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Evidence of sexuality in induced tetraploids of *Brachiaria brizantha* (Poaceae)

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Summary

Difficulties in obtaining new breeding lines of *Brachiaria* (Trin.) Griseb., an important forage grass in Brazil, are mostly related to differences in ploidy among the accessions, and to apomixis, an asexual mode of reproduction. Usually, sexual accessions are diploid while apomicts are polyploid. Induced tetraploids of *Brachiaria brizantha* (A. Rich.) Stapf have been successfully obtained and this paper presents the results of a study of their reproductive modes and fertility. Despite frequent meiotic aberrations during microspore development, the induced tetraploids produced viable pollen and produced progeny after controlled self-pollination. Similarly to the original diploid sexual progenitor, embryo sacs of the *Polygonum* type with confirmed meiotic origin were present in the induced tetraploids suggesting chromosome doubling did not alter the reproductive mode. The embryo sac of the *Polygonum* type was also observed in progenies obtained after self and open pollination. Nevertheless, embryo sacs of the *Polygonum* and the *Panicum* types within the same ovule were observed in some progenies obtained after open pollination, probably having resulted from hybridization with tetraploid, apomictic plants. Indeed, the compatibility of the progeny with tetraploid, apomictic *B. brizantha* was confirmed by the formation of mature caryopses after controlled pollination. Evidence is presented that the induced tetraploids and their progeny are sexual plants and that they are compatible with natural tetraploid *B. brizantha*. The induced tetraploids will be useful for analyses of apomictic inheritance as well as in the development of sexual tetraploid lines in *Brachiaria* breeding programs.

Introduction

Genus *Brachiaria* (Trin.) Griseb. belongs to the family Poaceae and some species of this genus have been introduced from Africa to Brazil where they are cultivated as forage grass on an estimated 30–70 millions hectares (Macedo, 1995; Miles & Valle, 1996). The existence of sexual and apomictic accessions of *Brachiaria* was detected through the analysis of the embryo sac structure in the accessions of different species (Valle, 1990). Apomixis is an asexual mode of reproduction that gives origin to plants genetically identical to the mother plant. It occurs through an autonomous development of the egg cell within an unreduced embryo sac in the gametophytic apomictic plants (Nogler, 1984; Asker & Jerling, 1992; Savidan, 2000; Koltunow & Grossniklaus, 2003; Bicknell & Koltunow, 2004).

Two ploidy levels are predominantly found in *Brachiaria*: diploid ($2n = 2x = 18$), associated with sexuality, and tetraploid ($2n = 4x = 36$), associated with apomixis. In *Brachiaria brizantha* (A. Rich.) Stapf, one of the most important tropical forage species

(Valle & Savidan, 1996), only one sexual accession, BRA002747, has been identified and characterized among 274 apomictic accessions (Carnahan & Hill, 1958; Valle, 1986; Valle, 1990; Valle & Glienke, 1991; Valle & Savidan, 1996; Penteado et al., 2000).

Segregation analysis in *Brachiaria* showed that the apomictic character is under the control of a single dominant locus while sexuality is recessive (Valle et al., 1994), similarly to other grasses (reviewed by Bicknell & Koltunow, 2004). The suggested genotype for tetraploid apomicts in *Brachiaria* is *Aaaa* and for diploid sexuals, *aa* (Valle et al., 1994; Miles & Escandon, 1997).

Morphological analyses of the embryo sac structure permit inferences on the reproductive mode in *Brachiaria* (Valle & Savidan, 1996). Sexual plants have an embryo sac of the *Polygonum* type, with eight nuclei within seven cells (Gobbe et al., 1982a; Lutts et al., 1994; Dusi & Willemse, 1999; Araujo et al., 2000). Apomictic plants have one or more aposporous embryo sacs of the *Panicum* type, with four cells: the egg cell, two synergids and the uninucleated central cell (Lutts et al., 1994; Dusi & Willemse, 1999; Araujo et al., 2000). Some *Brachiaria* apomictic accessions may have one embryo sac of the *Polygonum* type associated or not with aposporous embryo sacs, indicative of facultative apomixis (Valle & Savidan, 1996).

One of the main limitations in *Brachiaria* breeding programs is related to ploidy differences among the accessions and to the presence of an asexual mode of reproduction, which permits the apomictic accessions to be used only as pollen donors. Also, an apparent incompatibility among *Brachiaria* accessions is observed with high mortality of hybrids obtained after interspecific hybridization (Ndikumana, 1985; Ngendahayo, 1988; Valle & Savidan, 1996).

An important strategy to improve the *Brachiaria* breeding program was the development of an induced tetraploid, sexual *B. ruziziensis*, the current basis of the program (Swenne et al., 1981; Gobbe et al., 1982b; Valle & Savidan, 1996). Pinheiro et al. (2000) produced tetraploid *B. brizantha* using colchicine applied to *in vitro* plants of the diploid, sexual *B. brizantha* accession BRA002747 with the aim of increasing parental diversity in the *Brachiaria* breeding program and bringing in the agricultural advantages of *B. brizantha.*

The current paper discusses the reproductive mode and fertility of these induced tetraploid *B. brizantha* plants and their progeny. The results suggest that the tetraploids have maintained the sexual mode of reproduction of the diploids from which they originated, and indicate their self compatibility and compatibility with the natural tetraploid *B. brizantha*.

