Cytologia **66**: 341–348, 2001

Regeneration and Analysis of Genetic Variability in Wild Sorghum, *S. australiense* Garber and Snyder

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Accepted April 2, 2001

Summary *In vitro* techniques offer opportunity to broaden genetic variability by overcoming reproductive barriers between crop species. As an initial step for the exploitation of such techniques, we have established regeneration protocols for cultivated and wild sorghum species. The latter are important sources of insect resistance. The present study deals with immature inflorescence culture and cytogenetic stability of a wild *Parasorghum* species viz., *S. australiense* ($2n=20$). Regeneration was observed at high frequency (≥80%) on MS medium supplemented with kinetin (1.0 mg/l) and BAP (1.0 mg/l). Meiotic analysis of regenerants revealed somaclonal variation among regenerants from 12 month-old cultures. Chromosomal variations like aneuploids, hypodiploids, quadrivalent associations and tetraploids were found in the regenerated plants. RAPD analysis with PCR revealed polymorphism in these cytological variants. The protocol developed here might be used as a basis for achieving high frequency of regeneration and generating cytogenetic variants. Some of the variants might be useful in conventional breeding programs and for gene transfer studies from wild to cultivated species by somatic hybridization.

Key words *S. australiense*, Inflorescence culture, Meiotic, RAPD analysis, Somaclonal variation.

Sorghum australiense Garber and Snyder ($2n=20$) belongs to a polyploid complex of diploid and tetraploids among wild *Parasorghum* species. It is a good source of resistance for insect pests such as stem borers and shoot fly and therefore needs to be used in sorghum improvement program (Lazarides *et al.* 1991). Transfer of these desirable genes into the background of cultivated sorghum is difficult because of pre-zygotic reproductive barriers. Therefore, alternative approaches based on tissue culture like somatic hybridization and somaclonal variation would be useful additions to conventional breeding methods.

In vitro plant cell culture may lead to genetic and cytogenetics modifications in regenerated plants, a phenomenon termed as somaclonal variation (Larkin and Scowcroft 1981). These variations have been reported among regenerants in large number of species and their origin, cause and application in plant breeding has been widely investigated (Bajaj 1990, Phillips *et al.* 1994). The variations can be analyzed at phenotypic, chromosomal and molecular levels. In the genus *Sorghum*, there are few reports on somaclonal variation as a result of *in vitro* culture. Variation at phenotypic level for plants regenerated from callus cultures (Bhaskaran *et al.* 1987, Cai *et al.* 1990), variation in the chromosome number (Chourey *et al.* 1986) or genome organization (Kane *et al.* 1992) was reported. With wild sorghums chromosomal stability of the regenerants has been reported (Guo and Liang 1993, Mythili *et al.* 1999).

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Efficient regeneration protocols have already been established in cultivated sorghum (Cai *et al.* 1990, Sairam *et al.* 1999, Seetharama *et al.* 2000) and in some of the wild sorghum species (Guo and Liang 1993, Kuruvinashetty *et al.* 1998, Mythili *et al.* 1999). As a prerequisite to use of wild sorghum in crop improvement the present research was carried out to i) to establish a simple and reproducible regeneration protocol for *S. australiense* and ii) to evaluate the chromosomal stability of regenerants during *in vitro* culture and RAPD analysis of the cytological variants.

Materials and methods

Plant materials and tissue culture

The materials used were immature inflorescence collected from glasshouse grown plants of *S. australiense* (ICRISAT germplasm accession IS 18954). Based on our previous experience in sorghum and pearl millet, young panicles still enclosed in the boot measuring 4–6 cm were used for tissue culture studies. The inflorescence were sterilized with 0.1% mercuric chloride (w/v) for 5 min and rinsed with sterile distilled water. After removing the outer leaves, inflorescences along with the rachis were cut into pieces and placed on LS medium supplemented with 2 mg/l 2,4-D. Subculturing was done at the end of third week using the same medium. For regeneration, embryogenic calli at the end of 4, 8, 10 weeks (fresh cultures) and again at the end of 4, 6, 8 and 10 and 12 months (old cultures) were transferred on to regeneration medium (MS supplemented with Kinetin and BAP, 1.0 mg/l each). This combination of growth hormones was selected based on our previous experience in sorghum tissue culture (Mythili *et al.* 1999). Regenerated plants were transferred to glasshouse for establishment and were grown to maturity. The *in vitro* regenerants were designated as R0, and were self-pollinated to obtain R1 (Chaleff 1981) progeny.

