Plant Cell Reports (2000) 19:274–278

A.A. Pinheiro · M.T. Pozzobon · C.B. do Valle
M.I.O. Penteado · V.T.C. Carneiro

Duplication of the chromosome number of diploid *Brachiaria brizantha* plants using colchicine

Received: 9 Ocotber 1997 / Revision received: 9 December 1998 / Accepted: 15 June 1999

Abstract Some species of *Brachiaria*, generally tetraploid apomictic varieties, have become important forage grasses in the tropics. Breeding of *Brachiaria* depends on compatibility with the available apomitic tretraploid cultivars. This paper describes a procedure for chromosome duplication of two *Bracharia brizantha* diploid sexual accessions, using colchicine treatment of basal segments of in-vitro-grown plants. Explants were cultured on a medium containing 1 mg/l naphthaleneacetic acid, 3 mg/l kinetin and 0.01% colchicine for 48 h and transferred to the same medium without colchicine until shoot regeneration occurred. Regenerated plants were screened by flow cytometry, and chromosome number duplication was confirmed by cytological analysis of root tips.

Key words Apomixis · Flow cytometry · Grasses · Polyploidization · Tetraploids

Abbreviations *BAP*: 6-Benzylaminopurine · *KIN*: Kinetin · *NAA*: Naphthaleneacetic acid

Introduction

Brachiaria is a forage grass, native to the African tropical savannas and introduced to Brazil in 1952 (Serrão and Simão Neto 1971). It is well-adapted to poor soils and resistant to extended dry periods. *Brachiaria* is one of the most widely cultivated grasses in Brazil and is grown on 30 to 70 million hectares in the tropics (Miles et al. 1996). The two most important species of this genus, *Brachiaria brizantha* and *B. decumbens*, are polyploids ($2n = 4\times = 36$) and their reproductive mode has been characterized as aposporous apomixis (do Valle and Miles 1992). Fusion of female and male gametes does not occur and embryos develop by parthenogenesis (Nogler 1994). Seeds produced by apomictic plants are identical to the female parent, which makes breeding programs by hybridization techniques difficult (Hanna and Bashaw 1987). However, this mode of reproduction, which allows cloning by means of seeds, is of extreme interest to biotechnological programs since apomixis could potentially be used to perpetuate hybrids of interest by simple seed propagation (Vielle et al. 1996).

In *Brachiaria*, most sexual plants are diploids ($2n = 2\times = 18$), including sexual accessions of *B. brizantha* and *B. decumbens.* Plants carrying the same chromosome number and presenting different reproductive modes (sexual or apomictic) within *B. brizantha* or *B. decumbens* may allow intraspecific crosses which are required for studies of the inheritance of apomixis. New apomictic cultivars could be obtained by crossing a sexual maternal parent and an apomictic pollen parent, since apomictic plants produce viable reduced pollen.

Apomictic and sexual plants can be classified based on the morphology of the embryo sac (Koltunow 1993). The apomictic aposporic plants of *Brachiaria* produce a Panicum-type embryo sac while sexual plants produce a Polygonum-type embryo sac (do Valle and Savidan 1996).

In *B. ruziziensis* (Germain and Evrard 1953), among 36 accessions, none were identified as apomictic by embryo sac analysis (do Valle and Savidan 1996). However, duplication of the chromosome number was achieved by colchicine application to 3-day-old seedlings, with immersion of the entire plantlet in colchicine solutions at different concentrations (Swenne et al. 1981). These plants are the basis of the interespecific

Communicated by J.J. Finer

A.A. Pinheiro · M.T. Pozzobon · V.T.C. Carneiro (✉)
Centro Nacional de Pesquisa em Recursos Genéticos e Biotecnologia, CENARGEN/EMBRAPA, SAIN Parque Rural, Brasília, DF, Brazil

C.B. do Valle, M.I.O. Penteado
Centro Nacional de Pesquisa de Gado de Corte, CNPGC/DMBRAPA, Campo Grande, MS, Brazil

crosses in the *Brachiaria* breeding programs. Chromosome doubling has been obtained in various plant species by colchicine treatment of meristems (Lyrene and Perry 1982), suspension cells (Chavedej and Becker 1984), embryogenic calli (Leblanc et al. 1995) and haploid plants (Miyoshi and Asakura 1996). In this study, we established a protocol for multiple-shoot formation from basal segments of in-vitro-cultivated *Brachiaria* accessions. The chromosome number of *B. brizantha* plants was subsequently duplicated by the application of colchicine to in vitro explants.

Materials and Methods

Plant material

Plants of the following different *Brachiaria* species from a collection assembled at Embrapa were cultivated in a greenhouse and propagated vegetatively: diploid sexual *B. brizantha* (BRA 002747 and BRA 003417), tetraploid apomictic *B. brizantha* (BRA 000591) and tetraploid apomictic *B. decumbens* (BRA 001058). The ploidy level of these plants was verified by cytological analysis of root tips.

For in vitro culture, lateral meristems of greenhouse-grown plants were surface sterilized in 70% (vol/vol) ethanol for 2 min, 1% sodium hypochlorite for 20 min, and washed three times with sterile water.

Induction of regeneration

Meristems were isolated using a stereomicroscope and three meristems were cultured on 25 ml of LS medium (Linsmaier and Skoog 1965) in 9 cm-diameter petri dishes. Different combinations of growth regulators were tested as follows: 1 mg/l naphthaleneacetic acid (NAA), +3 mg/l kinetin (KIN) (NAA1/KIN3), and 1 mg/l (NAA)+3 mg/l 6-benzylaminopurine (BAP) (NAA1/BAP3). The number of shoots regenerated per meristem-derived callus was recorded. Regenerated shoots were transferred to B rooting medium (Bourgin et al. 1979) supplemented with 30 g/l sucrose. Plants were cultivated at 23 °C under a 16-h photoperiod (1.5 W m^{-2} daylight fluorescent illumination). For micropropagation, a 0.5-cm segment (called hereafter basal segment) just above the roots (Fig. 1A) was excised and inoculated on the same B rooting medium.

Colchicine treatment

Basal segments of BRA 002747 in vitro plants were inoculated in culture media supplemented with either 0.01, 0.05, or 0.1% (wt/vol) colchicine. A fresh colchicine stock solution was prepared just before use, filter-sterilized and added to the autoclaved culture media. The segments were inoculated for 48 h in LS medium supplied with NAA1/KIN3 and one of the three different colchicine concentrations. For each concentration, three replicates, each with 25 basal segments, were used. After colchicine treatment, explants were transferred to the same LS medium, without colchicine. Regenerated buds were transferred to B rooting medium (Bourgin et al. 1979) with 30 g/l sucrose. After four weeks, plants were moved to the greenhouse and planted in vermiculite for 1 week, before being transferred to soil.

Explants of the BRA 003417 genotype were incubated according to the same protocol using only a 0.01% (wt/vol) colchicine concentration.

Flow cytometric analysis

Flow cytometric analysis was carried out to determine ploidy levels of the original and the colchicine-treated plants. Approximately 0.5 cm^2 of leaf tissue from the target plants was cut and placed in a small petri dish, together with a similar amount of leaf tissue from the control plant, a known diploid accession of *B. decumbens*. A few drops of nuclei extraction buffer (Galbraith 1989), which uses bis-benzimidine as DNA stain, was immediately added and the leaves were chopped with a razor blade. The volume was adjusted to 2.5 ml with extraction buffer and the mixture was filtered, incubated for about 4 min and then run through a PARTEC CA II flow cytometer.

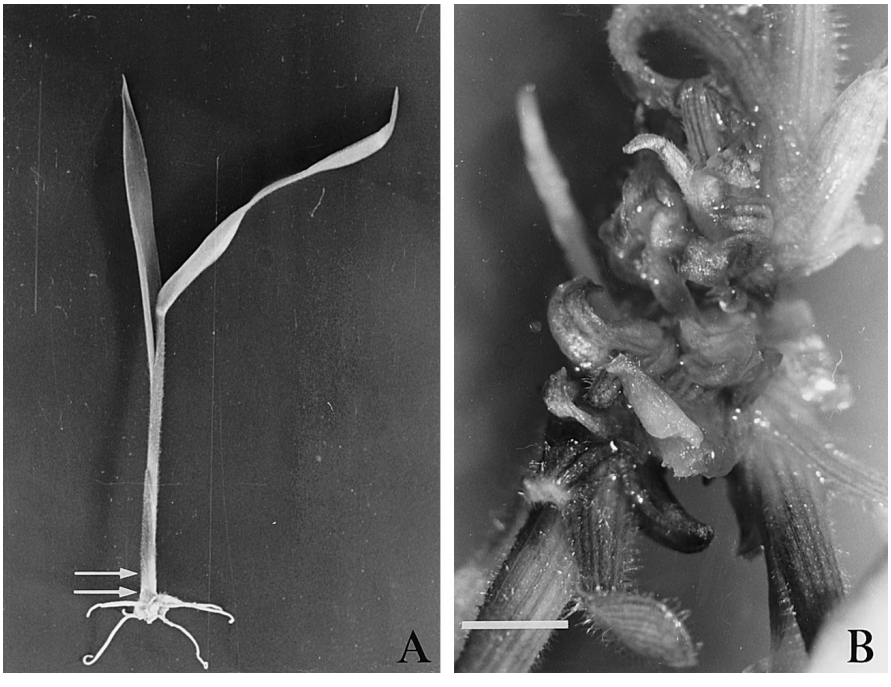

Fig. 1 Multiple shoot formation in *Brachiaria* **A** Plant of *B. brizantha*, cultured in vitro, showing basal segment (between arrows) used as explant. **B** Regenerated shoots from this same explant inoculated on LS medium supplemented with 1 mg/l NAA and 3 mg/l KIN (*bar* 8 mm)

Table 1 Effects of the growth regulators NAA, BAP, and KIN on multiple shoot formation in meristems of three *Brachiaria* genotypes. Within the same genotype, means with the same letter are not significantly different from each other ($P>0.05$)

Genotype	NAA1/KIN3					NAA1/BAP3				
	Number of cultured meristems	Number of meristems regenerating plants			Mean number	Number of cultured meristems	Number of meristems regenerating plants			Mean number shoots
		1–5 shoots	5–10 shoots	>10 shoots			1–5 shoots	5–10 shoots	>10	
BRA002747	30	9 (30%)	10 (33%)	11 (37%)	9.9a	26	7 (27%)	10 (38%)	9 (35%)	10.8a
BRA000591	24	7 (29%)	7 (29%)	10 (42%)	9.8a	30	10 (33%)	8 (27%)	12 (40%)	8.9a
BRA001058	25	0 (0%)	7 (28%)	18 (72%)	13.7a	23	1 (4%)	10 (43%)	12 (52%)	10.7b

Cytological Analysis

Mitotic chromosomes were counted on root meristems of all the donor plants and of the plants where duplication was verified by flow cytometry. The collected root tips were pre-treated in a saturated solution of 1-bromonaphthalene for 2 h at room temperature. Pre-treated root tips were fixed in a mixture of ethanol:acetic acid (3:1, vol/vol) for 2–24 h, hydrolyzed in 1 N HCl for 10 min at 60 °C, digested with 2% pectinase for 45 min, stained with Feulgen for 30 min (Pozzobon and Valls 1997) and then finally macerated with propionocarmine (0.6% carmine in propionic acid 45%) for slide preparation. Analyses were performed under a light microscope.

Results and discussion

Multiple shoot formation was obtained from basal segments of the sexual accession of *B. brizantha* (BRA 002747) in LS medium NAA1/KIN3 (Fig. 1B). High-efficiency shoot formation was important since colchicine should be applied at a precocious stage of culture, allowing the application of small quantities of this reagent to a large number of vegetative meristems. Both NAA1/KIN3 and NAA1/BAP3 resulted in high-efficiency multiple shoot formation from meristems of all three varieties, BRA 002747, BRA 000591, and BRA 001058 (Table 1).

All the inoculated explants regenerated in both culture media, with most explants forming more than six shoots/explant. Accession BRA 001058 responded significantly ($P<0.05$) more favorably to NAA1/KIN3, so this composition was used for colchicine treatment of BRA 003417.

The optimal mitotic activity of colchicine depends on its concentration and the duration of the treatment.

Table 2 Effect of colchicine on survival and chromosome duplication of in-vitro-regenerated BRA 002747 plants. Means with the same letter are not significantly different from each other ($P>0.05$)

Colchicine concentration (wt/vol)	Number of explants	Number of regenerated plants	Survival (%)	Number of duplicated plants
0.01%	75	44	58.7a	18
0.05%	75	0	0.0b	0
0.1%	75	2	2.7b	1

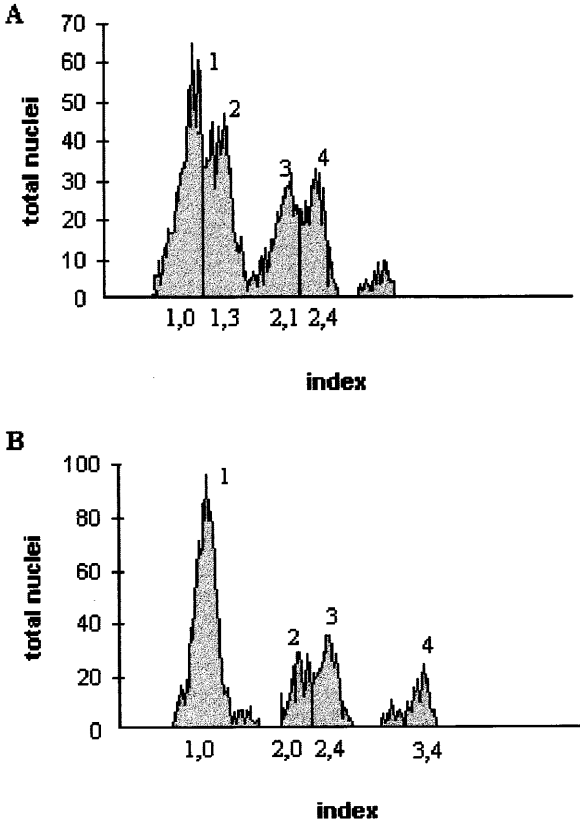

Fig. 2A, B Histograms obtained from flow cytometry of *Brachiaria* cells. **A** Peak *1* correspons to diploid *B. decumbens* plant, used as control;peak*2* corresponds to a diploid *B. brizantha* plant; peaks*3* and *4* correspond to cells of the same material, undergoing divisions. **B** Peak *1* corresponds to the same control plant used in **A**; peak *3* corresponds to a colchicine-treated plant of *B. brizantha*, indicating that duplication of the chromosome number was achieved; peaks *2* and *4* are cells from the same material undergoing divisions.

The treatment period was fixed for 48 h and the colchicine concentrations varied. The best colchicine treatment applied to 75 basal segments of BRA 002747 was 0.01% with approximately 60% of the explants regenerating shoots; 40% of these had duplicated chromosomes (Table 2), meaning that 24% of the inoculated explants produced duplicated plants. Only one triploid shoot was recovered. In long-term cultures, callus cells obtained from diploid plants may develop shoots of

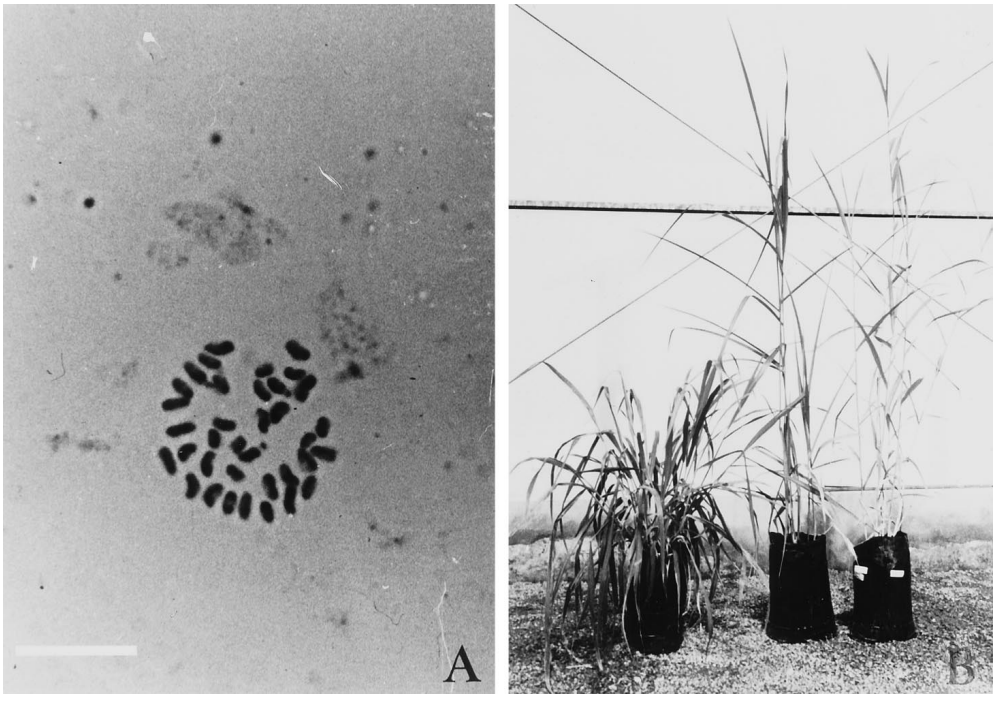

Fig. 3A, B Analyses of chromosome-duplicated plants of BRA 002747, *B. brizantha* sexual diploid. **A** Chromosomes (2n = 36) in root tip of colchicine-treated plant (*bar* 45 μm). **B** Duplicated plant in the *center*, an apomitic *B. brizantha* tetraploid plant BRA 000591 on the *left* and the original sexual plant BRA 0002747 on the *right*

different ploidy number (Chavadej and Becker 1984), which may be due to unequal migration of chromosomes during mitotic anaphase-telophase. Once a triploid cell is produced, it will multiply. Triploid somatic cells and plants regenerated from such cells will be triploid (Singh 1993). In our work, shoots were produced directly from basal segments in a short period of time, thus recovery of a high number of triploid plants was avoided. At the other colchicine concentrations (0.05% and 0.1%), survival of shoots was very low. BRA 003417 was therefore treated with 0.01% colchicine as described in the Materials and Methods. In this species, 88% of the explants survived, 40% of them being duplicated.

The ploidy level of leaves of the colchicine-treated plants was determined by flow cytometry and the chromosome number of the putative duplicated plants was counted using root tips. The results of both methods agreed, suggesting that no chimerical plant was obtained. Moreover, these results confer reliability to flow cytometry (Galbraith et al. 1983) as a practical and rapid tool for screening the ploidy levels of in-vitro-regenerated plants. Plants can be analysed as soon as the first leaves develop, in a precocious stage of the in vitro culture, before transfer to rooting medium. Similar results were also obtained in *Tripsacum* plants regenerated from colchicine-treated calli (Leblanc et al. 1995).

The cytohistogram (Fig. 2A) corresponding to a diploid *B. brizantha* non-duplicated plant, showed that this plant (peak 2) presents a higher DNA content than the diploid *B. decumbens* used as control (peak 1) as previously observed (Penteado et al. 1996). The cells undergoing mitosis yield duplicated peaks as shown in Fig. 2A, peaks 3 and 4.

B. brizantha plants (Fig. 2B) with a positive response to colchicine treatment (peak 3), showed a higher DNA content than *B. decumbens* plants (peak 1), demonstrating the duplication of the ploidy level of this plant. The number of dividing cells varied with the time of most harvest. Most of the cells were in metaphase, during the summer season, between 9^{00} and 10^{00} hours. Metaphase of BRA 002747 colchicine-treated plants had 36 mitotic chromosomes (Fig. 3A), confirming the flow cytometric data. The phenotype of the regenerated plants was similar to the explant donor sexual plant (Fig. 3B).

Chromosome duplication was achieved in the two *B. brizantha* accessions used in this work, BRA 002747 and BRA 003417. An efficient procedure to obtain *Brachiaria* plants with a duplicated chromosome number using tissue culture techniques, has thus been established.

Morphological analyses of embryo sacs of duplicated plants are being performed to investigate the relationship between the reproductive mode of *Brachiaria* and ploidy level. Agronomic evaluation of the duplicated plants will also be undertaken to determine their applicability in the *Brachiaria* breeding program.

Acknowledgements Alessandra A. Pinheiro thanks RHAE-CNPq program for an ITI fellowship. This work is partially financed by project no. 65.0090/94.4 PADCTII/SBIO from M.C.T., Brazil and project no. TS3-CT 93 0242 EU/STD.

References

Bourgin JP, Chupeau Y, Missonier C (1979) Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiol Plant 45:288–292

Chavadej S, Becker H (1984) Influence of colchicine treatment on chromosome number and growth rate of tissue cultures of *Valeriana Wallichii* DC. Plant Cell Tissue Organ Cult 3:265–272

Galbraith DW, Harkins KR, Maddox JM, Ayres NA, Sharma DP, Firoozabady E (1983) A rapid flow cytometric analysis of cell cycle in intact plant tissues. Science 220:1049–1051

Galbraith DW (1989) Analysis of nuclear DNA content in plant cell by flow cytometry. Biol Plant 31:113–120

Germain R, Evrard C (1953) Un nouveau *Brachiaria* de l'est du Congo. Belge Bull Jard Bot Etat 23:373–377

Hanna WW, Bashaw EC (1987) Apomixis: its identification and use in plant breeding. Crop Sci 27:1136–1139

Koltunow AM (1993) Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5:1425–1437

Leblanc O, Hernández M, Bello S, Garcia V, Bertaud J, Savidan Y (1995) Chromosome doubling in *Tripsacum*: the production of artificial, sexual tetraploid plants. Plant Breed 114:226–230

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. Physiol Plant 18:100–127

Lyrene PM, Perry JL (1982) Production and Selection of blueberry polyploids in vitro . J Hered 73:377–378

Miles JW, Maass BL, Valle CB do (1996) *Brachiaria*: biology, agronomy and improvement. Centro Internacional de Agricultura Tropical-CIAT, Cali, Colombia

Miyoshi K, Asakura N (1996) Callus induction, regeneration of haploid plants and chromosome doubling in ovule cultures of pot gerbera (*Gerbera jamesonii).* Plant Cell Rep 16:1–5

Nogler GA (1994) Genetics of gametophytic apomixis – a historical sketch. Pol Bot Stud 8:5–11

Penteado MIO, Hernández M, Savidan Y, Santos ACD, Valle CB do (1996) Variation on DNA content in several species of *Brachiaria*. Braz J Genet 19:1z–291

Pozzobon MT, Valls JM (1997) Chromosome number in germplasm accessions of *Paspalum notatum* (Gramineae). Braz J Genet 20:29–34

Serrão EAD, Simão Neto M (1971) Informações sobre duas espécies de gramíneas forrageiras do gênero *Brachiaria* na Amazônia: *B. decumbens* Stapf e *B. ruziziensis* Germain et Evrard. In: IPEAN série: Estudos sobre forrageiras na Amazônia. vol 2, no 1, Instituto de Pesquisa Agropecuária do Norte (IPEAN), Belém, PA, Brazil

Singh RJ (1993) Plant cytogenetics. CRC, Boca Raton, Fl

Swenne A, Louant B, Dujardin M (1981) Induction par la colchicine de formes autotetraploïdes chez *Brachiaria ruziziensis* Germain et Evrard (Graminée). Agron Trop 36:134–141

Valle CB do, Miles JW (1992) Breeding of apomictic species. Apomixis Newsl 5:37–47

Valle CB do, Savidan YH (1996) Genetics, cytogenetics and reproductive biology of *Brachiaria.* In Miles JW, Maass BL, do Valle CB (eds) *Brachiaria*: biology, agronomy and improvement. Centro Internacional de Agricultura Tropical-CIAT, Cali, Colombia pp 164–177

Vielle J-PC, Crane CF, Stelly DM (1996) Apomixis: the asexual revolution. Science 274:1322–1323