Material and methods

Plant material

Chromosome doubling of a diploid sexual accession BRA002747 of *B. brizantha* was accomplished by colchicine treatment of basal segments of *in vitro* grown plants (Pinheiro et al., 2000). The diploid, sexual accession BRA002747 of *B. brizantha*, the apomictic tetraploid accession BRA000591 – cultivar (cv.) Marandu – of *B. brizantha* and six individual induced tetraploid plants of *B. brizantha* ($2n = 4x = 36$) designated C14, C31, C35, C36, C41 and C48 are maintained in the experimental field in Embrapa Genetic Resources and Biotechnology, Brasília-DF, Brazil. Progeny was obtained from plant C14 after self-pollination (plants #1, 4, 9 and 12) and open pollination (plants #7, 8, 10, 11, 13–20). One progeny plant from open pollination of each induced tetraploid plants C31, C41, C42, C43 and C48 were kindly provided by Dr. C.B. do Valle, Embrapa Beef Cattle, Campo Grande, MS, Brazil and designated as plants #31, 41, 42, 43 and 48, respectively.

Morphology of mature embryo sacs in cleared ovules

Pistils were isolated from flowers collected at anthesis or 1 day after anthesis. Around 40 mature pistils of each induced tetraploid plant C31, C35, C36 and C41, 300 pistils each of C14 and C48, and around 30 mature pistils of plants #1, 4, 7, 10, 13, 14, 41–43 and 48 were isolated. Samples were fixed in a mixture of 40% formaldehyde:glacial acetic acid:50% ethanol (5:5:90, v/v) for 4–24 h at room temperature, and either cleared in the Herr's modified clearing solution (Pozzobon & Araujo, 1998) or phenol:methylsalicilate mixture gradually replaced over 3 days by a pure methylsalicilate. Pistils were whole-mounted on concave slides either in a drop of latic acid:phenol or methylsalicilate, depending on the method of clearing. Mature embryo sacs within the cleared ovules were morphologically characterized using the Normasky differential interference contrast microscopy (DIC) in a Zeiss Axiophot.

Morphology of the developing embryo sacs in sectioned ovules of the induced tetraploids C14 and C48

Ovules of the induced tetraploid plants C14 and C48 were collected during pistil development, at points corresponding to the stages of megasporogenesis and megametogenesis (stages I, II and III and IV, respectively – see Araujo et al., 2000 for details of the developmental stages). Around 20 isolated ovules at each stage of each plant were fixed in a mixture of 40% formaldehyde:glacial acetic acid:50% ethanol (5:5:90, v/v) for 4–24 h at room temperature, dehydrated in ethanol series (from 70% to 100%), infiltrated and embedded in JB4® plastic resin (Polysciences). Semi-thin sections (2–4 μm thick) of embedded ovules at different stages of the pistil development were obtained and stained with methylene blue-basic fuchsin for morphological assessment. Analyses and photography were done at the bright field microscopy using a Zeiss Axiophot.

Meiotic chromosome pairing in C14 and C48

Meiotic chromosomes were analyzed in young anthers fixed in a mixture of absolute ethanol and acetic acid (3:1, v/v) for 24 h at room temperature and stored in 70% ethanol at 4 °C, squashed in a drop of propionic-carmine solution (6% carmine in 45% propionic acid). Analyses of meiotic chromosomes were done in bright field and phase contrast using a Zeiss Axioskope.

Pollen grain analyses in C14 and C48

Pollen viability was estimated by the percentage of well-stained pollen grains after incubation in a glycerin:acetocarmine solution (1:1, v/v). Approximately 1000 pollen grains per plant were analyzed. Pollen size was determined by measuring the diameters of 170 pollen grains per plant. The diameters were determined using the LSM program in the Zeiss LSM 410 Laser Scanning Microscope, and mean values were calculated.

Chromosome counts

Chromosome numbers were determined in mitotic metaphases of root meristems. Young root tips were pre-treated with a saturated solution of 1-bromonaphtalene for 3 h at 16 °C, washed three times in water and fixed in a freshly prepared mixture of ethanol and glacial acetic acid (3:1, v/v) for 2 h, hydrolyzed in 5 N HCL for 20 min at room temperature, washed and incubated in 20% (w/v) Pectinase from *Rhizopus* sp (Sigma) and 2% (w/v) Cellulase from *Aspergillus niger* (Sigma) in a sodium citrate buffer, pH 4.8 for 40 min at room temperature. Such samples were stained with Feulgen for 30 min macerated on a microscope slide in a drop of propionic carmine and gently heated. Chromosome counts were done under the bright field and phase contrast in Zeiss Axiovert 135 M.

Estimates of plant fertility

Approximately 1000 florets containing flowers at anthesis in each induced tetraploid plant were tested. The racemes containing these flowers were isolated with paper bags for 12 days. Bagged flowers were harvested, counted and the number of fully filled caryopses was determined. The fertility was expressed as a ratio of the number of full caryopses to the total number of flowers scored. Among the progeny, a similar procedure was performed with 1511, 669, 325, 2273, 5926 and 3660 florets of plants #1, 4, 7, 14, 41 and 48, respectively. Approximately 154, 127, 189, 519, 1069, 423 self-pollinated flowers of plants #1, 4, 7, 14, 41 and 48, respectively, were also isolated and their fertility determined as above. Plants #14 and 41 (608 and 378 flowers in each, respectively) were pollinated with cv. Marandu to determine the fertility after cross-pollination.

Results

Morphology of mature embryo sacs in cleared ovules of the induced tetraploids

Analyses of cleared pistils of plants C14 and C48 showed 30% of aborted ovules, identified by their dehydrated, collapsed embryo sacs, or by ovules frequently showing oxalate crystals. Variations around this number were observed in all induced tetraploids, possibly as a consequence of different collection periods and locations.