Analysis of regenerants

The R0 and R1 plants were observed for the morphological, cytological and molecular variations. For studies on morphological variation, data on plant height, number of tillers and shape of the stem (cylindrical or flat) were collected from 50 randomly selected plants from each of R0 and R1 generations.

Cytological analysis

For cytological analysis, control plants used to initiate culture (10 plants), and regenerated plants from 4, 8, 10 weeks old callus (15 plants each from R0, R1) and 4, 6, 8, 10 and 12 months old callus (50 plants each from R0, R1) were used. Samples of immature tassels were fixed in Carnoy's solution (3 parts of alcohol, 1 part of glacial acetic acid) overnight and then stored in 70% alcohol for further analysis. Anthers were squashed in aceto-caramine for meiotic studies. A total of 100 cells were observed for screening.

DNA isolation and RAPD analysis

For RAPD analysis, R0 and R1 plants (from 12 months old calli) showing cytological variation were only used. For DNA extraction, sample of 20 independent R0 plants, 25 R1 plants and 5 samples each of calluses at 3 different stages (4 weeks after initiation, 4 weeks after subculture, 4 weeks after transferring to regeneration) were used. Tissues were harvested in liquid nitrogen and stored at −80°C. Frozen tissue from each sample was ground under liquid nitrogen in cold mortar and pestle and DNA was extracted following the CTAB (Hexadecyltrimethyl ammonium bromide) procedure (Saghai-Maroof *et al.* 1984).

We compared RAPD profiles obtained with DNA samples mentioned above using the protocol of Williams *et al.* (1990). A total of 8 primers (10-mers, OPJ-8, OPJ-18, OPJ-20, OPG-9, OPG-19, OPE-1, OPL-3, OPL-2) from Operon Technologies, USA were used. Amplification reactions were

performed with volumes of 25 μl containing 25 ng of genomic DNA, 1.0 μl dNTP (2.5 mM), 1.5 μl $MgCl_2$ (2.5 mM) and 0.4 μl thermostable DNA polymerase (5 units/μl), 2.5 μl of 10×PCR reaction buffer supplied with the Gibco-BRL kit. Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin Elmer) programmed for 40 cycles with the following temperature profile: denaturation at 94°C for 1 min; primer annealing at 40°C for 1 min; primer extension at 72°C for 2 min, except for the last cycle in which the primer extension lasted for 5 min. After amplification, the samples were mixed with 2 μl of 6× loading dye (25 mg xylene cyanol, 1.5 g ficoll type 400 in 10 ml). PCR products were separated by electrophoresis overnight on 1.8% agarose (Sigma) gels at a voltage of 25 V. The gels were stained with ethidium bromide (5 mg/l) and phographed under UV illumination. The polymorphism was scored as presence of a band (1) and its absence (0).

Cluster analysis

Similarity matrices were generated based on the proportion of common RAPD fragments among regenerants (Nei 1987) using $F=2M_{xy}/M_x+M_y$, where F is the similarity index, M_x is the number of bands in genotype x, M_y is the number of bands in accession y, and $2M_{xy}$ is the number of bands common to both x and y. For this analysis, plant entries showing polymorphism with at least four of the eight primers tested were only used (3 callus samples, 15 R0, 7 R1 plants). Cluster analysis was carried out using the statistical software package GENSTAT version-5.0.

