Theor Appl Genet (1985) 69:575–581

Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum

B. Swedlund and I. K. Vasil*

Department of Botany, University of Florida, Gainesville, FL 32611, USA

Received July 18, 1984; Accepted September 18, 1984
Communicated by P. L. Pfahler

Summary. Embryogenic calli were derived from cultured segments of immature inflorescences of *Pennisetum americanum* (pearl millet). The original explants as well as the embryogenic calli and the plants regenerated via somatic embryogenesis were examined cytogenetically. Embryogenic calli were predominantly diploid (2n = 14) after one month and six months in culture (92% and 76%, respectively). Tetraploid and aneuploid cells were observed in the original explant (2.5% and 1.2%) as well as in one (4.0% and 4.0%) and six-month-old calli (10.0% and 14.0%). Plants were regenerated from calli that had been in continuous culture for two, four and six months. Of the 101 regenerants, 100 were diploid and 1 was tetraploid. The tetraploid was an albino as were three of the diploid regenerants. Examination of 30 of the regenerants in meiotic diakinesis, anaphase I, anaphase II and quartet stages revealed no cytogenetic differences between control and regenerated plants. Gel electrophoresis for total protein content and alcohol dehydrogenase and malate dehydrogenase activity also did not reveal any differences between the controls and regenerants. The results of this study show that a slight shift toward aneuploidy and polyploidy may occur in embryogenic cultures, but there also is a strong selection in favor of plant regeneration from cytogenetically normal cells.

Key words: Cereals/grasses – Genetic variability – Pearl Millet – *Pennisetum americanum* – Plant tissue culture – Somatic embryogenesis

* To whom correspondence and reprint requests should be addressed

Introduction

Plant cells grown in vitro exhibit considerable cytological variability and at least some of this can be traced to the mixoploid nature of the explants used for culture (Bayliss 1980). However, evidence is also available to show that some of the variability arises in culture. Cytogenetic variability is common also in plants recovered from tissue cultures (Larkin and Scowcroft 1981).

It is now possible to regenerate plants from tissue cultures of most of the important species of cereals and grasses (Vasil 1982, 1983a, b). The regenerated plants of these species exhibit extensive cytogenetic variability in some cases (McCoy et al. 1982; Karp and Maddock 1984) and relative uniformity in others (Chen et al. 1981; Heyser and Nabors 1982; McCoy and Phillips 1982; Hanna et al. 1984).

Plants regenerated by somatic embryogenesis have been reported to be euploid and free of any noticeable morphological variability, and it has been suggested that embryogenic cultures of the Gramineae as well as the plants derived from them are cytogenetically normal and stable (Vasil 1982, 1983a, b; Hanna et al. 1984). However, no serious attempt has been made to correlate the nature of the cell cultures themselves, and/or the mode of plant regeneration, to the formation of cytologically normal or variant plants. This paper describes the results of a cytogenetic study of embryogenic cell cultures and plants of *Pennisetum americanum* (L.) K. Schum. (pearl millet) regenerated by somatic embryogenesis.

Materials and methods

Callus initiation, maintenance and plant regeneration

The methods of Vasil and Vasil (1981a) were followed for callus initiation and maintenance and plant regeneration from immature inflorescences of *Pennisetum americanum* cv.

'Gahi 3,' a cytoplasmic male sterile cultivar. Cultures were initiated from approximately 40 immature inflorescences and their culture lineages were maintained separately. At the end of each subculture period the embryogenic and nonembryogenic calli were separated and ca. 3×3 mm portions of embryogenic calli were placed on fresh media. After two, four and six months, embryogenic calli were transferred to Murashige and Skoog's (1962) medium (MS) with 0.25 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg/l kinetin and placed in the light. After embryoids began to green the 2,4-D level was reduced to 0.13 mg/l. Plantlets were transferred to ½ MS with 3% sucrose and 0.2 mg/l naphthalene acetic acid in 25×100 mm tubes to promote root growth. Plants were grown to maturity in a glasshouse.

Mitotic preparations

Material for mitotic examination was pretreated in a saturated solution of α-bromonaphthalene (1.5 h), placed in Farmer's fixative (3:1 ethanol:acetic acid) overnight and stored in 70% ethanol. Hydrolysis varied for a given tissue as follows: inflorescences – 18 min in 1N HCL at 60 °C; embryogenic callus – 10 min in 5N HCl at 26 °C; embryoids – 8 min in 5N HCl at 26 °C; root tips – 9 min in 5N HCl at 26 °C. Hydrolyzed material was stained in Schiff's reagent for 2–4 h. Observations of intact cells were made on a Zeiss phase contrast microscope at 800× and 1000× (oil immersion). Approximately 20 cells in each of 20 immature inflorescences, 100 cells each in one-month and six-month-old calli, and at least three cells in each of 10 embryoids were examined. For regenerated plants a minimum of three root tips were squashed for each regenerated plant, and at least three cells were examined for each root tip.

To prepare embryogenic callus for mitotic examination pieces of calli were taken 5–6 days following subculture and placed in 25 ml liquid MS maintenance medium with 1% sucrose in 125 ml flasks to reduce the amount of starch. These were placed in the dark at 26 °C on a gyrotory shaker (100 rpm). After 5–6 days the cultures were removed from the shaker and the flasks were placed in the cold (4 °C) for 24–36 h. The calli were then treated as described above.

Meiotic preparations

Emerging inflorescences were fixed in modified Carnoy's fixative (10:3:3 ethanol:acetic acid:chloroform). Anthers were excised and macerated in acetocarmine.

Meiotic cells were examined in 33 plants, 10 regenerants each from two, four and six-month-old calli and three control plants. A minimum of 50 cells were examined each in diakinesis, anaphase I and anaphase II and a minimum of 150 figures were examined in the quartet stage of each plant.

The regenerants and a control were crossed with cv. 'Tift 23 B' to obtain seeds. Two plantings (separated by time) of randomly selected two- and four-month regenerants and a control were made. The plantings were scored for germination and gross morphological characters.

Gel electrophoresis

Pieces of mature leaf tissue (400 mg) from 13 plants, four each from regenerants from two, four and six-month-old calli and one control plant were ground in sample buffer. Aliquots of 15 µl from each sample were run on 10% polyacrylamide gels (Laemmli 1970). Gels were silver stained for total proteins according to the method of Morrissey (1981).

For alcohol dehydrogenase (ADH), roots of nine randomly selected regenerants and a control were soaked in water overnight. Root tissue (400 mg) was ground in 1 ml of native sample buffer (pH 8.8) and 15 µl aliquots were run on 10% polyacrylamide native gels. Gels were developed in the dark at 26 °C for 2 h. (Schwartz and Endo 1966). For malate dehydrogenase (MDH) mature leaves (200 mg) of nine randomly selected regenerants and a control were ground in 1 ml of native sample buffer (pH 8.8) and run on gels as described for ADH. The staining solution differed by substituting 3.0 ml of 1M Na-L-Malate (pH 7.0) for the EtOH. The gels were developed in the dark at 45 °C for 2 h.

Controls

Control plants in all cases were seed propagated plants of *P. americanum* cv. 'Gahi 3'.

Results

Original explant and callus

Chromosome numbers observed in the original explant were found to be predominantly diploid (95.8%), with some tetraploid (2.5%) and aneuploid (1.2%) cells (Table 1).

Chromosome numbers in embryogenic calli were observed one month and six months after callus initiation. After one month the percentage of diploid cells was 92%, with 4% tetraploid and 4% aneuploid cells. After six months, diploidy had decreased to 76% while tetraploidy and aneuploidy had increased to 10% and 14%, respectively. The most frequently observed aneuploidy was that of 16 chromosomes per cell (9%,

Table 1. Variation in chromosome numbers in the original immature inflorescence explant and in the callus derived from it[a]

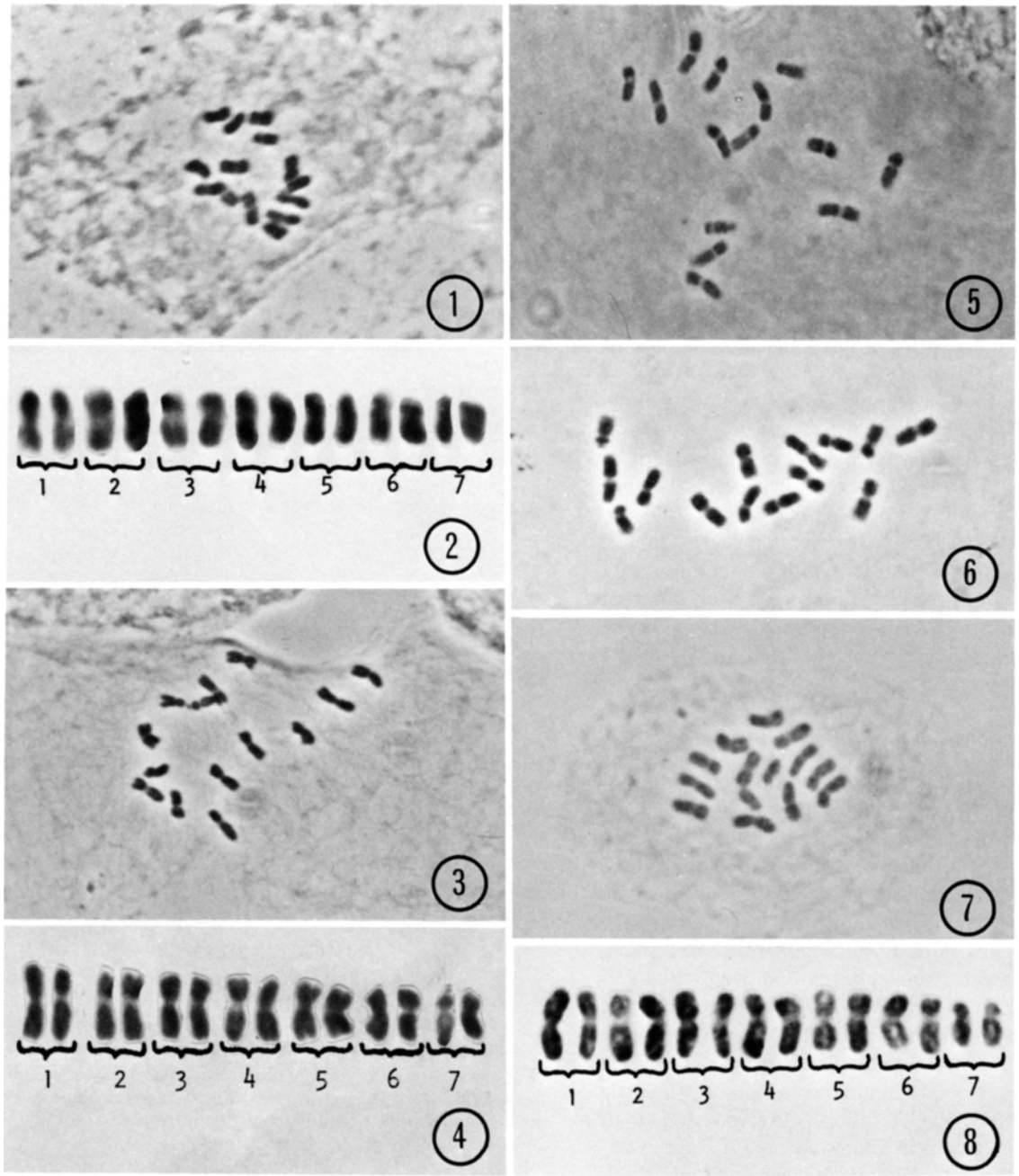
Age of callus	Chromosome nos observed (% of total)
Original explant	13 (0.6%)
	14 (95.8%)
	16 (0.3%)
	22 (0.3%)
	28 (2.5%)
1 month	13 (2.0%)
	14 (92.0%)
	16 (2.0%)
	28 (4.0%)
6 months[b]	13 (3.0%)
	14 (76.0%)
	16 (9.0%)
	17 (1.0%)
	20 (1.0%)
	28 (10.0%)

[a] Original explant data from 384 cells from 20 inflorescences. Callus data from 100 counts for each age examined

[b] Significant (compared to original explant) at the 0.01 level using the chi-square goodness of fit test. Data from all aneuploid cells were combined

Table 1). Some evidence of sectoring was seen in six-month callus as five of the ten tetraploid cells observed were seen in one preparation. Generally, however, diploid cells could also be found in preparations in which tetraploid cells were found. Although not all culture lineages were examined for ploidy, a random sampling of lineages indicated that all had similar proportions of ploidy types with each lineage being predominantly diploid. Figures 1 and 2 show a diploid cell and its karyotype from six-month callus.

Despite repeated attempts, mitotic events were never observed in nonembryogenic callus, indicating that the cells in this callus probably arose by enlargement, vacuolation or loss of starch from embryogenic cells, and not by cell divisions within the non-embryogenic callus.



Figs. 1 and 2 A diploid (2n = 14) cell from 6-month-old embryogenic callus (×4,000) and its karyotype (×10,000); **3 and 4** A diploid cell from a control plant (×4,000) and its karyotype (×10,000); **5–7** Root tip cells showing a diploid chromosome number in regenerated plants from 2-month (**5**), 4-month (**6**), and 6-month-old (**7**) callus (all ×4,000); **8** Karyotype of the cell in **Fig. 7** (×10,000)

Ten germinating embryoids were examined for chromosome numbers. Only 3–5 dividing cells per embryoid were observed but all showed 2n = 14 chromosomes.

Morphological aberrations among regenerants

Each lineage of callus produced approximately equal numbers of plantlets and the number of regenerants per callus piece did not appear to diminish with culture age, at least up to six months. Plantlets were randomly selected from petri dishes and transferred to tubes when their first leaves were greater than 2 cm long. Some of these plantlets had twisted, warped leaves when first transferred to culture tubes, but all outgrew their initial phenotypic aberrations when transferred to soil. Approximately 96% of plantlets transferred to soil survived. Three of these plants exhibited morphological aberrations. Two had fused primary stems, one of which split at the second most acropetal node, the other split at the tip of the inflorescence. No tillers of these two plants exhibited this phenomenon. The third plant developed small accessory inflorescences at the base of the main spike. This was associated with only the first three spikes formed. The next four spikes were morphologically normal. These two aberrations are also common among seed propagated plants grown out of season and are considered to be of physiological rather than a genetic origin.

Four of the 101 plants regenerated were albinos. Three of these arose from 4-month-old cultures and one from a 6-month-old culture; all were from different original explants. The albino plants could only be maintained by growing them in sterile culture on media supplemented with sucrose.

Chromosome numbers in regenerated plants

Mitotic analysis of regenerants from 2-, 4- and 6-month-old cultures showed that all of the green plants (97) were diploid. One of the albino plantlets was tetraploid (from 4-month-old callus) and the other three were diploid. No apparent correlation could be made between the ploidy of the callus and the ploidy of the regenerated plants since all but one plant were diploid. These plants arose from cultures that were predominantly diploid but which also contained aneuploid and tetraploid cells as shown in Table 1. The culture lineage from which the tetraploid plant arose (after four months) was also predominantly diploid after six months. Chromosome numbers observed and their percentages in controls and regenerants are summarized in Table 2. Among the regenerated plants occasional aneuploid (1.2%) or tetraploid (1.3%) cells were observed. These appeared at approximately the same frequency as in control plants (1.2% aneuploid, 1.2% tetraploid). All root tips were completely or predominantly diploid with the exception of the albino tetraploid plant in which all cells counted were tetraploid. No gross morphological changes were observed in any of the chromosomes of the regenerants (Figs. 3–8).

Meiosis

Control plants of 'Gahi 3' consistently showed seven bivalents at diakinesis. A low frequency of bridges and fragments in anaphase I and II, and of micronuclei in the quartet stage (Table 3), indicated the presence of a small paracentric inversion.

All regenerants also showed seven bivalents at diakinesis (Fig. 9) and at metaphase I (Fig. 10). Bridges

Table 2. Chromosome numbers in root tips of regenerated plants of *Pennisetum americanum*[a]

Age of callus from which plants were regenerated	Total no. regenerants	Total fields observed	Chromosome nos observed (% of total)
Control			14 (97.6%) 15 (0.6%) 16 (0.6%) 28 (1.2%)
2 months	26	504	13 (0.6%) 14 (97.4%) 15 (0.6%) 25 (0.2%) 28 (1.2%)
4 months	46	852	13 (0.5%) 14 (96.6%)[b] 15 (0.8%) 28 (2.0%)[c] 46 (0.1%)
6 months	31	639	13 (0.2%) 14 (98.9%)[d] 15 (0.3%) 16 (0.3%) 28 (0.3%)
Total for regenerants	101	1,995	13 (0.4%) 14 (97.5%) 15 (0.6%) 16 (0.1%) 25 (0.05%) 28 (1.3%) 46 (0.05%)

[a] For each plant at least three root tips were examined with an average of 20 cells being observed for each plant. Except for one tetraploid albino plantlet, all of the root tips examined were exclusively or predominantly diploid with only occasional polyploid or aneuploid cells. Control based on examination of 15 root tips of seed propagated plants

[b] Includes one diploid albino plantlet

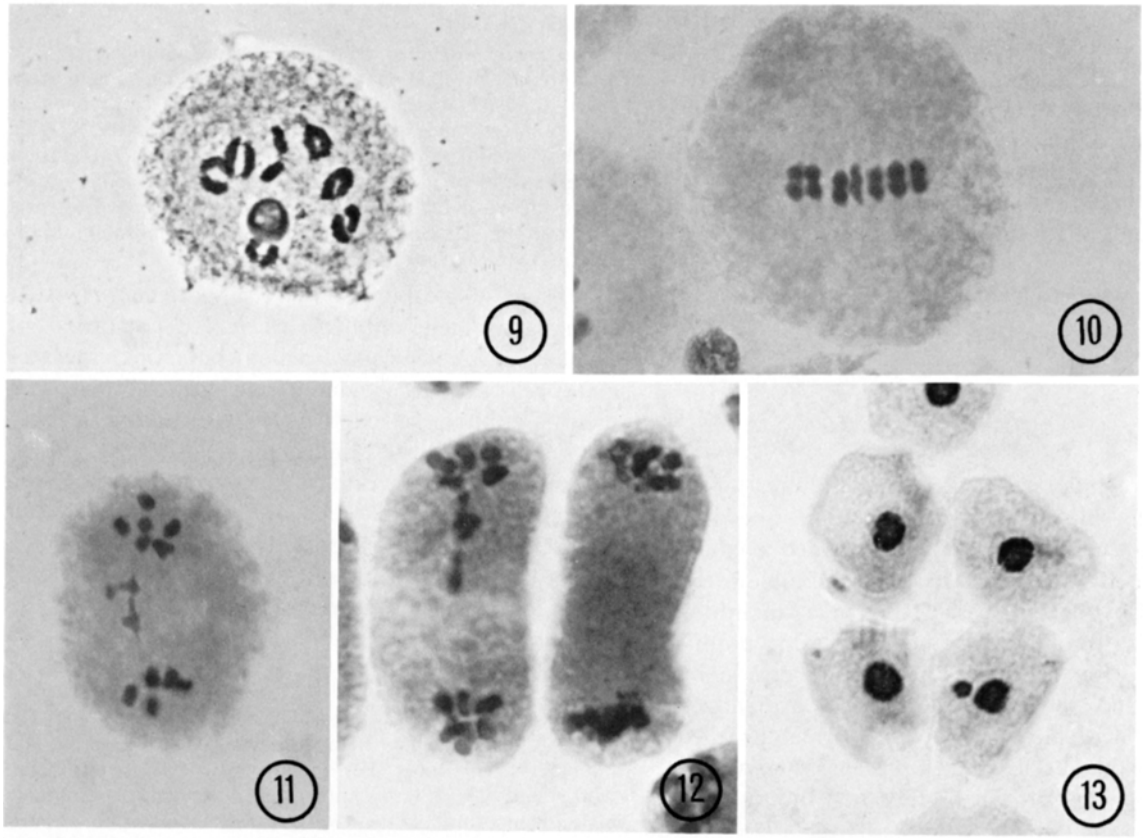
[c] Includes one tetraploid albino plantlet

[d] Includes two diploid albino plantlets

Table 3. Meiotic analysis of control and regenerated plants[a]

Age of callus from which plants were regenerated	No. plants examined	Diakinesis	Anaphase I % bridges and fragments	Anaphase II % bridges and fragments	Quartet % micronuclei
Controls	3	mean 0	3.63	3.90	4.37
		range 0	2.0–4.9	1.8–6.0	0–4.7
		s.d. 0	1.48	2.10	1.46
2 months	10	mean 0.2	3.76	3.78	3.17
		range 0–2.0	1.8–5.9	0–7.5	0–4.2
		s.d. 0.63	4.64	2.28	0.76
4 months	10	mean 0	4.87	3.14	2.82
		range 0	1.8–8.7	0–7.9	0–4.0
		s.d. 0	2.49	3.22	1.23
6 months	10	mean 0	2.39	2.58	3.05
		range 0	0–4.2	0–7.5	0–4.0
		s.d. 0	1.30	2.58	1.60

[a] The data for % micronuclei includes quartets with one micronucleus and quartets with two micronuclei. No more than two micronuclei were observed in any one quartet
s.d. = standard deviation



Figs. 9–13. Examples of meiotic figures in controls and regenerated plants: **9** Diakinesis in a regenerant with 7 bivalents (×2,800); **10** Metaphase I in a regenerant (×2,800); **11** Anaphase I in a regenerant showing a bridge indicative of a paracentric inversion (×2,800); **12** Anaphase II in a control plant, also indicative of a paracentric inversion (×5,600); **13** A quartet with a micronucleus in a regenerant (×4,000). Micronuclei were the result of bridges in anaphase I and/or II

and fragments in anaphase I (Fig. 11) and II (Fig. 12) and micronuclei in quartets (Fig. 13) persisted at the same frequency as in control plants (Table 3). One four-month regenerant showed an unusually high number of lagging chromosomes in anaphase I. In a second inflorescence from this same plant, laggards were not observed in anaphase I. The frequency of micronuclei in the quartet stage indicated that the laggards generally did not result in micronuclei. One cell in anaphase I of a six-month regenerant showed eight pairs of chromosomes. All pollen grains observed in controls and in regenerants were abortive (cv. 'Gahi 3' is cytoplasmic male sterile).

Progeny of regenerated plants

Seeds collected from crosses of regenerated plants with cv. 'Tift 23 B' all showed similar germination (92–100%, controls = 92%). Only green plants, without any noticeable morphological anomalies, were observed. Root tip counts from 20 of the progenies showed them all to be diploid.

Gel electrophoresis

Total protein gels showed no apparent qualitative differences between regenerants and controls. Zymograms of MDH activity in leaves showed several distinct bands in one group with several less pronounced bands in a more anodal group. Zymograms of ADH activity in roots showed the presence of two ADH-1 monomers and their intragenic dimer as well as two weak bands of the intergenic dimers of ADH-1 and ADH-2. There were no apparent differences between regenerants and controls for either ADH or MDH.

Discussion

The immature inflorecences used for culture, though predominantly diploid, did contain some aneuploid and polyploid cells. These apparently divided in culture and proliferated somewhat faster than the diploid cells as evidenced by their increased ratios in six-month-old callus. Culture conditions did not induce the production of cells with new chromosome numbers. In our experiments, no portion of the original explant was transferred during the first subculture. Thus the aneuploid and polyploid cells already present in the original explant participated in cell proliferation during callus initiation.

Diploidy in germinating embryoids and the regeneration of almost exclusively normal green diploid plants indicates that typically only diploid cells form embryoids and plants even though the proportion of aneuploid and polyploid cells increases in vitro. A small paracentric inversion in control plants was carried through culture to the regenerated plants and no new aberration appeared in meiosis. The meiotic analysis provides overwhelming evidence that the regenerants retain the cytogenetic characters of their parents, having gone through the culture process for as long as 6 months with no apparent chromosomal changes. The electrophoretic data and the examination of the progenies of the regenerants also show that these regenerants are normal.

Other reports in the Gramineae have described aneuploidy and/or mixoploidy (Heinz and Mee 1971; Ahloowahlia 1976; Bennici and D'Amato 1978; Orton 1980; McCoy et al. 1982). In these reports the mode of morphogenesis has either been through shoot morphogenesis or has not been described and is presumed to be via shoot morphogenesis.

The observations of the present study are in close agreement with preliminary reports of stability in regenerants from somatic embryos of *Panicum maximum* (Lu and Vasil 1982), *Pennisetum americanum* (Vasil and Vasil 1981a, b), *P. purpureum* (Wang and Vasil 1982), *P. americanum* × *P. purpureum* (Vasil and Vasil 1981a), *Sorghum arundinaceum* (Boyes and Vasil 1984) and *Triticum aestivum* (Ozias-Akins and Vasil 1982). In a recent detailed analysis, 20 plants derived via somatic embryogenesis from leaf callus of *Panicum maximum* were also shown to be cytologically and morphologically normal (Hanna et al. 1984).

In another detailed report (Karp and Maddock 1984), a high frequency of aneuploids (29%) were regenerated from wheat callus. Plants formed both by somatic embryogenesis and by adventitious shoot formation. The disparity between the above study and reports of normal plant regeneration from tissue cultures of wheat (Gosch-Wackerle et al. 1979; Ozias-Akins and Vasil 1982) is difficult to understand. At least part of it may be related to the presence of chromosome abnormalities in the explant material.

Results of the present study support the view that embryogenic cell cultures, which are comprised of small, richly ctyoplasmic, starch-containing, meristematic cells are largely stable cytogenetically and that there is a strong selection in favor of normal cells to form somatic embryos and plants (Vasil 1982, 1983a, b). Reports of regeneration of plants with cytogenetic variability are probably related to their origin from cell cultures consisting of large and vacuolated cells which do not have prominent starch grains (non-embryogenic cell cultures) and have been shown to be cytologically unstable.

Acknowledgements. We thank Dr. Wayne Hanna and Dr. Stanley Schank for their help with cytogenetic techniques and discussions, Dr. Robert Ferl for help with the electrophoretic work, and Dr. Prem Jauhar for discussions. Graduate assistantship through the Graduate School and the Department of Botany, University of Florida, supported Brad Swedlund through his master's program and are gratefully acknowledged. Florida Agriculture Experiment Station Journal Series No. 5476.

References

Ahloowahlia BS (1976) Chromosomal changes in parasexually produced ryegrass. In: Jones K, Brandham PE (eds) Current chromosome research. Elsevier/North Holland, Amsterdam, pp 115–122

Bayliss MW (1980) Chromosomal variation in plant tissues in culture. In: Vasil IK (ed) Perspectives in plant cell and tissue culture, suppl 11A. Academic Press, New York, pp 113–144

Bennici A, D'Amato F (1978) In vitro regeneration of Durum wheat plants. 1. Chromosome numbers of regenerated plantlets. Z Pflanzenzücht 81:305–311

Boyes CJ, Vasil IK (1984) Plant regeneration by somatic embryogenesis from cultured young inflorescence segments of *Sorghum arundinaceum* (Desv.) Stapf. var. 'sudanense'. Plant Sci Lett 35:153–157

Chen CH, Lo PF, Ross JG (1981) Cytological uniformity in callus culture-derived big bluestem plants (*Andropogon gerardii* Vitman). Proc S D Acad Sci 60:39–43

Gosch-Wackerle G, Avivi L, Galun E (1979) Induction, culture and differentiation of callus from immature rachises, seeds and embryos of *Triticum*. Z Pflanzenphysiol 91:267–278

Hanna WW, Lu CY, Vasil IK (1984) Uniformity of plants regenerated from somatic embryos of *Panicum maximum* Jacq. (guinea grass). Theor Appl Genet 67:155–159

Heinz DJ, Mee GWP (1971) Morphologic, cytogenetic, and enzymatic variation in *Saccharum* species hybrid clones derived from callus tissue. Am J Bot 58:257–262

Heyser JW, Nabors MW (1982) Regeneration of Proso millet from embryogenic calli derived from various plant parts. Crop Sci 22:1070–1074

Karp A, Maddock SE (1984) Chromosome variation in wheat plants regenerated from cultured immature embryos. Theor Appl Genet 67:249–255

Laemmli V (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Lu CY, Vasil IK (1982) Somatic embryogenesis and plant regeneration in tissue cultures of *Panicum maximum* Jacq. Am J Bot 69:77–81

McCoy TJ, Phillips RL (1982) Chromosome stability in maize (*Zea mays*) tissue cultures and sectoring in some regenerated plants. Can J Genet Cytol 24:559–565

McCoy TJ, Phillips RL, Rines HW (1982) Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue cultures; high frequency of partial chromosome loss. Can J Genet Cytol 24:37–50

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Orton TJ (1980) Chromosomal variability in tissue cultures and regenerated plants of *Hordeum*. Theor Appl Genet 56: 101–112

Ozias-Akins P, Vasil IK (1982) Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* (wheat): evidence for somatic embryogenesis. Protoplasma 110:95–105

Schwartz D, Endo T (1966) Alcohol dehydrogenase polymorphism in maize-simple and compound loci. Genetics 53: 709–715

Vasil IK (1982) Somatic embryogenesis and plant regeneration in cereals and grasses. In: Fujiwara A (ed) Plant tissue culture. Maruzen, Tokyo, pp 101–104

Vasil IK (1983a) Regeneration of plants from single cells of cereals and grasses. In: Lurquin P, Kleinhofs A (eds) Genetic engineering in eukaryotes. Plenum, New York, pp 233–252

Vasil IK (1983b) Toward the development of a single cell system for grasses. In: Cell and tissue culture techniques for cereal crop improvement. Science Press, Peking, pp 131–144

Vasil V, Vasil IK (1981a) Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum americanum* and *P. americanum* × *P. purpureum* hybrids. Am J Bot 68:864–872

Vasil V, Vasil IK (1981b) Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). Ann Bot 47:669–678

Wang DY, Vasil IK (1982) Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpureum* Schum. (Napier or elephant grass). Plant Sci Lett 25:147–154