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Plant Genetic Resources

Mode of reproduction and meiotic chromosome behavior in *Acroceras macrum*

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Abstract

Acroceras macrum Stapf is a high-quality C3 grass species adapted to the subtropics. It is a valuable forage resource in frequently flooded environments. The objectives were to analyze the type of polyploidy, mode of reproduction, and estimate seed and pollen fertility. A living collection of 22 tetraploid and five hexaploid genotypes of *A. macrum* was analyzed. Type of polyploidy was analyzed from chromosomal pairing at diakinesis and prometaphase I, and meiotic irregularities were evaluated during the microsporogenesis. Pollen viability was also examined. Bivalents predominated in tetraploids and trivalents in hexaploids. Meiotic abnormalities were more frequent in hexaploids than in tetraploids, and they were mainly related to mechanisms of chromosome nondisjunction. The reproductive mode was determined by cytological analysis of megasporogenesis and megagametogenesis, seed fertility under different pollination methods, and molecular progeny tests. In both tetraploid and hexaploid races, only the development of single meiotically derived embryo sacs was observed. Greater seed production in outcrossing compared with selfing indicated a primarily allogamous matting system. Progenies from crosses between genetically different tetraploid genotypes amplified male-specific molecular markers, indicating a hybrid origin and solid evidence of sexual reproduction. Population breeding approaches developed for sexual cross-pollinated species are suited for improving *A. macrum*.

1 | INTRODUCTION

Acroceras macrum Stapf is a grass species native to the tropics and subtropics of Africa. It is cultivated as a warm-season perennial forage in waterlogged soils throughout the world (Rhind & Goodenough, 1979). The species produces forage with high nutritional quality, a characteristic related to its C_3 carbon metabolism (Zuloaga, Morrone, & Saenz, 1987). Information about ploidy levels, cytogenetics, reproduction mode, and fertility of *A. macrum* is scarce. Chromosome counts included tetraploid ($2n = 4x = 36$) (Moffett & Hurcombe, 1949; Rhind & Goodenough, 1976, 1979), pentaploid ($2n = 5x = 45$), and hexaploid ($2n = 6x = 54$) cytotypes (Rhind & Goodenough, 1976, 1979).

The diversity of an *A. macrum* collection brought to Argentina in the 1990s was previously explored (Ferrari Usandizaga et al., 2014). Genetic diversity was studied by molecular (inter-simple sequence repeat [ISSR]) and phenotypic (morphological and agronomical) markers, and 27 genotypes were found and grouped, forming two clusters that corresponded to $4x$ and $6x$ cytotypes. The diversity found in the $4x$ germplasm proved to be suitable to initiate a breeding program. The mode of reproduction of *A. macrum*, required

Abbreviations: GD, genetic distances; ISSR, inter-simple sequence repeat; MMC, megaspore mother cell; PMC, pollen mother cell.

for conducting a breeding program, has not been determined through concluding assessing methods. Additionally, knowledge about its type of polyploidy and fertility needs to be acquired, since the most important breeding objectives are related to seed characteristics, such as seed fertility, retention, and yield.

Pathways to form seeds are classified according to the origin of the megagametophyte, the type of pollination, and fertilization of gametes. Those with a single embryo being from a megagametophyte originated by reductional meiosis, followed by double fertilization, have a sexual origin. Those with embryos from one or more megagametophyte originated from any of the nonreducing, nonhybridizing mechanisms are apomictic (Nogler, 1984; Ozias-Akins, 2006). The most direct and traditional form to study the reproduction mode is the observation of the embryo sacs and the processes of the megagametogenesis pathway (Koltunow, 1993). Among the Paniceae tribe, some of the apomictic pathways are detectable by microscopy methods by observing embryo sacs (Acuña, Martínez, & Quarin, 2005; Chapman & Busri, 1994; Kozub et al., 2017; Kumar, Chandra, Gupta, & Shukla, 2010; Naumova, Hayward, & Wagenvoort, 1999; Ortiz et al., 2013; Philipson, 1978; Quarin, 1994; Quarin & Burson, 1991; Quarin & Hanna, 1980; Warmke, 1954; Weiler et al., 2017). However, diplospory could be difficult to differentiate from sexual ones (Meier, Zappacosta, Selva, Pessino, & Echenique, 2011). Diversity analysis by phenotypic or genotypic traits is one of the current support tools to study the reproduction mode of populations and through a proper design of cross progeny test. Molecular genetic markers are the most reliable for the study of segregation among progeny to infer the mode of reproduction (Acuña et al., 2005; Daurelio, Espinoza, Quarin, & Pessino, 2004; Schranz el al., 2006; Meier et al., 2011; Barrett, 2013; Garcia, Vigna, Sousa, Jungmann, & Cidade, 2013; Kozub et al., 2017). Adequate markers would be unlinked and monogenically inherited.

Fertility is a determinant factor for a sexual species, and it depends in part on regular chromosome pairing and its segregation during meiosis. The more sets of chromosomes interact, the more susceptible they will be to adopting abnormal arrangements (Stebbins, 1947). The occurrence of meiocytes with unequal chromosome number, nonviable and heterogeneous pollen grains, or aneuploid gametes is common in newly formed polyploid (Ramsey & Schemske, 2002). Studying the chromosome associations, it is possible to infer about the type of polyploid, and its evolution degree, related to its stability and its fertility (de Wet, 1979).

The mating system (selfing vs. outcrossing rate of a plant) is a determinant factor of the genetic segregation pathway in its progeny. It is subject to genetic and environmental factors, so the same plant could have a wide range of variation (Acquaah, 2012; Phoelman & Sleeper, 1995; Vogel & Burson, 2004). In a system like a natural population, where selfing and/or a diversity of combinations are able to occur, and apomixis could not be excluded, modeling the mating system is possible (Brown, 1990; Barrett, 2003, 2013; Dart & Eckert, 2013) but should be complicated in very little known species like *A. macrum*.

Acroceras macrum has a hermaphrodite floret and a male one growing together in its spikelets (Zuloaga et al., 1987), and an adequate experimental approach to evaluate its mating system should be considered. The relation between both pollination systems, autogamy and allogamy, should be obtained with the adequate management of the emasculation or no emasculation and isolation of inflorescences to promote selfing or outcrossing (Dart & Eckert, 2013; Gutiérrez, Medan, & Pensiero, 2006; Novo, Montenegro Valls, Galdeano, Honfi, & Espinoza, 2016).

The objectives of this paper were (i) to determine the mode of reproduction of the tetraploid and hexaploid germplasm of *Acroceras macrum*, (ii) to analyze the type of polyploidy present in this species, and (iii) to estimate seed and pollen fertility.

2 | MATERIALS AND METHODS

2.1 | Vegetal material

A collection of 22 4*x* and five 6*x* genotypes of *Acroceras macrum* originally belonging to the Agricultural Research Council (ARC) in Cedara, South Africa was established at the Corrientes Experimental Station of the National Agricultural Technology Institute (INTA) in Argentina, as was described by Ferrari Usandizaga et al. (2014). Small cuttings of each material in the collection were planted in September 2012 in Corrientes, Argentina, at the field of the Agrarian Science School of the Northeast National University (UNNE) (27°27′27″ S, 58°49′23″ W) following a randomized complete block design with 27 genotypes and two replications. Genotypes A5–A24, A49, and A25 were tetraploid, and A26–A30 were hexaploid.