Results

Tissue culture

Callus initiation was observed from the cut ends of rachis and from the spikelet primordia within one week after culture initiation. At the end of 4 weeks, callus induction was almost 100%. Embryogenic callus was relatively friable and yellowish white. Somatic embryos initiated within 2–3 weeks after transferring the embryogenic callus on to regeneration medium. At least 80% (82.50±2.88) of calluses developing somatic embryos produced plantlets. When the plantlets were 2 cm long, they were transferred to MS medium without growth hormones (MS basal) for rooting and establishment. The established plants were transferred to greenhouse and grown to maturity. Up to 9 months after callus initiation regeneration frequency is very high and later it declined (60.00±4.00%). This could be partly due to pigmentation of calli in long-term cultures.

Analysis of regenerants

Analysis of the regenerants from 12 months old callus showed phenotypic and chromosomal variations. Among the R0 and R1 plants tested, 2 were morphological variants in R1 generation while others were similar to the control plants used to initiate culture. In contrast to the control plants, the culm is flat and wide in the variants where as in former the stem is cylindrical. In addition, yellow-green striping was observed on lamina at the seedling stage in the variants.

Results from meiotic chromosome behavior of regenerated plants after 4, 8, 12 weeks and 4, 6, 8, 10 and 12 months are summarized in Fig. 1 and Table 1. The meiotic behavior of the control plant was normal with 10 bivalents at diakinesis (Fig. 1a). A plant was classified as cytologically abnormal if it possessed one or more of the detectable variations. However, 20 out of the 50, R0 plants regenerated from 12 months old cultures showed variations in chromosome number like hypodiploids (Fig. 1b) aneuploids (Fig. 1c), tetraploids (Fig. 1i) and plants with multivalent associations (Fig. 1d–h)). In addition, at anaphase-I of meiosis, segregation was not normal which resulted in bridge and laggard formation (Fig. 1j–l). The number of laggards is varying from 1–4. Plants in the other category were chromosomal mosaics or mixoploid having different aneuploid and tetraploid numbers; anthers from the single spikelet showed cells with $2n$=20/40, 20/24, 20/22, 20/21.

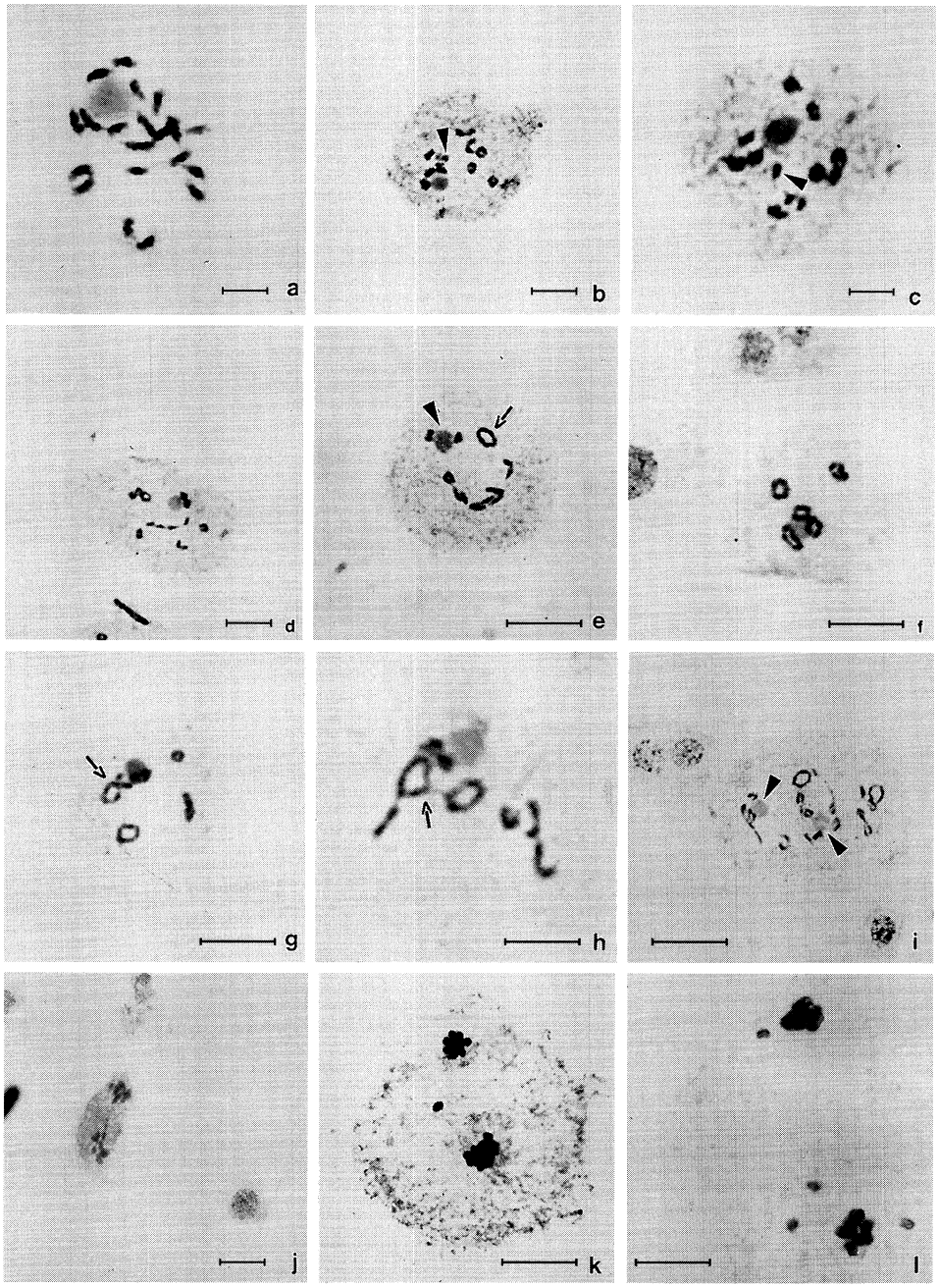

Fig. 1. Meiotic abnormalities in *S. australiense* immature inflorescence derived regenerants. a) Diakinesis in normal plant with 10 bivalents, b) Hypodiploid cell with $2n=18+1$ univalent, c) Aneuploid cell with $2n=20+1$ univalent, d, e) Diakinesis of variant with chain and ring of 4 chromosomes, f) Cell with 5 quadrivalent associations, g, h) Cells with complex associations, i) Tetraploid cell with 2 nucleoli, j) Anaphase stage showing bridge formation; k, l) Late anaphase stages with laggards. Bars represent 10 μm.

Multivalent association is the most common change observed in addition to change in chromosome number. Diakinesis of a plant with chain and ring of 4 chromosomes are shown in Fig. 1d–e. Regenerated plants heterozygous for this showed both ring and chain configurations. Up to 5 quadrivalent associations were observed in cells of 2 R1 generation plants (Fig. 1f). Fig. 1g–h is diakinesis cells with complex associations in 2 of the R1 plants. The number of chromosomes involved in these associations is yet to be estimated. Some of the plants were abnormal in addition to the chromosome variation. Such abnormalities included, the nucleolus being associated with more than one bivalent (Fig. 1e). In some of the tetraploid cells presence of 2 nucleoli was also observed (Fig. 1i). All the above abnormalities were also observed in R1 plants (25 out of 50). Among the 100 plants transferred to glasshouse, 2 plants that were chimeric for various aberrations and set only 2–6 seeds. Remaining plants appeared phenotypically normal.

Molecular analysis

Each of the 8 RAPD primers used in this study resulted in the amplification of 4–13 bands. Differences were observed between the RAPD profiles of regenerants with that of control plants used to initiate the culture. The polymorphism detected with primers OPE-1 and OPJ-20, is represented in Fig. 2a. Dendrogram based on degree of similarity of banding pattern from RAPD placed

Table 1. Number of cytologically normal and abnormal plants (R0, R1) regenerated from *S. australiense* immature inflorescence culture

Culture age		Normal		Chromosomal variations							
				Aneuploid		Quadrivalents		Tetraploid		Others	
		R0	R1	R0	R1	R0	R1	R0	R1	R0	R1
4, 8, 10	weeks	10	10	0	0	0	0	0	0	0	0
4, 6, 8	months	10	10	0	0	0	0	0	0	0	0
10	months	50	50	0	0	0	0	0	0	0	0
12	months	30	25	6	7	7	9	5	7	2	2

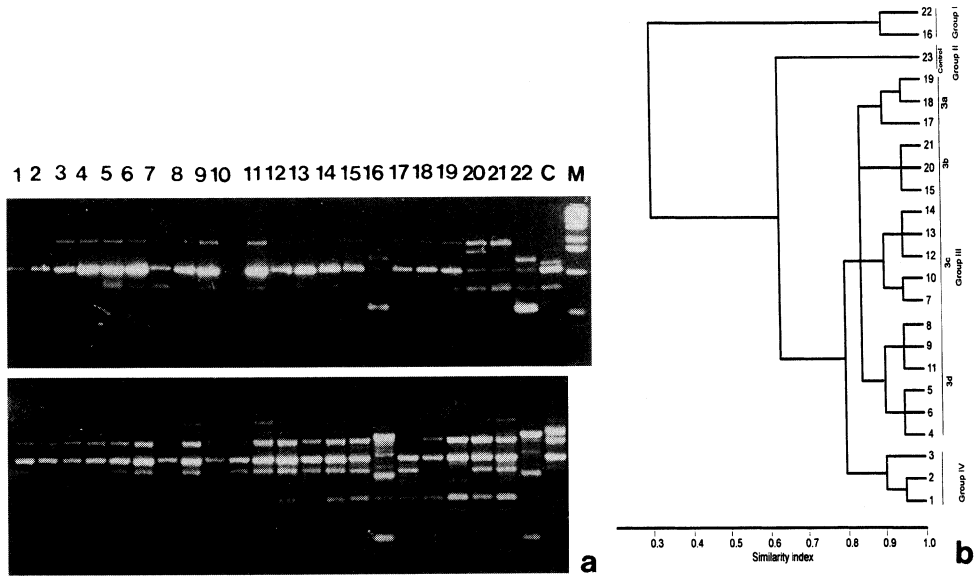

Fig. 2. a) Amplification of genomic DNA of the variants with primers OPE-1 (top) and OPJ-20 (Lanes 1–3 Calluses, 4–15 R0 plants, 16–22 R1 plants; C Control, M Marker). b) Dendrogram of Control, R0 and R1 plants based on RAPD analysis.

the regenerants into 4 different groups with more than 0.8 similarity index (Fig. 2b). The 2 morphologically different R1 plants that are chimeric formed Group I and were most diverse compared to the others. Out of the remaining, entry 23 that is the control (non-tissue culture-derived; designated as Group II) was distinct from all the chromosomal variants. The regenerants formed 2 divisions, namely Group III and Group IV. Group III can be further subdivided into 4 subgroups out of which 2 (3C, 3D) consisted of R0 plants, while all R1 plants formed 2 subgroups 3A and 3B with a single exception of entry 15, which is a R0 plant. The last group- (Group IV) represented calluses sampled for this study.

Discussion

Wild species such as *S. australiense* with high (Type II) callus induction and regeneration efficiency will be of use in further tissue culture studies such as somaclonal variation and for somatic hybridization. The present study revealed wide cytological variation among the regenerants, although the mode of regeneration was through somatic embryogenesis. Somatic chromosome number in the control plant is $2n=20$ without any changes in the number in the different samples examined. Plants regenerated from the successive subcultures (12 months old) had varied numbers. These variations included hypo-diploids, aneuploidy, tetraploidy, interchanges, laggards and association of nucleolus with more than one bivalent. On average, among the R0 regenerants 12% were aneuploid, 14% tetraploid and 4% were showing multivalent associations. Similar chromosomal instability in the regenerants has been reported in a number of *Graminaceous* species like oats (McCoy *et al.* 1982), *Triticale* (Armstrong *et al.* 1983), *Lolium* (Ahloowalia 1983), maize (Molina and Garcia 1998, Rhodes *et al.* 1986, Lee and Phillips 1987, 1988) and wheat. The occurrence of plants with multivalent associations in the present study might indicate segmental homologies. Similar results have been reported in cultivated and wild *S. bicolor* ($2n=20$) collected from Nigeria (Morakinyo and Olorode 1988) and in an auto-tetraploid sorghum (Hoang and Liang 1988, Luo *et al.* 1992). Presence of mixoploids with $2n=20/40$, 20/24, 20/22, 20/21 suggested that some of these might have arisen from chromosomal loss in the higher polyploids. Pollen fertility and seed set of the regenerated aneuploids and tetraploids was almost similar to the controls. The complex mosaic nature of some of the plants (2 of the R1 plants) might have enhanced sterility as very few seeds were obtained in these plants.

In recent years DNA-based marker technologies have made a major contribution to detect somaclonal variation. In the present study, RAPD analysis using 10-mer oligonucleotide primers revealed polymorphism between the R0 and R1 plants compared to the control. RAPD markers have been used for detecting genetic stability of the tissue culture derived plants in grass species like *Festuca pratensis* (Valles *et al.* 1993), sugarcane (Taylor *et al.* 1995), *Lolium* (Wang *et al.* 1993) and *Triticum* (Brown *et al.* 1993). Similarly other techniques like RFLP were used to detect somaclonal variants in *Beta vulagaris* (Sabir *et al.* 1992) rice (Muller *et al.* 1990) and wild barley (Shimron-Abarbanell and Breiman 1991). In the present study, R0 and R1 regenerants formed separate groups (with a single exception) in the cluster analysis, which could be due to the changes at molecular level, and which might not have been documented through cytological analysis. The grouping of entries in the dendrogram (Fig. 2b) corresponds well to the cytological analysis. However, further studies with large sample and more molecular markers are required to confirm the above observation.

In conclusion, the present study demonstrates potential for generating cytogenetic variants through *in vitro* culture in this wild species. Meiotic analysis was useful to detect the chromosomal aberrations. The important feature of this species is its resistance to insect pests and therefore transfer of such genes to the cultivated species is desirable. As successful crossability has been reported between *S. bicolor* and the tetraploid species, *S. halepense* ($2n=40$), some of the variants like the

tetraploids might be useful as bridge species in breeding programs for the improvement of sorghum. In future it should be possible to use the protocol for producing aneuploids, which can be used for mapping specific probes (genes) on chromosomes. We continue working with these variant plants for establishing cell suspension system using our protocol (Mythili *et al.* 1999) and for the isolation of micro-protoplasts (Ramulu *et al.* 1996) for partial genome transfer to the cultivated sorghum through somatic hybridization.

Acknowledgements

We thank Drs. K. K. Sharma and K. Kameswara Rao for critical reviewing of the manuscript. Technical assistance from C. Lakshminarayana, L. Vidyasagar (photography) and K. D. V. Prasad (cluster analysis) are gratefully acknowledged.

References

Ahloowalia, B. S. 1983. Spectrum of variation in somaclones of triploid rye grass. Crop Sci. **23**: 1141–1147.

Armstrong, K. C., Nakamura, C. and Keller, W. A. 1983. Karyotypic instability in tissue culture regenerants of triticale (*Triticosecale* Wittmack) cv. 'Welsh' from 6 months old callus cultures. Z. Pflanzenzuecht **91**: 233–245.

Bajaj, Y. P. S. 1990. Somaclonal Variation-origin, Induction, Cryopreservation and Implication in Plant Breeding. In: Bajaj, Y. P. S. (ed.) Biotechnology in Agriculture and Forestry, Vol 11, Somaclonal Variation in Crop Improvement. Springer Verlag, Berlin. pp. 3–48.

Bhaskaran, S., Smith, R., Paliwal, S. and Schertz, K. F. 1987. Somaclonal variation from *Sorghum bicolor* (L.) Moench cell cultures. Plant Cell Tiss. Org. Cult. **9**: 189–196.

Brown, P. T. H., Lange, F. D., Krang, E. and Lorz, H. 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. Gen. Genet. **237**: 311–317.

Cai, T., Ejeta, G., Axtell, J. D. and Butler, L. G. 1990. Somaclonal variation in high tannin sorghums. Theor. Appl. Genet. **79**: 737–747.

Chaleff, R. S. 1981. Genetics of Higher Plants. Cambridge University Press, London.

Chourey, P. S., Lloyd, R. E., Sharpe, D. Z. and Isola, N. R. 1986: Molecular Analysis of Hyper Variability in the Mitochondrial Genome of Tissue Cultured Cells of Maize and Sorghum. In: Mantell, S. H., Chapman, G. P. and Street, P. F. S. (eds.) The Chondriome-chloroplast and Mitochondrial Genomes. Wiley and Sons, New York. pp. 171–191.

Guo, J. H. and Liang, G. H 1993. Callus induction and plant regeneration of cultivated and wild sorghums. Cytologia **58**: 203–210.

Hoang, T. and Liang, G. H. 1988. The genomic relationship between cultivated sorghum [*Sorghum bicolor* (L.) Moench] and Johnson grass (*S. halepense* [L.] Pers.): A re-evaluation. Theor. Appl. Genet. **76**: 277–284.

Kane, E. J., Wilson, A. J. and Chourey, P. S. 1992. Mitochondrial genome variability in sorghum cell culture protoclones. Theor. Appl. Gent. **83**: 799–806.

Kuruvinashetty, M. S., Pahil, V. M., Bhat, S. and Hedge, M. 1998. High frequency plant regeneration from embryogenic callus culture in the genus *Sorghum*. Ind. J. Agr. Sci. **68**: 27–28.

Larkin, P. J. and Scowcroft, W. R. 1981. Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. **60**: 197–214.

Lazarides, M., Hacker, J. B. and Andrew, M. H. 1991. Taxonomy, cytology and ecology of indigenous Australian sorghums (*Sorghum* Moench. Andropogoneae: Poaceae). Aust. Syst. Bot. **4**: 591–635.

Lee, M. and Phillips, R. L. 1987. Genomic rearrangements in maize induced by tissue culture. Genome **29**: 122–128.

— and — 1988. Genetic and cytogenetic variation in plants regenerated from organogenic and friable embryogenic tissue cultures of maize. Crop Sci. **28**: 363–369.

Luo, Y. W., Yen, X. C., Zhang, G. Y. and Liang, G. H. 1992. Agronomic traits and chromosome behavior of autotetraploid sorghums. Plant Breed. **109**: 46–53.

McCoy, T. J., Phillips, R. L. and Rines, H. W. 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.

Molina, D. C. and Garcia, M. D. 1998. Analysis of genetic variability in long term callus cultures and regenerated plants of maize. Cytologia **63**: 183–190.

Morakinyo, J. A. and Olorode, O. 1988. Cytogenetic studies in *Sorghum bicolor* (L.) Moench. Cytologia **53**: 653–658.

Muller, F., Brown, P. T. H., Hartke, S. and Lorz, H. 1990. DNA variation in tissue culture derived rice plants. Theor. App. Genet. **80**: 673–679.

Mythili, P. K., Seetharama, N. and Reddy, V. D. 1999. Plant regeneration from embryogenic cell suspension cultures in wild species of sorghum, *S. dimidiatum* (Stapf). Plant Cell Rep. **18**: 424–428.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Phillips, R. L., Kaepler, S. M. and Olhoft, P. 1994. Genetic instability of plant tissue cultures: breakdown of normal controls. Proc. