Cytological Alterations in *Eragrostis* Regenerants

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Summary Weeping lovegrass, *Eragrostis curvula* (Schrad.) Nees, is an important apomictic forage grass for semiarid regions. The aim of this work was to study the effect of *in vitro* culture in the mitotic and meiotic behaviour of regenerated weeping lovegrass plants. Thirty two plants were obtained from *in vitro* culture of inflorescences segments in MS medium with different concentrations of BAP (0.01, 0.1, 1, 2, 5 mg⁻¹) and 2,4-D (1, 2, 4, 6, 8 mg⁻¹). Ten of these plants were taken at random in order to determine the chromosome number and analyze the meiotic behaviour. In the regenerated plants it was possible to observe mixoploidy and meiotic disturbances like alterations in chromatin condensation, meiocytes degeneration, diminution in pollen mother cells and pollen grains quantity, abnormal pairing, bridges and fragments and no pollen formation. Because the apomictic nature of this grass and the uniformity of this cultivar, stable genetic variability that involve phenotypical and biochemical traits due to karyological variation could be potentially used for crop improvement.

Key words Weeping lovegrass, Tissue culture, Chromosome instability, Meiotic behaviour.

Plants regenerated from relatively undifferentiated callus cultures possess a vast array of genetic changes (Phillips *et al.* 1994). Such variations can result in useful agricultural products or may be undersirables, for example, when using cultured cells for genetic engineering.

Weeping lovegrass is an important forage grass for semiarid regions (Covas 1991). The reproductive mode of this species, mainly apomictic, restricts the use of conventional breeding methods. The search for variability through hybridization procedures involves the availability of sexual and self-sterile germoplasm (Voigt and Bashaw 1971). The response to hybridization is also variable, in some instances with low seed production (Voigt 1991). The alternative of using plant tissue culture techniques in addition to conventional breeding methods would be useful, specially in obligate apomictic cultivars.

Normal cell behaviour is the result of a complex cascade of genetic programs that is sensitive to disruption by biotic and abiotic stresses (Phillips *et al.* 1994). *In vitro* culture *per se* can be extremely stressful on plant cells and involve highly mutagenic processes during explant establishment of callus induction, maintenance, embryo induction, and plant regeneration (Lörz *et al.* 1988).

Because broad spectrum variation is available through either nuclear or cytoplasmic sources, all types of variation could potentially be recovered and used for crop improvement (Elkonin *et al.* 1994, Skirvin *et al.* 1994). Stable genetic alterations involve phenotypic and biochemical traits due to karyological variations (gene or chromosome aberrations) (Lörz *et al.* 1988). Heritable somaclonal variation involves either single or multiple gene changes, including alterations in DNA bases, chromosomes, or the entire genome, single base-pair changes, paracentric and pericentric inversions, deletions, translocations, and ploidy changes (Duncan 1997). Abnormal microsporogenesis affecting pollen sterility may be common and genotype dependent (Garcia *et al.* 1994).

The objective of this work was to study the effect of *in vitro* culture in the mitotic and meiotic

behaviour of regenerated weeping lovegrass plants as a way to evaluate the increase of variability in this species.

Materials and methods

Plant material

One representative plant of *Eragrostis curvula* (Schrad.) Nees, cv. Morpa, was taken as explant donor and it was also used as control.

Inflorescence culture

It was done as previously reported (Echenique *et al.* 1996a), briefly: inflorescences just emerging from the flat leaf were sterilized with 70% ethanol for 1 min, followed by commercial bleaching solution (0.8% of active chlorine) for 15 min, and were washed three times with sterilized distilled water. Inflorescence segments (with 10 to 20 spikelets) were cultured on Murashige and Skoog's medium (1962)+2,4-D (1, 2, 4, 6, 8 mg l⁻¹)+BAP (0.01, 0.1, 1, 2, 5 mg l⁻¹). Three samples by treatment were carried out. The cultures were kept for 4 weeks in the dark at 25°C and then transferred to a growth chamber with a photoperiod of 16 h (dim light: 7–11 μmol m⁻² s⁻¹). Then the cultures were transferred to a culture room with the same photoperiod and temperature but 66 μmol m⁻² s⁻¹ for 6 months. The regenerated plants (R₀) were transferred to 101 pots with soil and were kept in a greenhouse with high humidity.

Cytogenetic analysis

Immature panicles were fixed in Clarke's solution for 24 h at room temperature and were preserved in 70% ethanol at 4°C. Staining was done with Feulgen technique. The hydrolysis was carried out with 1 N hydrochloric acid at 60°C during 15 min. The staining was strengthened with 45% hematoxylin solution and iron acetate as mordant (Nuñez 1968). For chromosome counting 15 metaphasic plates from 10 roots per plant were taken. For meiotic behaviour analysis 30 flowers from each plant were considered. Ten R₀ (primary regenerants) plants were taken at random.

Results

Thirty two plants were obtained by embryogenesis and organogenesis, both responses in the same callus (Echenique *et al.* 1996a). These plants showed morphological and isozymic changes (Echenique *et al.* 1996b).

The somatic number of the control plant was 2n=4x=40, as was previously reported for this cultivar (Poverene *et al.* 1985), with three supernumerary or B chromosomes. These B chromosomes were constant in all the analyzed cells (Fig. 1a, b). In a previous paper (Poverene *et al.* 1985) these structures were mentioned as chromosome breakage for another *Eragrostis curvula* cultivar. The constancy in all the plates conforms that this supernumerary chromosomes are B chromosomes.

Two of the regenerated plants exhibited instability of the somatic number or mixoploidy (P₅ and P₈), having normal cells mixed with abnormal ones, with different aneuploid numbers (Fig. 1c). Chromatin clumps in the cytoplasm (Fig. 1e) and normal (4x) and polyploid cells mixed in the same root (Fig. 1d) were observed. The other eight plants showed normal somatic number.

The meiotic behaviour of the control plant was normal. In diakinesis the pairing was mainly as bivalents and, in a lower ratio, quadrivalents. No univalents were observed. Metaphase I was synchronous but non-cooriented bivalents were appeared (in 8% of meiocytes in this stage). The disjunction in Anaphase I was normal and this situation persisted until the end of the cell division (Fig. 2a). Pollen grains shape, size and quantity were normal (Fig. 2b).

Fig. 1. *Analysis in somatic tissue.* Control plant. a) Metaphase $2n=40+3B$. b) Prometaphase with three B chromosome (arrow). Regenerated plant. c) Mixoploidy (P_5). d) Polyploid cell (P_8). e) Chromatin clumps. Interphase (P_5). Bar = $10\mu m$.

One of the regenerated plants taken for analysis did not flower (P_{10}) and another one (P_9) was in an advanced pollen stage, even though the size and the age of the spikelets were characteristic of a less mature plant (Table 1).

All of the other 8 plants showed meiotic abnormalities (Table 2). These abnormalities were: alterations in chromatin condensation (5 plants) with a range of 4 to 52%, meiocytes degeneration (4 plants) (Fig. 2c), diminution in pollen mother cells (PMC's) and pollen grains quantity (4 plants). During Prophase I (Zygotene to Diplotene) cytomixis was observed (Fig. 2d). This alteration involves chromatin migration between 2 cells. It was detected in 3 plants with percentages ranging 16 to 29%. This plants produced pollen grains with different shapes and sizes (Fig. 2e). It was also possible to observe total or partial desynapsis (in 3 plants, ranging 14 to 25%).

In one of the analyzed plants, Table 2 (*) no pollen grains were found and the callose surrounding the meiocytes was deposited in a layer thicker than that in control plants (Fig. 2f).

The organization of the spindle microtubules was also affected in 5 plants (14 to 65%) resulting in non centromeric coorientation in MI followed by anarchic chromosome distribution in the equatorial plate. Abnormal spindles were also observed in MII (Fig. 2g).

Two regenerated plants showed fragments and bridges in AI (Fig. 2h) and AII. Twenty two percent of the regenerated plants did not flower. Instead of the normal flowering observed in the most

Fig. 2. *Meiotic behaviour*. Control plant. a) Tetrads. b) Normal pollen grain. Regenerated plant. c) Meioocytes degeneration. d) Cytomictic pollen mother cells. Prophase I. e) Abnormal pollen grain. f) Aberrant callose deposition in tetrads. g) Result of tripolar spindle. h) Fragments and bridge. Anaphase I. Bar=10 μ m.

Table 1.

	P ₉	Control
Secnd secondary branch length from the panicle base	25 mm	25 mm
Third spikelet length from the secondary branch base	4 mm	4 mm
Spikelet's 2nd floret anthers length	0.9 mm	0.8 mm
Meiotic stage	Pollen grain	Pachytene

Table 2. Meiotic anomalies in eight regenerated plants

	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₇	P ₈	Control
Chromatin condensation alteration	7/114 6%	32/227 14%	15/130 12%	43/82 52%				7/181 4%	
Cytomixis		52/329 16%	37/130 29%	20/82 24%					
Desynapsis	31/150 21%				43/174 25%	12/89 14%			
Meiocytes degeneration									
Spindle failure	44/68 65%				77/229 34%	25/180 14%	207/412 50%	112/323 35%	
Different pollen size and shapes									
Scarce pollen								(*)	
Aberrant callosa deposition									
PMCs scored	205	331	211	150	422	445	658	556	754

P₁ to P₈=Primary regenerated plants (R₀). (*) Absence of pollen grains. White spaces mean normality. Painted sectors indicate abnormalities observed in different plants. Alteration in chromatin condensation: represent the total of meiocytes that have this alteration from the beginning of cigotene to the end of diakinesis divided by the total number of meiocytes in the same period. Desynapsis: from Cigotene to the end of metaphase I. Cytomixis: from Leptotene to the end of Diakinesis. Spindle failure: from Metaphase I to the appearance of microspores.

of them, some plants had not seeds.

Discussion

The isolation of plant cells to callus formation, and the subsequent resetting of the genome may inflict to a cell a series of traumatic experiences, giving rise to chromosome restructurings that result in altered phenotypic expressions (Mc Clintock 1984). One of the first responses observed in this study was mixoploidy, indicating a multicellular origin for some of these plants that came from calli where embryogenesis and organogenesis were observed simultaneously (Echenique *et al.* 1996a). Chromatin clumps in the cytoplasm could be the origin of the aneuploidy observed, reflecting chromosome losses related with them. Events leading to chromosome breakage, and in some instances subsequent exchange or reunion of fragments also results in chromatin loss.

The majority papers conducted with structural chromosomic changes under tissue culture conditions were made in species with easily checked karyotypes like maize (Green and Phillips 1975). In this grass chromosome size was critical to detect chromosomic aberrations but some considerations could be made comparing with the control plant, and through meiotic analysis, that offer the additional advantage of evaluating pairing relationships among homologous chromosomes and chromosome behaviour through several stages of meiosis. This approach often allow detection of subtle variation that would be more difficult to observe through analysis of mitotic cells. Analysis of meiotic cells has made an important contribution to the understanding of the cytological variation associated with tissue culture.

The behaviour of the control plant was normal like in the other tetraploids of the *Eragrostis curvula* complex (Vorster and Liebenberg 1977). In the regenerated ones it was possible to observe alterations in chromatin condensation, meiocytes degeneration, diminution in PMC's and pollen grains quantity, abnormal pairing, bridges and fragments in Anaphase and no pollen grains formation. Chromosome aberrations found in plants regenerated from tissue culture are frequently the result of chromosome breakage events (Phillips *et al.* 1994). The appearance of bridges and fragments in Anaphase I and II involves the presence of paracentric inversions in these plants. In maize, Rhoades and Dempsey (1971, 1973) proposed a model of late replicating heterochromatin as the

basis for chromosome breakage in A chromosomes in the presence of B chromosomes. Cytological and genetic observations have shown that breakage occurs between the knob and the centromere. Labeling studies have subsequently demonstrate that knob heterochromatin was the last to replicate in root-tip cells containing B chromosomes (Pryor *et al.* 1980). As was mentioned above it was possible to detect B chromosomes in these plants, and *in vitro* culture could increase the frequency of these breakages. All the fragments generated could be rearranged or lost, contributing in some instances to increase variability.

The herbicide mode of action of 2,4-D is not well understood but apparently involves a substantial general increase in transcription. The events laeding to the increased transcription may alter the chromatin structure (Phillips *et al.* 1994), in the way that was possible to observe in this study.

With this level of analysis it is not possible to determine the stability of these variations. Evidences indicate some genetic origin, being all the disturbances the consequence of ruptures, looses and exchanges. The presence of abnormal spindles in meiocytes of regenerated plants resembles mutations like those observed by Oakley and Morris (1981) in *Aspergillus nidulans*.

Instead of the normal flowering it was possible to detect pollen disminution and absence of seeds in some regenerated plants. Could be possible here a phenomenon similar to that observed by Staiger and Cande (1993) in maize, mentioned as "exit from meiosis". This phenomenon results in meiocytes degeneration, like it was possible to observe in this plants, meaning that the stress inflicted by *in vitro* culture could direct the plant to stop this event. For a sexual plant this should be detrimental for variability. The no dissolution of callose observed may contribute to the pollen absence. Callose dissolution is a necessary step for the normal formation of pollen grains (Heslop-Harrison 1972, Abad *et al.* 1995).

The important feature for this grass, an apomictic one, is to generate variability that could be potentially expressed phenotypically and recovered in the progeny. It was possible here to detect changes. We continue working with this plants and the progeny to detect if that changes are important to generate useful genetic variants.

Meiotic analysis was more useful to detect anomalies in this material. Studying mitosis we only conclude that 2 plants out of 10 had anomalies but all of them had some aberrations, detectables at some meiotic stage.

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