2.2 | Megasporogenesis and megagametogenesis

From December 2012 to February 2013, both replications of the assay were sampled for inflorescences from eight 4*x* and two 6*x* genotypes, between 8:00 and 9:00 AM. These 10 genotypes were randomly selected from the germplasm bank attempting to represent the contained diversity (Ferrari Usandizaga et al., 2014). Inflorescences containing spikelets at different developmental stages were fixed for 24 h in FAA (formalin–glacial acetic acid–70% ethanol, 1:1:18 v/v/v) and stored for at least 24 h in 70% ethanol at 4°C. Fertile (upper) florets from different positions in the panicle were

dissected to sample the distinct maturity stages of pistils. Young pistils were obtained from panicles where < 50% had emerged, in surrounding positions from the source of anthers with prophase meiotic stage pollen mother cells (PMCs). Mature ovaries were obtained from florets at anthesis. Pistils at immature stages came from florets on branches below the source of mature pistils and above the source of young pistils. Pistils were clarified following the instructions described in Zilli et al. (2015) with some modifications, and embryo structures were observed. Modifications were as follows: pistils were kept 30 min in a 10% H_2O_2 solution for a first bleaching. Then, the solution was renewed and pistils were kept in it for 1–2 h for effective discoloration. As a last step, pistils remained in 100% methyl salicylate for 90 min before being observed. If they were not observed on the clearing day, they were kept in 100% methyl salicylate to be observed the next day. Pistils were mounted on a glass slide for microscopic observation of embryo structures, using a light microscope with a differential interference contrast device, and then they were photographed with a Leica EC3 camera. The process of embryo sac formation was followed in ovules at different stages of maturity. At least 30 pistils of each stage and each genotype were observed. When the pistil had an ovule rotated 90–45° with respect to the axis of the floret, it was considered a young pistil (Supplemental Table S1). The ovule was considered intermedium-mature when an angle of 45° with respect to the floret axis was observed (Supplemental Table S1).

2.3 | Male meiosis and gametogenesis

Young nonemergent inflorescences were collected and fixed in an ethanol and acetic acid 3:1 solution for 24 h. Fixed inflorescences were stored in 70% ethanol at 4°C. The meiotic stage of the spikelets was screened along the inflorescence: from the younger spikelet sited on the base to the most mature on the tip. Anthers were removed and gently squashed on a drop of 2% acetocarmine over a glass slide to liberate and stain the PMCs. The PMCs were observed in a light microscope checking the chromosomal status. Analyses of meiotic chromosomes were performed at pachytene, diakinesis, metaphase I, and anaphase II. Preparations with chromosome at diakinesis or metaphase were used to study the meiotic chromosome behavior. The regularity of the pollen formation processes was also observed.

2.4 | Chromosome associations

Where the stage was diakinesis or prometaphase, meiotic preparations were observed for analyzing the chromosomic associations. At least 15 meiotic cells were studied, in four tetraploid hybrids and two hexaploids. To determine the polyploid types, present chromosomic associations were analyzed, in both cytotypes (Espinasse, 1982). Meiotic chromosome configurations were observed under 100 × oil magnification. The frequency of univalents, bivalents, and polyvalents was calculated in each cell, and the most frequent arrangement was recorded for both ploidy levels.

2.5 | Pollen fertility

Three flowers per genotype per replication were sampled, just before the anthesis occurred. Pollen preparations consisted of fresh staining of pollen grains liberated by squashing of anthers on a 2% I_2–KI solution. Samples of 1000 grains were evaluated under a microscope. The viability of pollen grains was estimated for each genotype from their stainability.

2.6 | Pollination system and seed set

Seed setting in outcrossing (open-pollination and crosses between selected genotypes) and selfing were assayed. Crosses between different genotypes were made with two approaches: without emasculation in the field and with emasculation in a greenhouse. The combinations of parents were randomly selected, attempting to make the maximum number of crosses during the flowering season. Self- and open-pollination were performed in the field. The field assay had two replications of each genotype.

2.6.1 | Open-pollination

Three inflorescences per genotype and per replication were randomly selected from the field assay on the first day of blooming. Inflorescences were enclosed in sulfite paper bags a day after anthesis was observed in all florets, remaining enclosed for 20–30 d, to ensure complete seed maturation.

2.6.2 | Crosses without emasculation

At least three inflorescences randomly selected per female parent and per replication were dusted with pollen from each selected male parent. Panicles were bagged a day before anthesis. The following day, these inflorescences were hand-pollinated with pollen from the selected donor and immediately bagged to avoid contamination. The treatment was repeated to the end of blooming.

2.6.3 | Directed crosses with emasculation

Selected female parents were grown in pots with three replications in a greenhouse. Crosses were made with the help of

a humidity chamber. Three panicles in its first day of blooming or with < 10% of bloomed flowers were selected for each cross. Plants were placed into the chamber in the afternoon of the day before anthesis. Flowers were emasculated just after anthesis and pollinated with pollen from the selected male parent. Inflorescences were immediately bagged after pollination. The procedure was repeated for 3–5 d until blooming occurred in all florets of inflorescences. Inflorescences remained enclosed for 20–30 d until harvest.

2.6.4 | Self-Pollination

Three inflorescences per genotype and per replication of the field assay were randomly selected on the first day of blooming. A day before anthesis, inflorescences were enclosed in sulfite bags and remained that way for 20–30 d before harvest. Seed set was estimated as the fraction of spikelets that effectively develops a mature caryopsis. The reproductive efficiency of each combination was calculated as the percentage of pollinated spikelets that resulted in plants. Caryopses were sown in sterilized soil, and the obtained plants were counted.

2.7 | Statistical analyses

InfoStat 2016 software (Di Rienzo, Casanoves, Balzarini, González, & Tablada, 2016) was used for the Shapiro and Wilks test (modified) for testing the normal distribution of the data. Data were normalized when necessary, and the ANOVA and the Tukey honestly significant difference test were used for multiple mean comparison ($p < .05$).

2.8 | Progeny test

Seed from four homoploid tetraploid crosses with no emasculation (performed at the field) was used to assess the mode of reproduction. Crosses were between the genotypes A15 × A9, A16 × A12, A19 × A8, and A24 × A9. It was considered that female parents were exposed to their own pollen, and to pollen from the selected male parent. The hybrid origin was tested by a paternity test with ISSR molecular markers. The ISSR primers detected as polymorphic in *A. macrum* by Ferrari Usandizaga et al. (2014) were assayed to detect specific markers of the male parent in each family. The DNA was isolated from the young leaf of any putative hybrid and its parents. The DNA extraction procedure and ISSR protocols and screening methods were described in Ferrari Usandizaga et al. (2014). The amplification of at least two male-specific markers was required to identify plants as hybrids.

3 | RESULTS

3.1 | Megasporogenesis and megagametogenesis

Figure 1 represents the process from the megaspore mother cell (MMC) differentiation to the maturation of the embryo sacs.

3.1.1 | Megasporogenesis

The ovule rotated in an angle of ~90° to the axis of the floret, and the MMC became elongated and was surrounded by a layer of cells greatly enlarged (Figure 1a). Outer and internal integuments grew occupying most of the surface of nucellus (Figures 1a–1d). The ovule gradually turned from the angle of 90° to the axis of the floret and continued its rotation to become completely anatropous at the end of megasporogenesis (Figure 1b–1e). A linear triad or tetrad of megaspores was visible (Figure 1b). Two or three megaspores nearest to the micropyle degenerated, leaving the chalazal member as the functional megaspore (Figures 1b–1d).

Most ovules from younger pistils were in Stage 1 (MMC is visible, Figure 1a) (Figure 2a). Some of them were in Stage 2 (four megaspores, Figure 1b; or functional megaspore plus the three degrades ones, Figure 1d) (Figure 2a).

3.1.2 | Megagametogenesis

A binucleate bipolar stage resulted from successive mitotic divisions of the functional megaspore (Figure 1e). A four-nucleate (Figure 1f) and an eight-nucleate stage were observed. Nuclei were separated by a central vacuole. In both cytotypes (4*x* and 6*x*), it developed into a meiotic embryo sac characterized by an egg apparatus with the egg cell and two synergids, a large central cell with two polar nuclei, and a clump of antipodal cells that crowd at the chalazal end (Figures 1e–1h). Embryo sacs had from 3.1–4.5 antipodal cells. The overall mean of antipodal cells was 3.6, the same for both cytotypes.

However, female gametogenesis could be interrupted at different points. The functional megaspore could lag its enlargement and eventually degenerated, whereas the ovule continued its rotation. On average, 8.7% of pistils had a visible MMC that did not initiate division (Supplemental Table S1). Otherwise, most pistils from intermedium maturity were at Stage 3 (bi- or four-nucleate structures, Figure 1e–1g) or Stage 4 (earliest or mature eight-nucleate embryo sac, Figure 1h–1k) (Figure 2b). It seemed they had reverted arrests or had not suffered one. The arrest followed by degradation was seen after the formation of the MMC and before maturation of

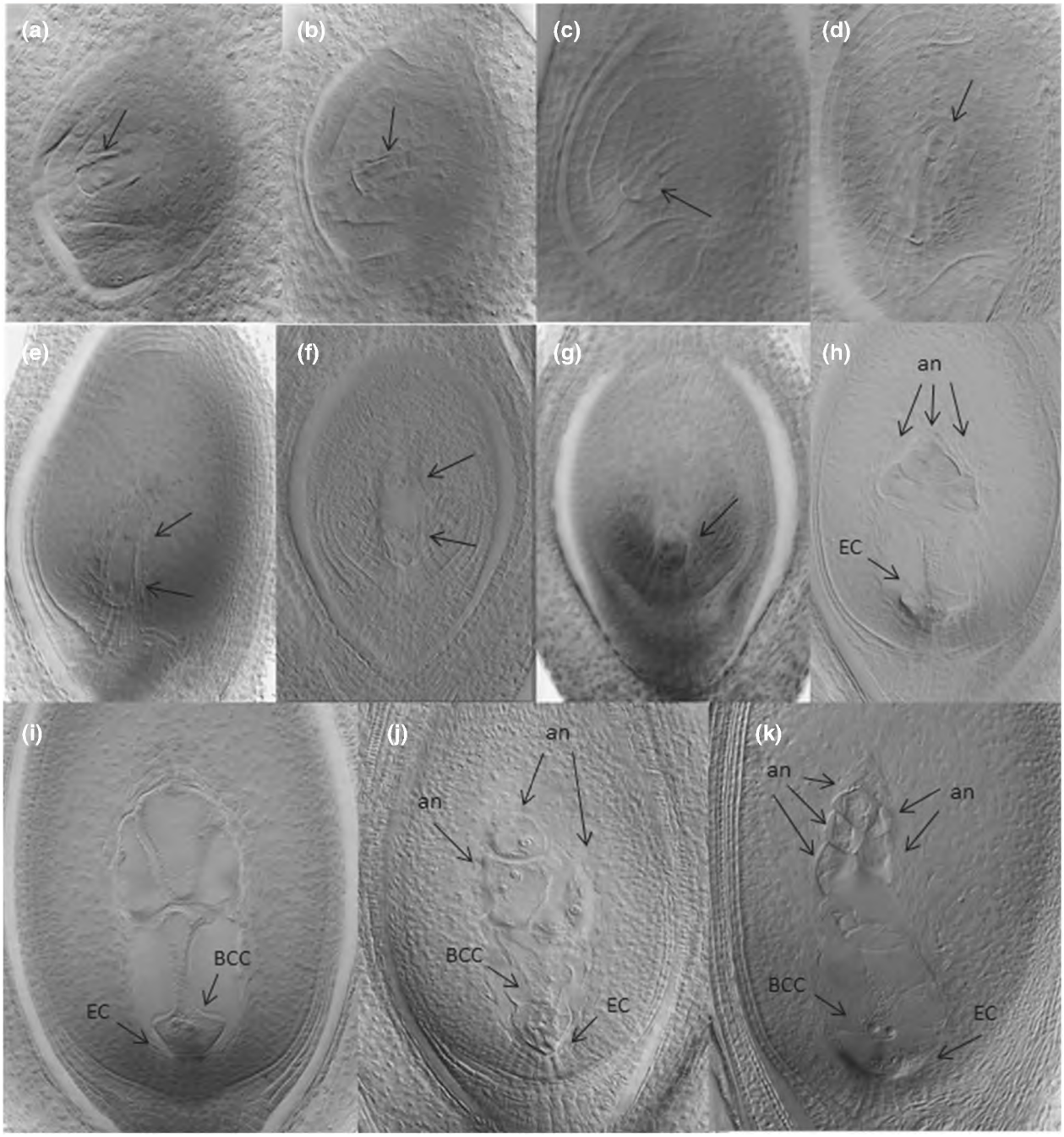

FIGURE 1 Development of the embryo sac at clarified ovaries. (a) Megaspore mother cell (MMC) at prophase (arrow). (b) Megaspores produced at meiosis of the MMC (arrow). (c) Degradation of micropylar megaspore (arrow). (d) Functional megaspore (arrow) and remaining of micropylar megasporocytes. (e) Binucleate stage (first functional megaspore mitosis). (f) Tetranucleated stage (second mitosis) and polarization of nuclei (arrows). (g) Nuclei grouped at micropylar pole (arrow). (h–k) Embryo sac. (h) Maturing embryo sac. (i) *Polygonum*-like embryo sac. (j) Nuclear proliferation of antipodes. (k) Poaceae like embryo sac, containing five antipodal cells. an, antipodal cell; EC, egg cell; BCC, binucleate central cell

the embryo sac. These cases were considered as abortions. In addition, the primordial embryo sac could lag its normal evolution and be aborted, shrinking the cytoplasm, mostly at the four- or eight-nucleate stages. Abortions in 4*x* genotypes were significantly lower than in the 6*x* ones: 8.1 vs. 22.2% (Figure 2c).

3.2 | Meiosis and microgametogenesis

Anthers at different stages of maturity were used, and PMCs at different stages of meiosis I and meiosis II were studied. Megasporogenesis and microsporogenesis occurred in neighboring positioned florets in the same panicle,