All well developed ovules of the six *B. brizantha* induced tetraploids had mature embryo sac of the *Polygonum* type, morphologically similar to that present in the diploid, sexual plant (Figure 1a). No embryo sac of the *Panicum* type (Figure 1b), as found in natural tetraploid apomicts of *B. brizantha*, was observed among the induced tetraploids. Only one embryo sac of the *Polygonum* type was present in each ovule, located close to the micropyle pole and containing the egg cell,

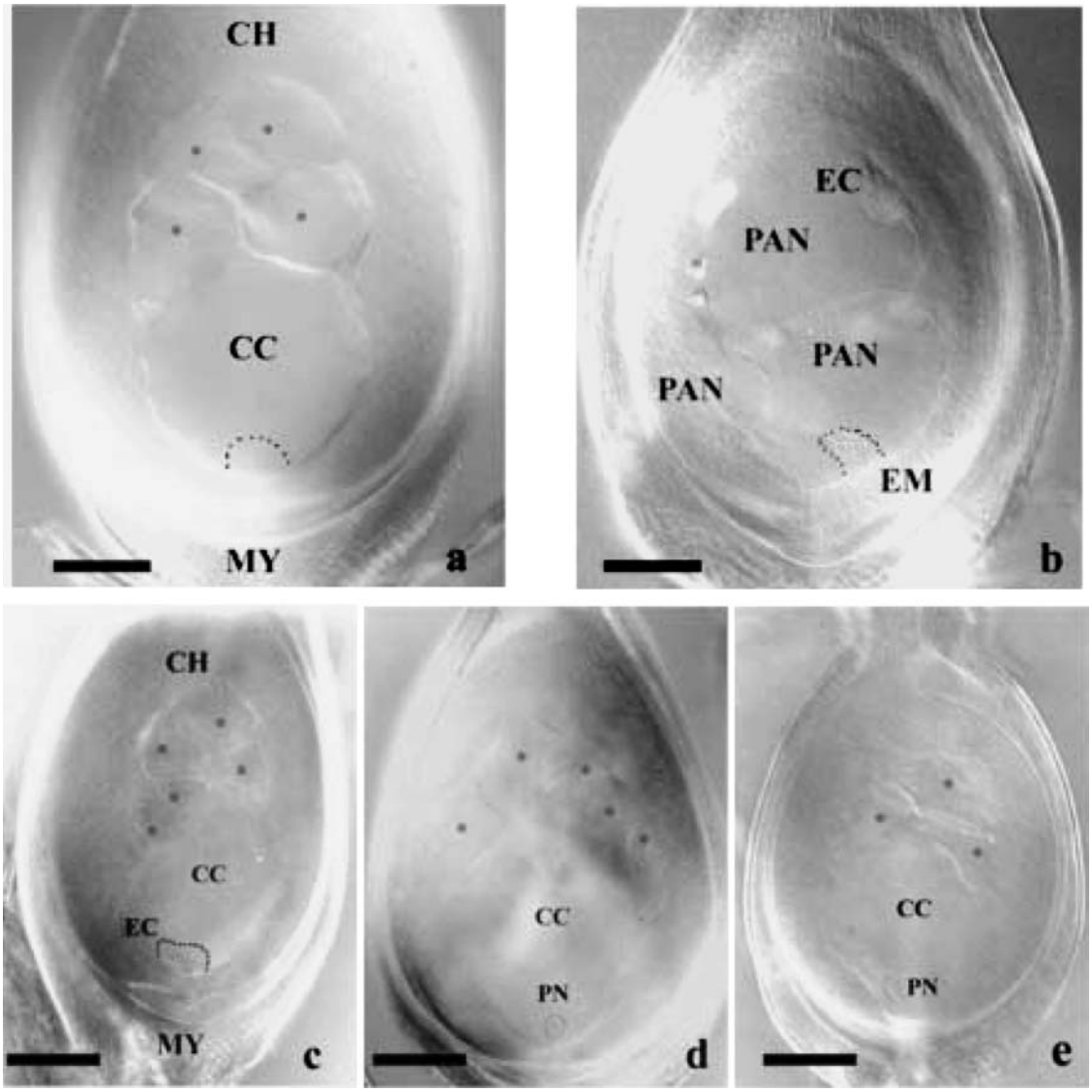

Figure 1. Micrographs of whole mounted cleared ovules of *Brachiaria brizantha* in showing the embryo sac. Micropyle (MY). Chalaza (CH). Egg cell apparatus (EC). Central cell (CC). Antipodal cells (*). (a) Diploid, sexual accession BRA002747 showing one embryo sac of the *Polygonum* type with four of the six antipodal cells in this plane of focus (bar corresponds to 600 μm). (b) Tetraploid, apomictic accession BRA000591 showing three embryo sacs of the *Panicum* type (PAN). Note the presence of an embryo (EM) within the embryo sac close to the micropyle pole, while in the chalazal embryo sac, the presence of the egg cell, identified by its cytoplasm is observed (bar corresponds to 600 μm). (c) Plant C35 (induced tetraploid) showing one embryo sac of the *Polygonum* type with five of the six antipodal cells in this plane of focus (bar corresponds to 500 μm). (d) Plant C48 (induced tetraploid) showing one embryo sac of the *Polygonum* type with two polar nuclei (PN) within the central cell and five of the six antipodal cells (bar corresponds to 500 μm). (e) Plant C41 (induced tetraploid) showing one embryo sac of the *Polygonum* type with two polar nuclei (PN) of the central cell and three of the six antipodal cells (bar corresponds to 600 μm).

two synergids, a large bi-nucleated central cell and six antipodal cells (Figures 1c–1e). The egg cell apparatus was always situated at the micropyle pole with the cytoplasm of the egg cell and the synergids evident while the nuclei were not easily detectable. Within the central cell, a large vacuole and two evident polar nuclei surrounded by dense cytoplasm were present in all plants (Figures 1d and 1e). Antipodal cells extended from the chalazal pole to the middle region of the embryo sac (Figures 1a and 1c–1e, Figures 2a and 2c).

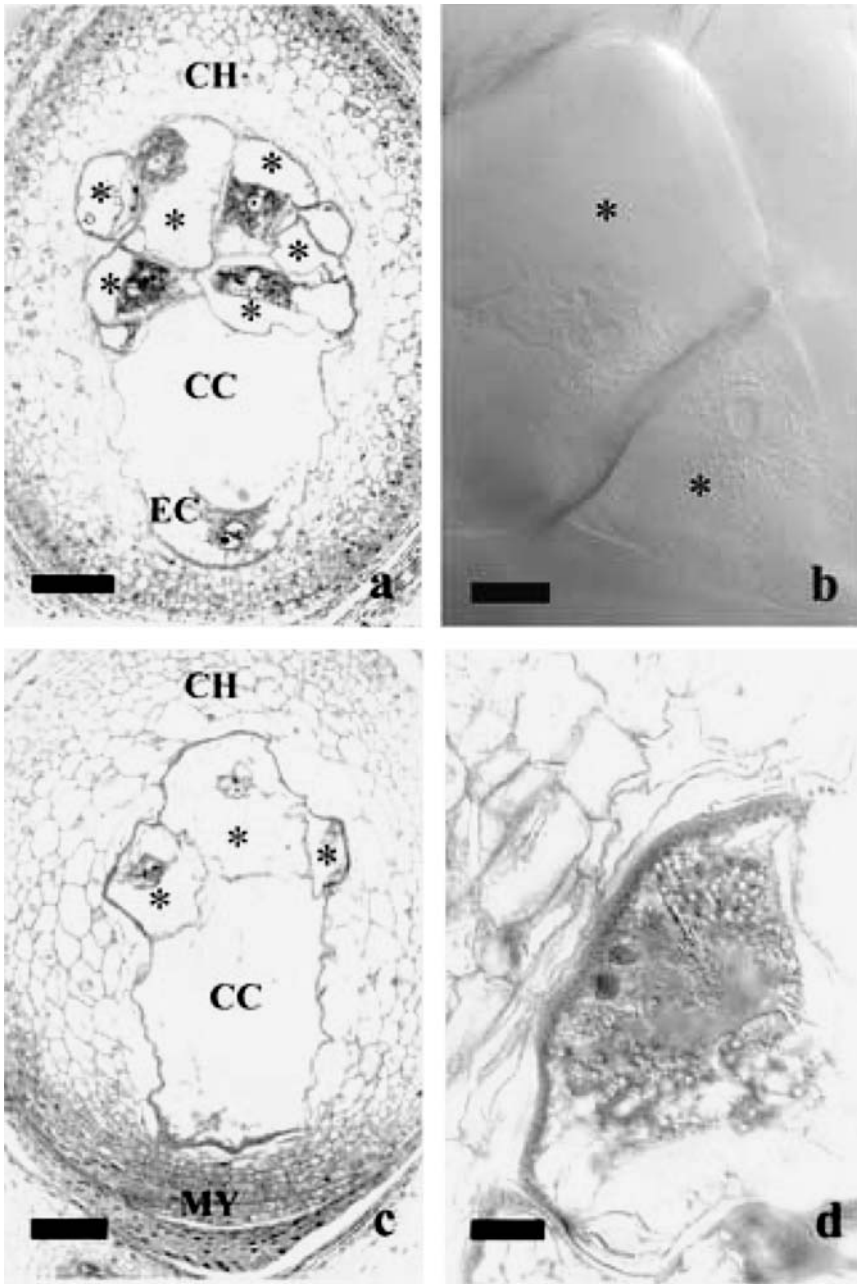

Figure 2. Micrographs of semi-thin sections of *B. brizantha* ovules (a, c and d) and a cleared ovule (b). Micropyle (MY). Chalaza (CH). Egg cell (EC). Central cell (CC). Antipodal cells (*). (a) Embryo sac of the *Polygonum* type in the diploid, sexual accession BRA002747 (bar corresponds to 200 μm). (b) Detail of two antipodal cells in the *Polygonum* type of embryo sac showing multiple nuclei surrounded by dense nucleoplasm and many vacuoles in the cell cytoplasm (bar corresponds to 200 μm). (c) Embryo sac of the *Polygonum* type in plant C48 (induced tetraploid) (bar corresponds to 400 μm). (d) Detail of one antipodal cell of plant C48 (bar corresponds to 400 μm).

Nuclei of these cells were different in shape and volume due to frequent nuclear divisions (Figures 2b and 2d). The nuclei were always surrounded by dense cytoplasm (Figure 2). During earlier stages of the embryo sac development the antipodal cells in plants C14 and C48 had larger volumes relative to the diploid, sexual *B. brizantha*. However, the number of antipodal cells was always six and they showed similar volume, shape

and spatial distribution in mature embryo sacs of the diploid and in the induced tetraploid plants (Figures 1 and 2).

Morphology of the developing embryo sac in sectioned ovules of plants C14 and C48

Sectioned ovules in the early stages of megasporogenesis showed one megaspore mother cell (MMC) identified as a large uninucleated cell with thick cell walls (Figure 3a). Bi-nucleated MMC and the resulting cells after meiosis, the megaspores, could be observed first as dyad and then as megaspore tetrads (Figure 3b). Degeneration of these cells was indicated by the presence of a dense and large intensely stained region (Figure 3c) where viable megaspores could be detected at earlier stages of ovule development. Indications of degenerating cells were associated with a large cell situated chalazally, corresponding to the surviving, or functional megaspore (Figures 3c and 3d). This megaspore, which was larger than the adjacent cells, had a large vacuole and thick cell wall (Figures 3e–3g). Only one surviving megaspore was present in each tetrad and there was no indication of the presence of initial cells of the aposporous embryo sac of the *Panicum* type. During megagametogenesis, the functional megaspore enlarged and divided mitotically, forming a multinucleated structure containing a large vacuole. Observations of differentiation of this structure into a typical embryo sac of the *Polygonum* type (Figures 3f–3i) confirm the meiotic origin of the embryo sacs, with each embryo sac easily identified as of the *Polygonum* type by the presence of a bi-nucleated central cell and dividing antipodal cells in the chalaza (Figures 3f and 3g). In other ovules, the abortion of the female reproductive structure could be detected from early until late stages of development.

Meiosis in plants C14 and C48

Analyses of the metaphases I showed primarily bivalent (Figure 4a) and quadrivalent associations (Figure 4b). Univalents were sporadically observed in both plants while trivalents were observed only in C48. Although quadrivalents tend to be frequent in autopolyploids, in the analyzed plants bivalents predominated, with 34% of the microsporocytes in both plants having 18 bivalents (Table 1). Unequal chromosome segregation and micronuclei were observed both at anaphase and telophase I and II (Figures 4c and 4d). Micronuclei with polarity disorder (Figure 4c) and multipolar spindles (Figure 4d) were present, the later leading to the formation of polyads. In addition, precocious chromosome migration, chromosome stickiness, lagging chromosomes and the presence of bridges at anaphase and telophase I were also observed. Asynchronous chromosome distribution resulting in the displacement of the equatorial plate chromosome was observed at metaphase I and II. These abnormalities resulted in irregular cytokinesis, giving rise to tri-nucleated cells (Figure 4e). Also, morphologically altered microspore tetrads, numerical deviations in the microspores with polyads, cells with irregular sizes, failure of the cell wall development and unbalanced meiotic products were present (Figure 4f). These irregularities notwithstanding, regular microspore tetrads outnumbered the abnormal ones.

Pollen grains and fertility of the induced tetraploids

Pollen grains of plants C14 and C48 had mean diameters of $42.21 \pm 10.41\,\mu$m and $42.15 \pm 5.28\,\mu$m, respectively and 85% of the pollen grains showed intense acetocarmine stainability. Among the tested flowers of induced tetraploids C14, C38 and C48 about 17% developed fully filled caryopses. In C36 and C41, the corresponding percentage was about 23%. C35 had an even higher fertility, with about 34% of fully filled caryopses.

Chromosome counts among progeny

Chromosome counts were done in four plants from controlled self pollination and in sixteen plants from

Table 1. Meiotic chromosome associations in the induced tetraploid plants C14 and C48 of *Brachiaria brizantha* at diakinesis and metaphase I

			Average chromosome associations (range)			
Plant	No. of chromosomes	No. of cells analyed	I	II	III	IV
C14	36	47	0.04 (0–2)	15.25 (8–18)	0	1.36 (0–5)
C48	36	63	0.14 (0–2)	15.63 (8–18)	0.05 (0–1)	1.11 (0–5)

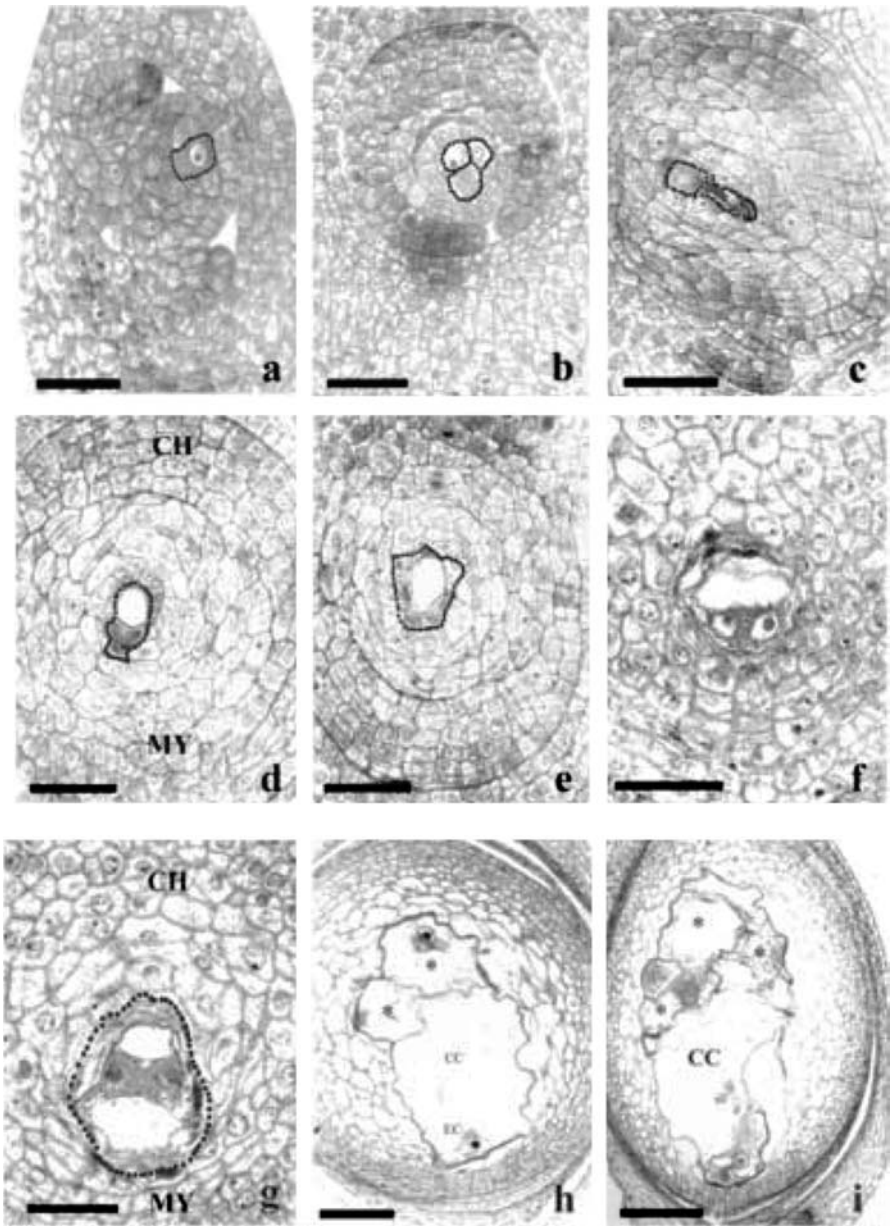

Figure 3. Micrographs of semi-thin sections of the induced tetraploid plants of *B. brizantha* ovules collected during megasporogenesis (a–e) and megagametogenesis (f–i). Micropyle (MY). Chalaza (CH). (a) An ovule of plant C14 collected at stage I showing the megaspore mother cell (the larger uninucleate cell with thick cell walls) (bar corresponds to 200 μm). (b) An ovule of plant C14 collected at stage I showing three of the four reduced megaspores which are not linearly displaced (bar corresponds to 200 μm). (c) An ovule of plant C48 collected at stage II showing degeneration of the megaspores (an irregularly-shaped intensely stained structure situated in the same place where megaspores are found at the earlier stages of development). Note one large cell with dense cytoplasm corresponding to the surviving megaspore (bar corresponds to 150 μm). (d) An ovule of plant C14 collected at stage II, showing the vacuole within the enlarged surviving megaspore and three degenerating megaspores (bar corresponds to 200 μm). (e) An ovule of plant C48 collected at stage II, showing the enlarged surviving megaspore surrounded by degenerating nucellar cells (bar corresponds to 100 μm). (f, g) Ovules of plant C14 collected at stage III showing the large functional megaspore with two of the four nuclei resulting from the mitosis. Note the presence of the vacuoles and the adjacent degenerating nucellar cells (bars correspond to 200 μm). (h) An ovule of plant C14 collected at stage IV showing one developing embryo sac of the *Polygonum* type containing the egg cell (EC), the central cell (CC) and the antipodal cells (*) (bar corresponds to 100 μm). (i) An ovule of plant C48 collected at stage IV showing one developing embryo sac of the *Polygonum* type with the egg cell apparatus, the central cell (CC) and the antipodal cells (*) (bar corresponds to 100 μm).

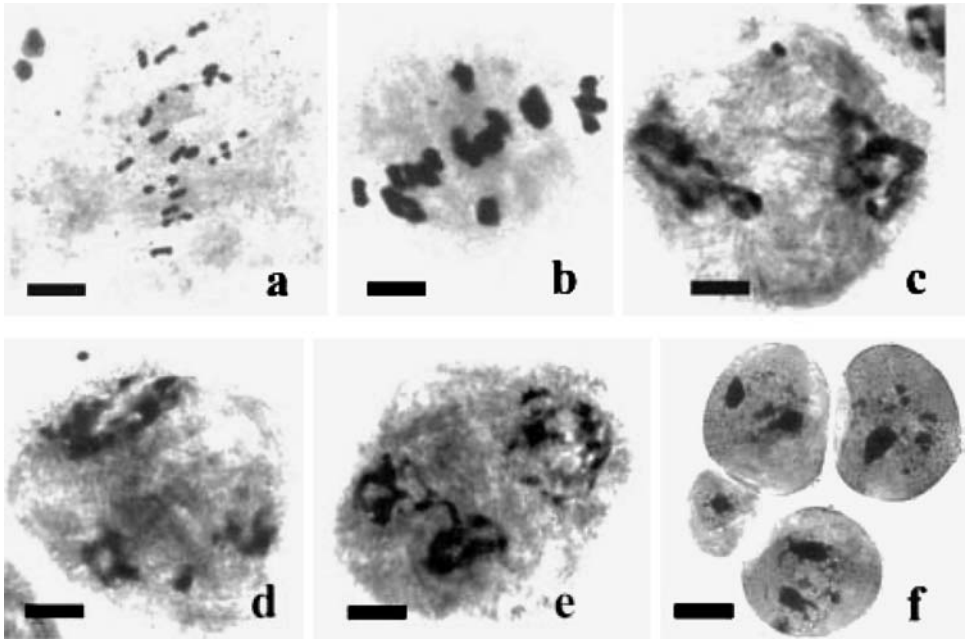

Figure 4. Meiosis in the *B. brizantha* induced tetraploid plants C14 and C48. Bivalent (a) and quadrivalent (b) associations (bars corresponds to 700 μm). (c) Micronuclei with polarity disorder (bar corresponds to 800 μm). (d) Multipolar spindles (bar corresponds to 800 μm). (e) One tri-nucleated cell (bar corresponds to 800 μm). (f) Cells with irregular size and polyads (bar corresponds to 800 μm).

open pollination. Among the progeny from self pollination one was tetraploid, three were triploid and none was diploid while among those from open pollination, six were tetraploid (Figure 5a), nine were triploid (Figure 5b) and one was diploid (Figure 5c).

Observations of mature embryo sacs in cleared ovules

Progeny plants obtained from the induced tetraploids showed 25–35% of aborted ovules. All well developed ovules of plants #1, 4, 7, 10, 43 and 48 contained mature embryo sacs of the *Polygonum* type, morphologically similar to those found in the diploid, sexual plant and in the progenitor tetraploid plants (Figure 5d). No embryo sacs of the *Panicum* type were observed in these ovules. The well developed ovules of plants #13, 14, 41 and C42 that were produced by open pollination, showed within the same ovule one embryo sac of the *Polygonum* and one or more embryo sacs of the *Panicum* type (Figure 5e). The embryo sacs of the *Panicum* type showed the egg cell, two synergids and the uninucleated central cell, morphologically similar to the one found in apomictic *Brachiaria* sp.

Estimates of fertility

Fertility after open pollination was higher in C41 than in any other plant. Around 32% of the identified flowers in C41 showed fully filled caryopses while in plant #1, the corresponding rate was 6%, in plants #4 and 7 it was 2%, in plant #14 it was 5%, and in #48, 3%. The fertility of plant #41 was also higher in self pollination. In plants #14 and 1, seed set was 2% and 1%, respectively. In plants #4, 7 and 48, no seeds were obtained after self-pollination. After controlled cross pollination (with cv. Marandu), plant #41 and 14 had 18% and 5% seed set, respectively.

Discussion

The inheritance of apomixis in *Brachiaria* has been reported to be simple and the trait to be conferred by a single dominant factor (Valle et al., 1994; Miles & Escandon, 1997). Similar type of inheritance was reported in other aposporous apomictic grasses such as *Pennisetum* (Sherwood et al., 1994) and *Panicum* (Savidan, 1982), diplosporous such as *Eragrostis curvula* (Voigt & Burson, 1981) and *Tripsacum dactyloides* (Leblanc et al., 1995) and in

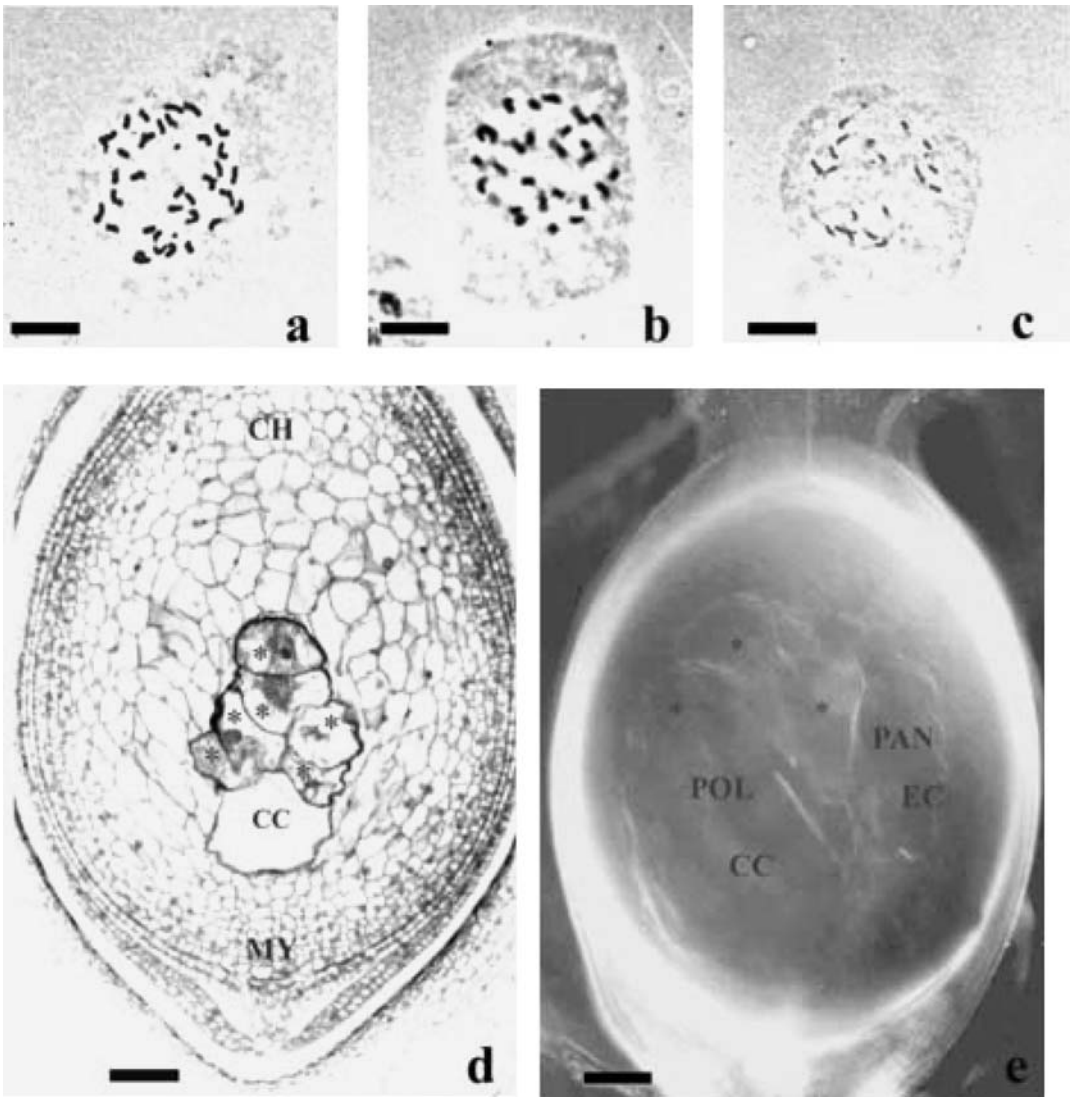

Figure 5. Mitotic metaphases of root meristems (a–c) and ovules of the progeny of the induced tetraploid *B. brizantha* (d, e). Embryo sac of the *Panicum* type (PAN) and *Polygonum* type (POL). Micropyle (MY). Chalaza (CH). Egg-cell apparatus (EC). Central cell (CC). Antipodal cells (*).Tetraploid plant #14 (a), triploid plant #7 (b) diploidy plant #16 (c). (d) Semi-thin section of the ovule of plant #4 with one embryo sac of the *Polygonum* type (POL). (e) Cleared ovule in plant #14 with one embryo sac of the *Panicum* type (PAN) and one of the *Polygonum* type (POL). Three of the six antipodal cells are visible within the POL and the EC is observed within PAN (bars correspond to 700 μm).

dicotyledonous genera such as *Ranunculus* (Nogler, 1984) and *Hieracium* (Bicknell et al., 2000).

An association of apomixis with polyploidy is evident (Carman, 1997), although the apomictic mode of reproduction is not exclusive of polyploids. Rare diploid apomicts have been reported (reviewed by Savidan, 2000; Bicknell & Koltunow, 2004). An analysis of induced autotetraploids *Paspalum hexastachyum* (Quarin & Hanna, 1980), *P. notatum* (Quarin et al., 2001), and *P. rufum* (Quarin et al., 1998) indicated changes in the structure of the embryo sac, from the *Polygonum* to the *Panicum* type and, consequently, a possible change in the reproductive mode after chromosome doubling. Studies of induced tetraploid *Paspalum* suggested that genetic factors related to apomixis were present in the diploid genotype although expressed only at the tetraploid level. The failure of inducing apomixis in tetraploid *Brachiaria* could be due to the fact that although the genetic factors of apomixis may be present in a cryptic form at the diploid level, these

might not have been present in the accessions used for chromosome doubling (Swenne et al., 1981; Pinheiro et al., 2000).

Based on the hypothesis that the apomictic character in *Brachiaria* is under the control of a single dominant allele, which is not present in sexual plants (Valle et al., 1994), apomixis would not be expected after the chromosome doubling of a sexual genotype (*aa*). Here we confirm the presence and the meiotic origin of the embryo sac of the *Polygonum* type in the induced tetraploids of *B. brizantha*. Maintenance of the embryo sac structure and of the sexual reproductive mode was reported in induced tetraploids of *B. ruziziensis* (Gobbe et al., 1982a; Lutts et al., 1994). However, in *B. ruziziensis*, no natural tetraploid and apomictic accessions are known.

It is generally accepted that there is an overlap between apomixis and sexual development, and that the apomictic characteristics are an outcome of temporally and spatially altered regulation of the gene expression pathways (Koltunow & Grossniklaus, 2003; Tucker et al., 2003). Indeed, in *Brachiaria*, the presence of the *Polygonum* type embryo sac, suggesting the occurrence of facultative apomixis (Valle et al., 1994; Valle & Savidan, 1996; Araujo et al., 2000) may be indicative of this overlap. Additionally, similarities of the carbohydrate metabolism patterns (Dusi & Willemse, 1999b) and the presence of common cDNA sequences in different steps of the female reproductive development of apomictic and sexual *Brachiaria* were identified (Rodrigues et al., 2003).

A precocious enlargement of the antipodal cells was observed during the development of the embryo sac in the induced tetraploid relative to the sexual diploid accession, BRA002747 of *B. brizantha*. However, at maturity, the cells of the embryo sacs were identical in size, spatial distribution and shape.

The preservation of the embryo sac of the *Polygonum* type in induced tetraploids of *B. brizantha* as well as in their progeny indicate that these plants set seeds by a sexual process, similarly to the diploid accession BRA002747 from which they originated. Therefore, in contrast to the induced tetraploids of *Paspalum* sp (Quarin et al., 2001), in *Brachiaria,* the increase in gene dosage following chromosome doubling cannot by itself trigger the expression of the apomictic characteristics such as the formation of the aposporous embryo sac of the *Panicum* type.

Despite meiotic irregularities during pollen formation the majority of pollen mother cells showed bivalent associations, suggesting that fertile pollen could be produced. Additionally, the size of pollen grains and their viability, as measured by the acetocarmine stainability in the induced tetraploids C14 and C48, were similar to the fertile pollen in the diploid, sexual BRA002747 and in cv. Marandu (Alves, 2000). The ultimate evidence of fertility of the induced tetraploids was their seed set accomplished using these pollen grains. Irregular male meiosis of the induced tetraploids was similar to that observed in natural tetraploids of *Brachiaria* (Valle & Savidan, 1996).

Notwithstanding the abnormalities, previous studies in *B. brizantha* cv. Marandu showed high levels of stained and regularly shaped pollen (Alves, 2000). The presence of viable pollen grains in the apomictic accessions of *Brachiaria* might dictate a strategy for the success of seed development, since these plants are pseudogamous apomicts. Endosperm formation depends on the fertilization of the central cell as demonstrated by the failure of caryopsis development in pistils which stigmas were incised before anthesis by Ngendahayo (1998). This was later confirmed by Alves et al. (2001) with the observations of the nuclear fusion and the endosperm ploidy levels. In fact, cv. Marandu propagates by seeds (Hopkinson et al., 1996), which are likely originated from self-pollination.

The similarity in the meiotic chromosome behavior during microsporogenesis and microgametogenesis and the pollen grain viability in the natural and induced tetraploids of *B. brizantha* suggest that the evolutionary origin of the natural tetraploids could be through self-polyploidization. This was suggested for *P. rufum* based on a cytogenetic analysis of triploid and tetraploid hybrids and of induced tetraploids (Quarin et al., 1998). Genetic analyses using molecular markers will be conducted to verify the origin of the tetraploid genome in *B. brizantha*.

A progeny showing the *Panicum* and the *Polygonum* type embryo sacs within the same ovule might be a result of intraspecific hybridization with an apomictic tetraploid. Cv. Marandu represents a potential pollen donor since it produced large amounts of viable pollen grain (Alves et al., 2000) during the flowering period of the induced tetraploid. Indeed, fully filled caryopses were obtained after controlled pollinations of the progeny with the cv. Marandu pollen, confirming the fertility and the sexuality of the progeny and suggesting an intraspecific compatibility. These plants are currently being used as females to generate a hybrid population for the analyses of the segregation of apomixis. The production of stable tetraploid, sexual *B. brizantha* represents a new perspective for the

Brachiaria breeding program, where all known natural tetraploids are apomictic and only one induced tetraploid, sexual *B. ruziziensis* is available (Swenne et al., 1981).

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