis the presence of tri- and tetravalents involving non-homologous chromosomes reinforces the hypothesis of intergenomic recombination. Moreover, the tendency for chromosome nondisjunction and a high association of certain groups of chromosomes (with different size and morphology) revealed a high homology between some chromosome regions, which difficult their

normal dissociation during meiosis. Furthermore, the chromosome organization presented in Fig. 1k can be a clue to triploid *L. algarvense* hybrid origin. In this late anaphase I derived from a tripolar fuse, three distinct chromosome groups of six, nine and ten chromosomes were clearly visible, perhaps pointing to three genomes involved in this species formation. This meiotic behaviour strongly suggest parental genome differences for triploid *L. algarvense*, which may imply allopolyploid origin (i.e., attained by hybridization). Distinct genomes usually have several differences at chromosome level as well as modifications in sequence, structure, and/or gene order that difficult or inhibit homologous pairing (Ramsey and Schemske, 2002). These chromosome irregularities during the first meiotic division can be better explained by a broad FDR-type of meiotic restitution (De Storme and Geelen, 2013) or an indeterminate-type meiotic restitution (IMR-type) (Lim et al., 2001). In this latter case, meiotic non-reduction involved a reductional division of bivalents together with an equational segregation of univalents (Lim et al., 2001). Compared to diploid *L. ovalifolium*, triploid *L. algarvense* frequently showed fused nuclei at first division and second division, and dyads, triads, tetrads and polyads. In our study, co-existence of fused dyads and fused tetrads in the same nuclei spread is a strong indication of the occurrence of meiotic restitution. FDR- and/or SDR-type meiotic restitution were considered as important processes for polyploid formation in e.g., *Triticeae* (Jauhar, 2007; Ressurreição et al., 2012), *Solanum* (den Nijs and Peloquin, 1977), *Arachis* (Lavia et al., 2011), and *Taraxacum* (Van Dijk et al., 1999). These processes lead to unreduced gametes formation (Bretagnolle and Thompson, 1995; Brownfield and Köhler, 2011; De Storme and Geelen, 2013).

Callose is an essential barrier between meiocytes and defects on its deposition could lead to an ectopic genome doubling and cell fusion (Spielman et al 1997; Yang et al., 2003; De Storme and Geelen, 2013). Our results showed that nuclear fusion might occur before callose deposition, since throughout anther development its deposition was regular in both species. Another parallel phenomenon that occurred in both species was cytomixis that consists in the movement of the nuclei content between cells (Singhal et al., 2010; Kaur and Singhal, 2012; Mandal et al., 2013), and may lead to unreduced gametes. Although it was not obvious at which stage of meiosis cytomixis occurred, it probably took place at meiosis I before callose deposition. This phenomenon can be one of the precursors of chromatin bridges, micronuclei, triads and polyads, as found in *Spergularia diandra* (Kaur and Singhal, 2012).

Compared to the diploid species that produced regularly sized pollen (Róis et al., 2012), a great diversity of pollen morphology and size was revealed in the triploid species. As previously observed for diploid (*L. ovalifolium*) and tetraploid (*L. multiflorum*) *Limonium* species (Róis et al., 2012), our study supports that pollen size and ploidy are not correlated in the *Limonium* system. Moreover, a direct correlation seems to exist between pollen grain morphology, viability and pollen tube germination, since such processes were only observed in grains predominantly with three *colpi*. In *Limonium*, pollen viability appeared to be high in diploids whereas in polyploids, low to high fertility was reported (Erben, 1978). A high pollen viability was observed in triploid *Turnera sidoides*, which had irregular meiotic behaviour (Kovalsky et al., 2018).

In both diploid and triploid species studied here spontaneous seed production occurred, as insect pollinations were not frequent in our greenhouse. Both species showed a high percentage of seeds per scape, with moderate to high germination. The exact chromosome numbers and DNA ploidy level of the progenitor plants were determined in a previous study by combined flow cytometry and chromosome counting (Caperta et al., 2017). In the present study, flow cytometric seed screening investigations demonstrated that in both species only one DNA peak was found, which corresponds to the embryo peak, since mature seeds were characterized by one embryo and a well-developed starchy endosperm without nuclei (Róis et al., 2012). No quantitative variation in seed ploidy was found in the progeny of diploid or triploid plants and thus *L. ovalifolium* only produced a diploid progeny whereas *L. algarvense* originated a triploid progeny.

CONCLUSIONS

Triploid *L. algarvense* plants displayed extremely unbalanced meiotic cell division, probably originating non-functional aneuploid gametes. However, as found in other natural triploids (Ramsey and Schemske, 1998), these plants may also generate small numbers of euploid (x, 2x) gametes and 3x gametes via non-reduction. Even if the importance of these triploids as pollen donors is limited, they spontaneously produce viable seeds. Although, the reproductive mode of triploid *L. algarvense* is not yet determined, this species show stable populations widespread in the Iberian Peninsula and in Morocco (Caperta et al., 2017), probably originated by apomixis (asexual seed production).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Ana Paula Paes (LEAF/ISA) for help in plant maintenance and Wanda Viegas for useful comments and suggestions (LEAF/ISA).

FUNDING

This work was funded by Portuguese national funds through Fundação para a Ciência e a Tecnologia (FCT, www.fct.pt/) projects PTDC/AGRPRO/4285/2014 and UID/AGR/04129/2013, grants PTDC/AGRPRO/4285/BM/2014, BPD/UID/AGR/04129/2013 attributed to SIRC and ADC.

REFERENCES

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. Curr Opin Plant Biol 8(2): 135–141.

Alexander MP. 1969. Differential staining of aborted and nonaborted pollen. Stain Technol 44(3): 117–122

Arrigoni PV, Diana S. 1993. Contribution à la connaissance du genre *Limonium* en Corse. Candollea 48(2): 631–677

Bartolo G, Brullo S, Pavone P. 1981. Números cromosomáticos de plantas occidentales, 138-156. In: Anales del Jardín Botánico de Madrid (Vol. 38, No. 1), Real Jardín Botánico. Madrid, p. 288–299

Bretagnolle F, Thompson JD. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129(1): 1–22

Brownfield L, Köhler C. 2010. Unreduced gamete formation in plants: mechanisms and prospects. J Exp Bot 62(5): 1659–1688

Caperta AD, Castro S, Loureiro J, Róis AS, Conceição S, Costa J, Rhazi L, Espírito Santo D, Arsénio, P. 2017. Biogeographical, ecological and ploidy variation in related asexual and sexual *Limonium* taxa (Plumbaginaceae). Bot J Linn Soc 183(1): 75–93.

Castro M, Rosselló JA. 2007. Karyology of *Limonium* (Plumbaginaceae) species from the Balearic Islands and the western Iberian Peninsula. Bot J Linn Soc 155(2): 257–272.

Cowan R, Ingrouille M, Lledó M. 1998. The taxonomic treatment of agamosperms in the genus *Limonium* Mill. (Plumbaginaceae). Folia Geobot 33(3): 353–366.

D'Amato F. 1940. Contributo all'embriologia delle Plumbaginaceae. Nuovo Giorn Bot Ital 47(2): 349–382.

D'Amato F. 1949. Triploidia e apomissia in *Statice oleaefolia* Scop.var. *confusa* Godr. Caryologia 2(2): 71–84.

De Storme N, Geelen D. 2013. Pre-meiotic endomitosis in the cytokinesis-defective tomato mutant *pmcd1* generates tetraploid meiocytes and diploid gametes. J Exp Bot 64(8): 2345–2358.

De Storme N, Mason A. 2014. Plant speciation through chromosome instability and ploidy change: cellular mechanisms, molecular factors and evolutionary relevance. Curr Plant Biol 1: 10–33.

Den Nijs TPM, Peloquin SJ. 1977. Polyploid evolution via 2n gametes. Am Potato J 54(8): 377–386.

Erben M. 1978. Die Gattung *Limonium* im südwestmediterranen Raum. Mitt. Bot. Staatssamml. Münch. 14:361–626

Erben M. 1979. Karyotype differentiation and its consequences in Mediterranean *Limonium*. Webbia 34: 409–417.

Erben M. 1993. *Limonium* Mill. In: Castroviejo S, Aedo C, Cirujano S, Laínz M, Montserrat P, Morales R, Garmendia FM, Navarro C, Paiva J, Soriano C, editors. Flora Iberica ,Vol.3, Real Jardín Botánico, Madrid, CSIC p. 2–143

Fennane M, Ibn-Tattou M, El Oualidi J. 2014. Flore pratique du Maroc. Manuel de détermination des plantes vasculaires, Vol. 3. Travaux de l'Institut Scientifique, Série Botanique No 40. Rabat: Université Mohammed V de Rabat.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049-1051.

Greilhuber J, Doležel J, Lysak MA, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms "genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95: 255-260.

Henry IM, Dilkes BP, Young K, Watson B, Wu H, Comai L. 2005. Aneuploidy and genetic variation in the *Arabidopsis thaliana* triploid response. Genetics 170(4): 1979–1988.

Jauhar PP. 2007. Meiotic restitution in wheat polyhaploids (amphihaploids): A potent evolutionary force. J Hered 98(2): 188–193.

Kaur D, Singhal VK. 2012. Phenomenon of cytomixis and intraspecific polyploidy (2x, 4x) in *Spergularia diandra* (Guss.) Heldr. & Sart. in the cold desert regions of Kinnaur District (Himachal Pradesh). Cytologia (Tokyo) 77(2): 163–171.

Khan A, Rayner GD. 2003. Robustness to non-normality of common tests for the many-sample location problem. JAMDS 7(4): 187-206.

Kovalsky IE, Luque JMR, Elías G, Fernández SA, Neffa VGS. 2018. The role of triploids in the origin and

evolution of polyploids of *Turnera sidoides* complex (Passifloraceae, Turneroideae). J Plant Res 131(1): 77–89.

Lavia GI, Ortiz AM, Robledo G, Fernández A, Seijo G. 2011. Origin of triploid *Arachis pintoi* (Leguminosae) by autopolyploidy evidenced by FISH and meiotic behaviour. Ann Bot 108(1): 103–111.

Lim KB, Ramanna MS, de Jong JH, Jacobsen E, van Tuyl JM. 2001. Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103(2-3): 219–230.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann Bot 100: 875–888.

Mandal A, Datta, AK, Gupta S, Paul R., Saha A., Ghosh BK., Bhattacharya A, Iqbal M. 2013. Cytomixis — a unique phenomenon in animal and plant. Protoplasma 250(5): 985–996.

McClintock, B. 1929. A cytological and genetical study of triploid maize. Genetics 14(2): 180–222.

Matzk F, Meister A, Schubert I. 2000. An efficient screen for reproductive pathways using mature seeds of monocots and dicots. Plant J 21(1):97–108.

Musiał K, Kościńska-Pająk M, Antolec R, Joachimiak AJ. 2015. Deposition of callose in young ovules of two *Taraxacum* species varying in the mode of reproduction. Protoplasma 252(1): 135–144.

Ramanna MS, Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement - A review. Euphytica 133(1): 3–18.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29(1):467–501.

Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants. Annu Rev Ecol Syst 33(1): 589–639

Ressurreição F, Barão A, Viegas W, Delgado M. 2012. Haploid independent unreductional meiosis in hexaploid wheat. In: Andrew Swan, editor. Meiosis-molecular mechanisms and cytogenetic diversity. Dublin: Tech Press; p. 321–330.

Rim YW, Beuselinck PR. 1996. Cytology of 2N pollen formation and pollen morphology in diploid *Lotus tenuis* (Fabaceae). Am J Bot 83: 1057–1062.

Rodrigues MJ, Neves V, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L. 2016. *In vitro* antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: A comparison with green tea (*Camellia sinensis*). Food Chem 200: 322–329.

Rodrigues MJ, Soszynski A, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L. 2015. Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*. Ind. Crops Prod 77: 315–322.

Róis AS, Teixeira G, Sharbel TF, Fuchs J, Martins S, Espírito-Santo D, Caperta AD. 2012. Male fertility versus sterility, cytotype, and DNA quantitative variation in seed production in diploid and tetraploid sea lavenders (*Limonium* sp., Plumbaginaceae) reveal diversity in reproduction modes. Sexual Pl Reprod 25(4): 305–318.

Róis AS, Sádio F, Paulo OS, Teixeira G, Paes AP, Espírito-Santo D, Sharbel TF, Caperta AD. 2016. Phylogeography and modes of reproduction in diploid and tetraploid halophytes of *Limonium* species (Plumbaginaceae): evidence for a pattern of geographical parthenogenesis. Ann Bot 117(1): 37–50

Róis AS, Castro S, Loureiro J, Sádio F, Rhazi L, Guara-Requena M, Caperta AD. 2018. Genome sizes and phylogenetic relationships suggest recent divergence of closely related species of the *Limonium vulgare* complex (Plumbaginaceae). Plant Syst Evol 304: 955.

Singhal VK, Kaur S, Kumar P. 2010. Aberrant male meiosis, pollen sterility and variable sized pollen grains in *Clematis montana* Buch.-Ham. Ex DC. from Dalhousie hills, Himachal Pradesh. Cytologia 75(1): 31–36.

Spielman M, Preuss D, Li F, Browne WE, Scott RJ, Dickinson HG. 1997. TETRASPORE is required for male meiotic cytokinesis in *Arabidopsis thaliana*. Development 124(13):2645–2657

Van Dijk PJ, Tas ICQ, Falque M, Bakx-Schotman T. 1999. Crosses between sexual and apomictic dandelions (*Taraxacum*). II. The breakdown of apomixis. Heredity 83(6): 715–721.

Yang CY, Spielman M, Coles JP, Li Y, Ghelani, S, Bourdon V, Brown RC, Lemmon BE, Scott RJ, Dickinson HG. 2003. TETRASPORE encodes a kinesin required for male meiotic cytokinesis in *Arabidopsis*. Plant J 34(2): 229–240.

Zhang C, Fountain DW, Morgan ER. 1997. In vitro germination of the trinucleate pollen of *Limonium perezii*. Grana 36(5): 284–288.