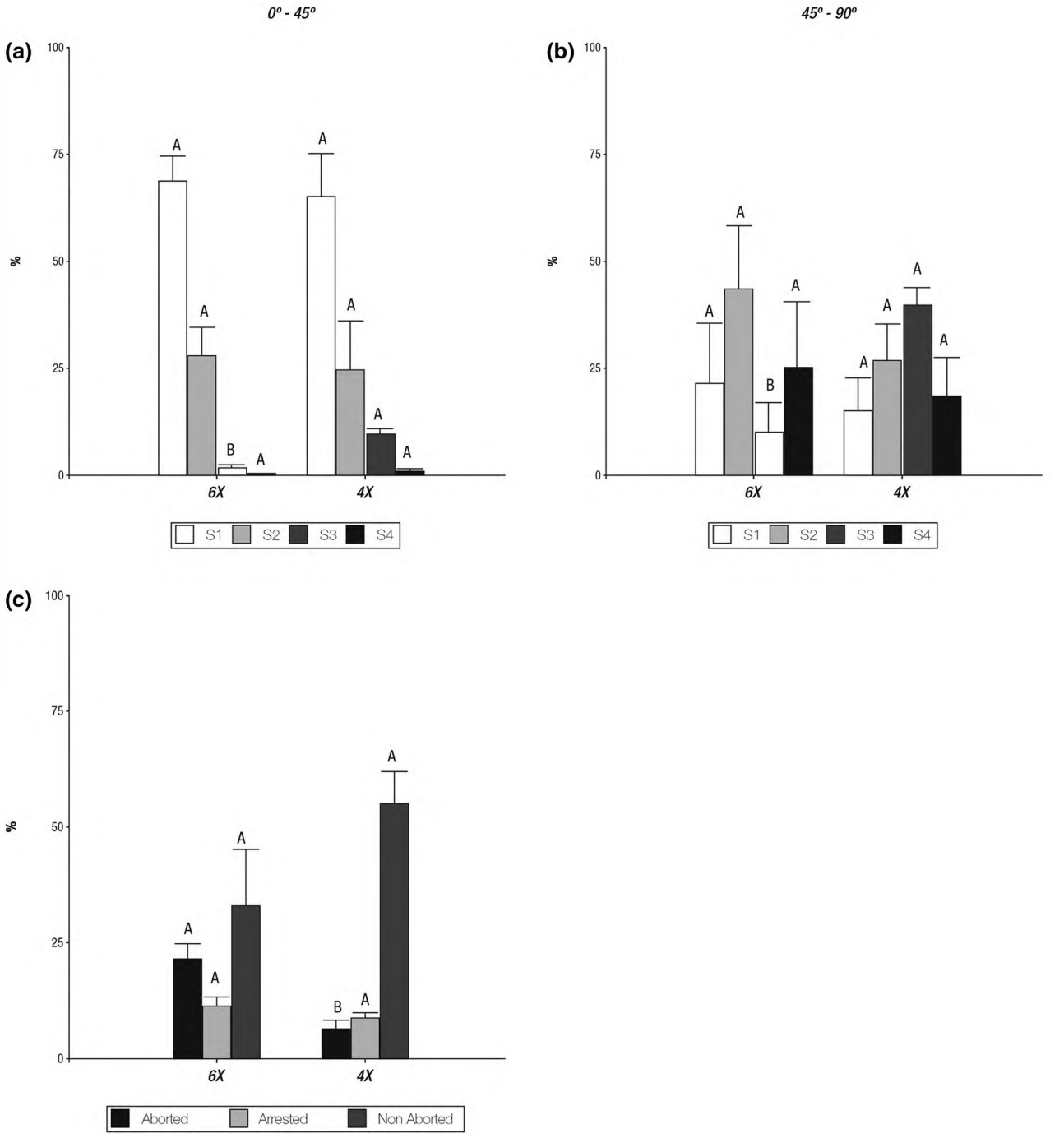

FIGURE 2 Observations of cleared pistils from six tetraploid (4*x*) and two hexaploid (6*x*) genotypes of *Acroceras macrum*. (a) Comparison between stages observed in 4*x* and 6*x* young pistils (those with a rotation angle from 0–45°). (b) Comparison between stages observed in 4*x* and 6*x* medium maturity and mature pistils (those with a rotation angle from 45–90°). Stage 1 (S1): a prophasic megaspore mother cell was observed. Stage 2 (S2): four megaspores or one functional megaspore and the remaining of the degraded other three were observed. Stage 3 (S3): the steps of cellularization in megagametogenesis where the number of cells was from two to eight. Stage 4 (S4): the last steps where a primordial sac or a mature sac is present. (c) Comparison between tetraploid (4*x*) and hexaploid (6*x*) means of arrest, abortions, and nonaborted pistils, observed for the genotypes and stages studied. Different letters indicate significant differences according to Tukey test ($p < .05$)

indicating that both processes took place at similar times. The PMCs from anthers of the same flower were mainly in the same stage of meiosis. Arrangement of chromosome associations was analyzed at diakinesis, and abnormalities were analyzed among of all the meiotic process. The meiotic behavior of chromosome PMCs from floral buds of 4*x* plants is represented in Figure 3, and the one from 6*x* is represented in Figure 4. Frequencies of the distinct

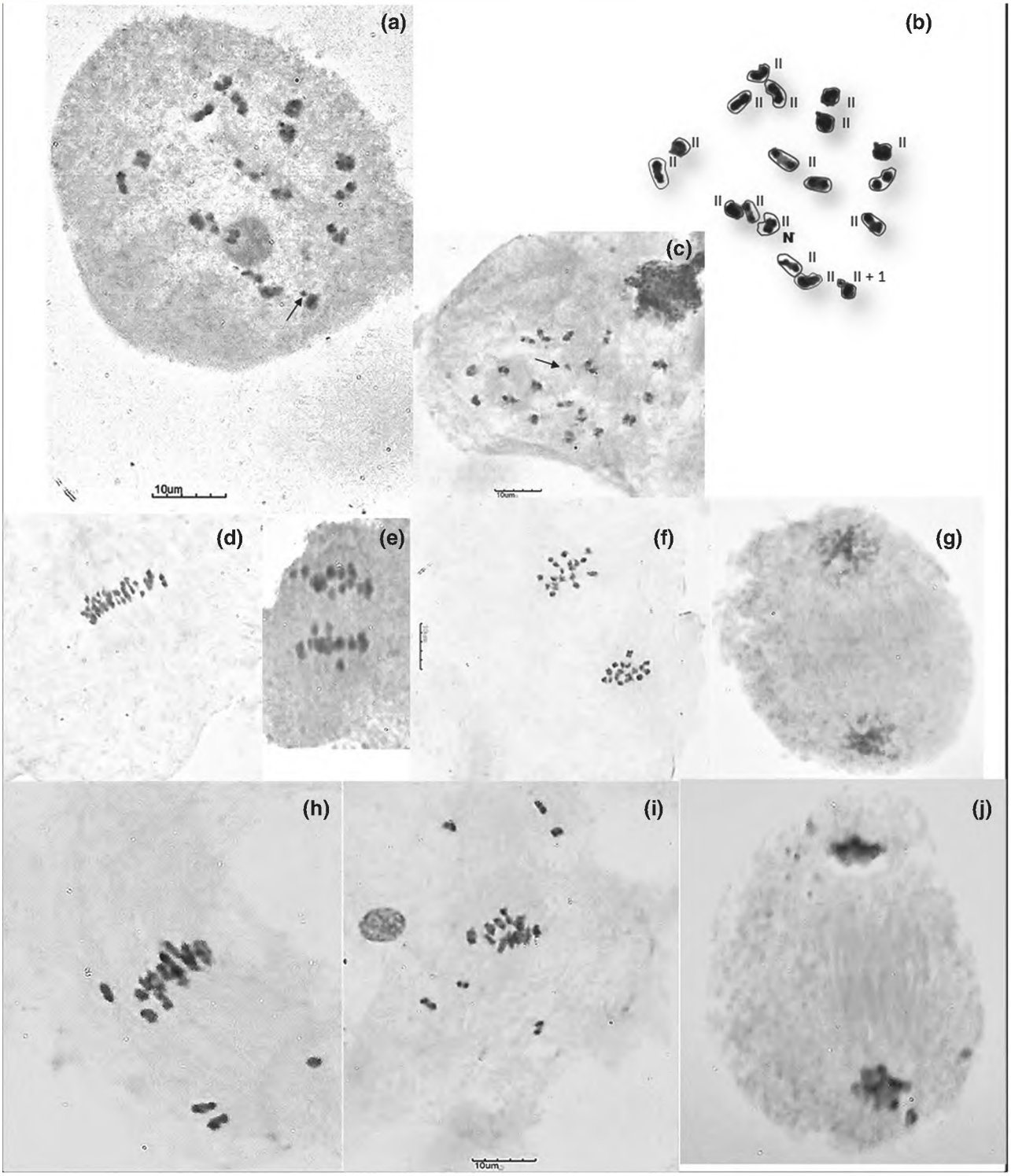

FIGURE 3 *Acroceras macrum* tetraploid cytotype microsporogenesis ($2n = 4x = 36$). (a–c) Pollen mother cell (PMC) chromosomal associations. The arrows indicate the presence of a putative B chromosome. (a) PMC at diakinesis. (b) Schematic copy of (a). (c) PMC at prometaphase I. (d–g) Regular meiotic stages. (d) Metaphase I. (e and f) Anaphase I; at (e), 18 chromosomes were at poles. (g) Telophase I. (h–k) Meiotic abnormalities. (h) PMC at metaphase I with lagged bivalents. (i) PMC at anaphase I with difficulties of bivalent disjunction. (j) Laggard chromosomes (arrows) at telophase I. In (b), II = bivalent. The bar scale indicates 10 µm

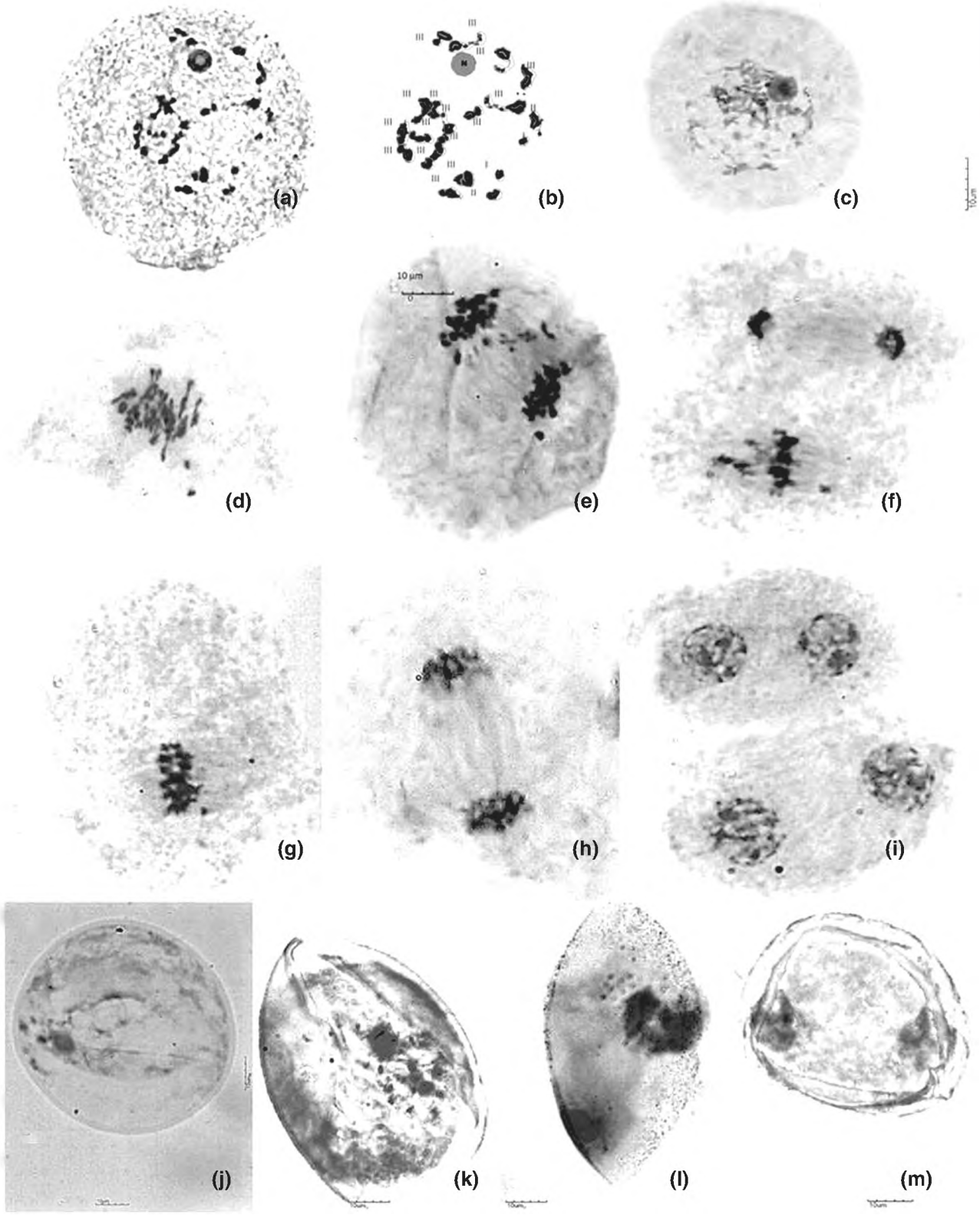

FIGURE 4 *Acroceras macrum* hexaploid cytotype microsporogenesis ($2n = 6x = 54$). (a) Pollen mother cell (PMC) at diakinesis with diverse types of chromosomal associations. (b) Schematic copy of (a). (c–f) Meiotic abnormalities. (c) PMC at prophase I with disorganized chromosomes, after the disappearance of the nucleus. (d) PMC at anaphase I and chromosomic disjunction difficulties: lagged univalents and remaining chromosomes at the equatorial plane, not pairing, wrong orientation, and stickiness of chromosomes. (e) PMC at anaphase I with lagging chromosomes. (f) Asynchronic dyads at metaphase II and lagging chromosomes. (g–i) Regular meiotic stages. (g) Metaphase I. (h) Anaphase I. (i) Telophase II. (j–m) Microgametogenesis. In (b), I = univalent, II = bivalent, III = trivalent. The bar scale indicates 10 µm

TABLE 1 Chromosomal associations at meiosis of tetraploid (4*x*) and hexaploid (6*x*) cytotypes of a germplasm collection of *Acroceras macrum*

Ploidy level	No. studied PMCs[a]	Average (range)[b]					
		I	II	III	IV	V	VI
4*x*	65	0.17 (0–2)	16.09 (12–18)	0.11 (0–1)	0.83 (0–3)	0	0
6*x*	30	0.30 (0–3)	2.20 (0–6)	13.26 (8–18)	0.27 (0–2)	0.33 (0–1)	1.13 (0–4)

[a]PMC, pollen mother cell.

[b]I, univalent; II, bivalent; III, trivalent; IV, tetravalent; V, pentavalent; VI, hexavalent.

chromosomal associations at diakinesis and prometaphase from first meiotic division are summarized in Table 1 for both cytotypes.

Most of diakinetic PMCs from 4*x* plants had 18 bivalent chromosome associations (Figure 3b). To a lesser extent, 16 bivalents plus one quadrivalent or 14 bivalents plus two quadrivalent associations were observed. Unpaired chromosomes, trivalent associations, or associations of more than four chromosomes were unusual. Trivalent chromosome associations were the most usual for the 6*x* cytotype, in a range from 8–18. Also common were bivalent and hexavalent associations, present in variable quantity (with a maximum of six or four, respectively). Arrangements with univalents, quadrivalents, or pentavalents had a lesser presence (Table 1).

Meiotic abnormalities were detected in both genotypes, with higher frequency in PMCs for 6*x* plants. The general frequency of meiotic irregularities in PMCs from 4*x* plants was 0.48, and in 6*x* was 0.78. The microspores were spherical, had a large central nucleus, and were beginning to develop their callose wall. Mitotic microgametocytes were also observed. Their walls seemed thicker than microspore walls, and some of them had two peripheral nuclei in a position opposite from the pore (Figures 4j–4m).

Male meiotic abnormalities in 4*x* were observed mainly when the PMCs elapsed from prophase to metaphase and from metaphase to telophase—in either, the first or the second meiotic division. One to five lagging bivalent associations with misguided orientation in the equatorial plate (Figures 3h–3i) were the most frequently observed abnormalities. A small, spherical, solitary, and well-staining structure, putatively a B chromosome, was frequently observed in tetraploids PMC (Figures 3a–3c).

Abnormalities were observed among all the meiotic process in the 6*x* microsporogenesis. Some PMCs failed to organize the chromosomes in prophase I, even when the nuclear membrane had completely disappeared (Figure 4c). The most frequent metaphase aberrations were wrongly oriented, lagging, or out-of-equatorial-plane chromosomes. Adhesive or precocious migrating chromosomes were observed at anaphase (Figures 4d–4f). Differently sized dyads after meiosis I, asynchronous sister dyads at meiosis II, and varying sized post-meiotic microsporocytes were also observed.

3.3 | Pollen and seed fertility

The mean viable pollen for all the collection was 40.8% (Figure 5a). Fertility of the pollen grains showed highly significant differences both within and between cytotypes (Figure 5b). The mean pollen viability on the 4*x* cytotype was 43.3%, which was significantly higher than the mean of 26.2% of the 6*x* cytotype. The viability of pollen grains from different 4*x* genotypes presented a wide range of values from 8.9–69.6% (Figure 5b). The viability of pollen grains from 6*x* genotypes was less variable, with a range from 22.3–32.9% (Figure 5b).

Seed fertility was remarkably different in function from the pollination system considered. Seed set resulting from self-pollination was low, with a general mean value of 4%. The 6*x* genotypes did not set seed, and 4*x* cytotypes did it with a significantly higher mean value of 5.1, but no variation ($p > .05$) among the 4*x* cytotypes was observed (Figure 5a). Seed set on open-pollination was significantly different within and between both ploidy levels. The 4*x* genotypes had a significantly higher seed set with a mean of 34.5% and a range from 3.7–69%, in comparison with the 6*x* genotypes, where the mean was 1.5% and the values ranged from 0.2–3.7% (Figure 5a).

The pollen viability of the 4*x* cytotype was positively correlated with seed set under open-pollination ($r = .43, p = .0034$). No correlation between these variables was found for the 6*x* cytotype.

3.4 | Crosses

A total of 1157 spikelets were hand-pollinized in 15 crosses with emasculation, 13 tetraploid homoploid crosses (4*x* × 4*x*), and two heteroploid ones (one 4*x* × 6*x*, and one 6*x* × 4*x*) (Figure 6a). Crosses had different genetic distances (GD) obtained from Ferrari Usandizaga et al. (2014). Homoploids tetraploids crosses were in a range of 0.57–0.68 Jaccard units, and heteroploids ones had a GD of 0.71 Jaccard units (Figure 6a).

A total of 1725 spikelets were hand-pollinized in 22 crosses with no emasculation, 10 tetraploid homoploid crosses (4*x* × 4*x*), two hexaploids homoploids (6*x* × 6*x*), and

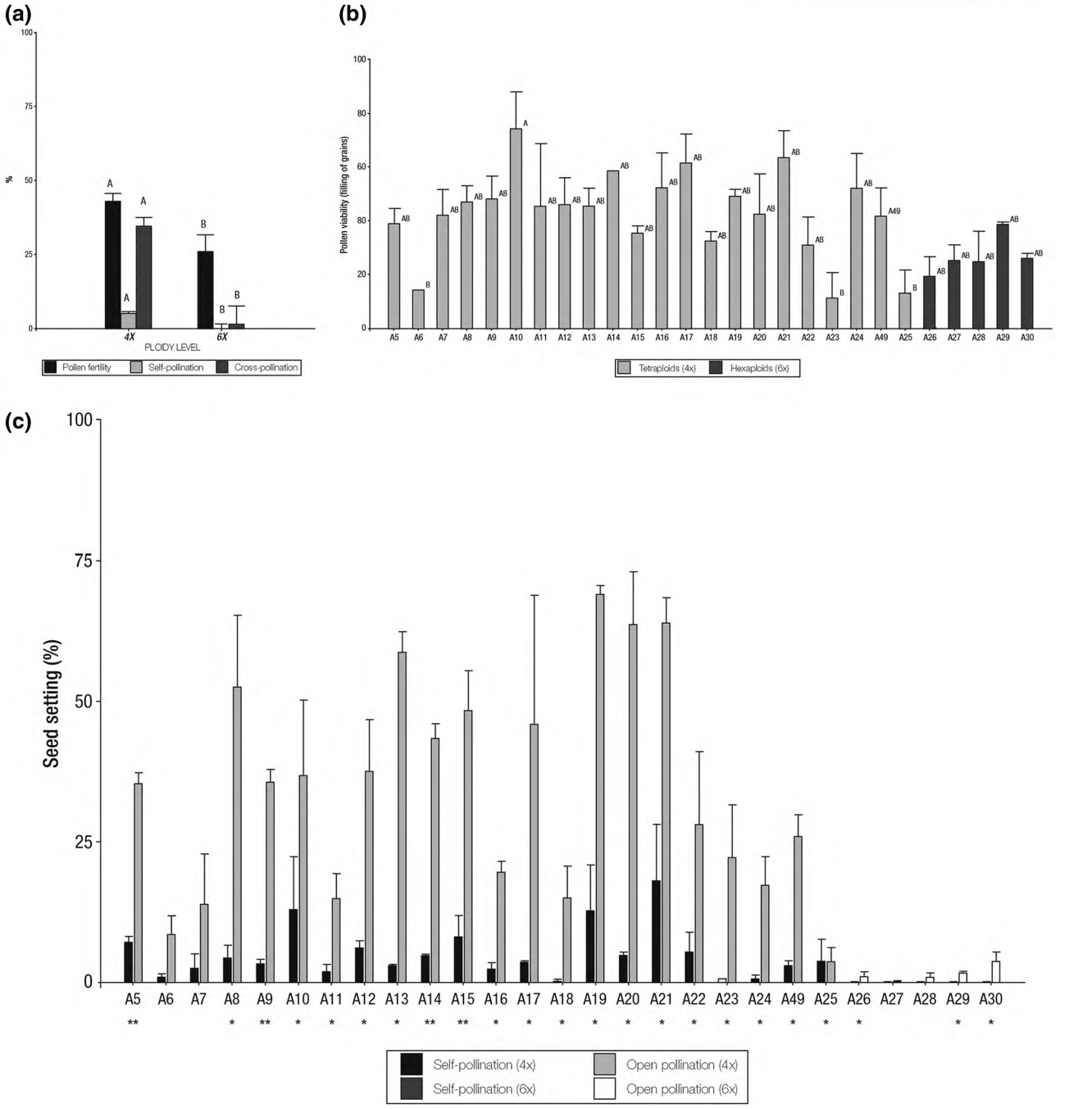

FIGURE 5 Percentage of viable pollen grains (determined by filling of grains) and seed set, on self-pollination and open-pollination conditions of a collection of 27 genotypes of *Acroceras macrum* tetraploids (4*x*) and hexaploids (6*x*). (a) Pollen fertility, self- and open-pollination comparison between tetraploid and hexaploid cytotypes. (b) Filling of pollen grains means comparison between the studied genotypes. Different letters indicate significant differences according to Tukey test ($p < .05$). (c) Comparison between self- and open-pollination seed setting. *$p < .05$, and **$p < .01$

10 heteroploids (four 4*x* × 6*x*, and six 6*x* × 4*x*) (Figure 6a). Crosses had different GD obtained from Ferrari Usandizaga et al. (2014). Homoploids tetraploids crosses were in a range of 0.48–0.67 Jaccard units, homoploids hexaploids ones were 0.22 and 0.61 Jaccard units, and all the hetroploids had a GD of 0.71 Jaccard units (Figure 6a).

Reproductive efficiency was remarkably different in function of the cytotypes involved in crosses, depending on the genotype combination. Homoploid crosses between 4*x* parents were the most productive. They resulted in an average reproductive efficiency of 7% (Figure 6b). When the female parent was 6*x*, no seed was produced regardless if the nature of the crossing was homoploid or heteroploid. Heteroploid

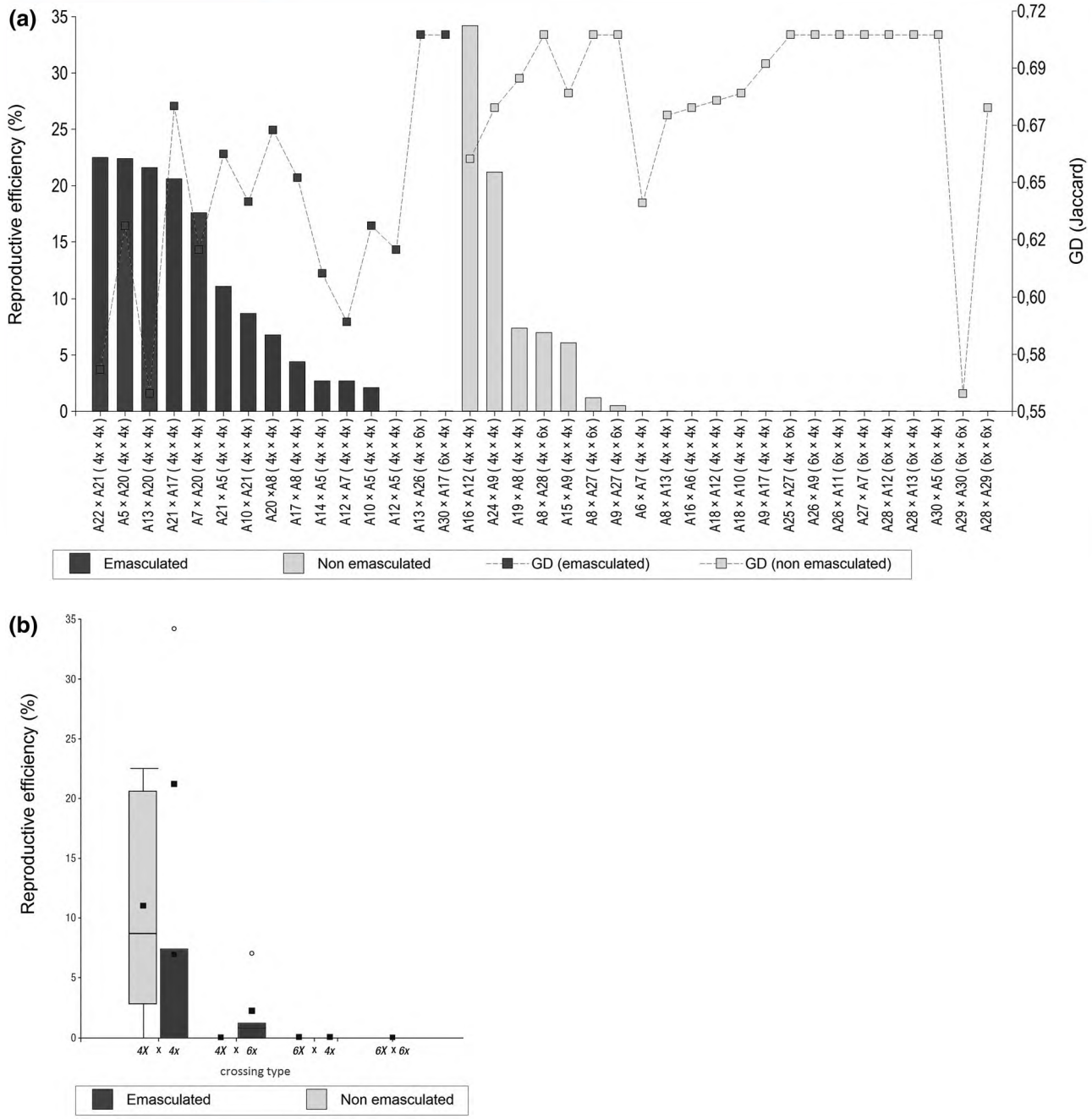

FIGURE 6 (a) Reproductive efficiency at controlled crosses between specific genotypes of *A. macrum* and its genetic distances (GD) in Jaccard units. Crosses were made without emasculation in the field, or with emasculation in a greenhouse. Crosses were homoploid tetraploid ($4x \times 4x$), heteroploid ($4x \times 6x$ or $6x \times 4x$), or homoploid hexaploid ($6x \times 6x$). (b) Boxplot indicating distribution and atypical values of the reproductive efficiency obtained in different types of crosses by both techniques. GDs are from Ferrari Usandizaga et al. (2014)

crosses with a nonemasculated $4x$ female parent resulted in a reproductive efficiency of 4% (Figure 6b). There was not a significant correlation between reproductive efficiency, open-pollination seed set of female parents, and pollen viability of male parents.

3.5 | Progeny test

The A15 × A9 and A16 × A12 crosses resulted in 100% hybrid plants, and the A19 × A8 and A24 × A9 crosses have 88% confirmed hybrids each (Table 2), with only one plant each

TABLE 2 Progeny test in four families of *A. macrum*. The putative hybrids of their progenies are indicated by letters. The number of male parent specific markers is indicated at right of the analyzed plant

	Cross combinations			
Hybrid	**A24 × A9**	**A16 × A12**	**A19 × A8**	**A15 × A9**
A	3	2	4	6
B	1	3	1	5
C	3	4	2	4
D	7	4	2	4
E	3	3	3	5
F	5	4	5	5
G	4	2	6	5
H	7	5	+ [a]	6
I	+	3	+	3
% Hybrids[b]	87.5	100	87.5	100

[a]+, there is not a plant for the letter.

[b]The percentage of corroborated hybrids (% Hybrids) is indicated by crossing; it includes only individuals for whom two or more markers of the putative father were found.

that could not be confirmed as hybrid in the progeny. The total percentage of plants that could not be confirmed as hybrids (~6%) is similar to the general percentage of 4*x* seed at autopollination (5.1%). These not-confirmed plants had at least one putative male-specific marker and various unique markers that were absent in both the female and the male.

4 | DISCUSSION

The observed chromosome associations in *Acroceras macrum* may indicate but not confirm a strict allotetraploid and allohexaploid origin, with predominance of bivalents in the tetraploid cytotype, and trivalents and bivalents in the hexaploid cytotype. The chromosome pairing frequencies may indicate that the hexaploid originated from the 4*x* cytotype. Assuming that the 4*x* is a strict allotetraploid (AABB) because it forms 18 bivalents, a hexaploid $2n = 54$ (AAABBB) may result from the combination of a nonreduced gamete ($2n = 36$, AABB) and a reduced one ($n = 18$, AB). This putative allohexaploid would be expected to form mainly trivalents in meiosis, but no other multivalents. However, the multivalent chromosome associations observed in tetraploids (up to three quadrivalents) suggests that it may be a segmental allotetraploid ($A_1A_1A_2A_2$). The presence of pentavalents and hexavalents in the putative allohexaploid ($A_1A_1A_1A_2A_2A_2$) would be explained by a low frequency of allosindetic pairing between chromosomes of both subgenomes based in residual homology.

Despite the abundance of bivalents, meiosis behaves irregularly with several alterations, like sticky and lagging chromosomes. As the number of chromosomes was increased, more irregularities were involved in meiosis. These irregularities had a narrow correlation with pollen fertility, as observed in other grasses (Hunziker, Zuloaga, Morrone, & Escobar, 1998; Nath, Swaminathan, & Mehra, 1970), and do not seem to be a limiting factor for the fertility of crosses. The origin of the lagging synaptic chromosomes requires further analysis with complementary methods. Judging by its meiotic behavior, *A. macrum* does not fit requirements of stabilized polyploids, which suggests it might have a recent origin as described in Comai (2005).

The results of this research also indicate sexual reproduction for *A. macrum*. Cytological events observed during megasporogenesis and megagametogenesis, added to the structure of the resulting embryo sac, all corresponded with a sexual process. The very low seed set when bagged panicles were impeded to outcrossing but selfing was possible was an indication of self-incompatibility, but even more, of no-apomixis. When hybrid origin was tested, all the progeny segregated in all crosses. That was the concluding indication of a predominantly outcrossing conducted sexual reproduction. In addition, no evidence of an apomixis-related process (Leblanc & Mazzucato, 2001) was found, since we did not observe multiple seedlings or multiple embryo sacs, high homogenous or not segregating progeny, or the skill of reproductive assurance despite the low pollen fertility.

The predominant mating system in our material was cross-pollination. The fertility was dependent on the combination, and it was moderately high in some cases. In several another grass species, gene-based systems were described as for self-incompatibility reactions (Baumann, Juttner, Xueyu, & Langridge, 2000).

Embryo sacs with aposporic features (Chen & Kozono, 1994; Savidan, 2001) were not observed in any mature pistil. Also, no indicium for a second or multiple embryo sacs appeared in young pistils or in the intermedium stages. In young ovaries of *A. macrum*, the functional megaspore was distinguished as an enlarged cell after to the degradation of the nonfunctional megaspores. Nevertheless, no vacuolated enlarged nucellar cell was distinguishable near of the micropylar end or surrounding areas. Just one type of structure of embryo sac and only single sacs were observed in mature ovaries of *A. macrum*. The observed of embryo sacs correspond to a *Polygonum* type, which has a typical structure of Paniceae (Anton & Cocucci, 1984), similar to the sexual sacs of *Panicum elephantipes* Nees (Urbani, 1990). Constitutions of these mature embryo sacs are almost indistinguishable from diplosporic unreduced ones by structural observation (Meier et al., 2011), but the wide extent of hybrids among progeny, as we found with the hybrid origin test on the four crosses in *A. macrum*, is attributable to a sexual origin of all of the 33 members of their progeny. Although a small fraction of the putative hybrids could not be demonstrated to have

derived from the cross, the presence of a unique band, absent in both the mother and the putative father, is strong evidence that these putative nonhybrids did not result from an apomictic origin.

Polyploidy is frequent in long-lived perennials that propagate mostly by vegetative means, conserving their intrinsic heterozygosity (Barrett, Colautti, & Eckert, 2008; Hilu, 1993). Fertility in polyploids diminishes as the frequency of irregularities rises at meiosis (Sotomayor-Ríos & Schank, 2000). Frequently, allopolyploids could become more fertile than autopolyploids, due the diploidization process that allows for a diploid-like meiotic behavior and segregation (Dar, Beigh, & Wani, 2017). However, that is not true of neo-allopolyploids, which could have various meiotic aberrations that make them almost infertile (Comai, 2005; Ramsey & Schemske, 2002). Although maximum homologous chromosome pairing is the expected condition, it seems that polyploids may change their meiotic behavior, and it could evolve by fertility selection, in both auto- and allopolyploids (Ramsey & Ramsey, 2014). That is a good reason for studying the evolution of the meiotic behavior and chromosome pairing from now on, in a breeding program attempting to increase the seed set.

The predominant matting system in *A. macrum* was characteristic of cross-pollinated species. Some members of Paniceae such as *Panicum dichotomiflorum* Michx. (Urbani, 1996), *Setaria magna* Griseb. (Aliscioni, Gómiz, Torretta, & Pensiero, 2011), and *S. pflanzii* Pensiero (Caponio & Pensiero, 2002) are mainly autogamous self-compatible grasses. They do not have statistically different averages of seed setting under self- and open-pollination. Conversely, in *A. macrum*, we found very significant differences between treatments. Average 4*x* seed production was 5.0 and 34.5% under self- and open-pollination, respectively, and average 6*x* seed setting was 0.0 and 1.5% under self- and open-pollination, respectively. That is similar to the cases of self-incompatible, mainly in allogamous grasses as *Bromus auleticus* Trin. ex Nees (Gutiérrez et al., 2006) *Panicum spathellosum* Döll (Vega, 1996), and diploid *Paspalum fasciculatum* Willd. ex Flueggé (Urbani, 1996). Among sexual grasses, a self-incompatibility system could ensure allogamy (Baumann et al., 2000). These are homomorphic systems based on the genotypic constitution of the pollen or the megagametophyte (Ávila Castañeda & Cruz García, 2011; Vallejo Cabrera & Estrada Salazar, 2002).

The significantly low seed production under induced self-pollination compared with cross-pollination strongly suggests the presence of self-incompatibility mechanisms. The progeny test did result in a broad majority of corroborate hybrids and, similar to the seed set assay under self-pollination seed set, offered strong evidence that *A. macrum* has a self-incompatibility system that stimulates it to cross-pollinate.

Heteroploid crosses resulted in seed setting similar to the average value of self-pollination of the 4*x* female parents, and none of the heteroploid crosses resulted in seeds when the female parent was emasculated or was 6*x*. Thus, it is possible that the seed obtained at heteroploid crosses with a not-emasculated 4*x* female parent came mainly from remaining self-pollination like a stimulated mentor effect (Hörandl & Temsch, 2009), and the 6*x* races seem to be sterile, perhaps due to endosperm unbalance, meiotic abnormalities, and cross-incompatibilities.

5 | CONCLUSIONS

Tetraploid and hexaploid plant materials of *Acroceras macrum* reproduce sexually. That conclusion comes from the cytological observations of mature embryo sacs and its process of generation, strongly supported by the results of the progeny test. Furthermore, from the results of the seed production at open- and self-pollination and the support of the progeny test, we could conclude that *A. macrum* is predominantly allogamous. Chromosome associations observed during the PMC (male) meiosis may indicate that *A. macrum* has allopolyploid 4*x* and 6*x* races. However, it will be necessary to use complementary techniques, such as genomic in situ hybridization (GISH), to resolve the type of polyploidy in this species. Meiotic alterations were seen during the male meiosis, and they perhaps are the primary reason for the diminishing fertility in the 4*x* cytotype and almost null fertility in the 6*x* cytotype. Nonetheless, we found that adequate 4*x* homoploid combinations between genetically compatible parental genotypes could enhance fertility, at least among 4*x* genotypes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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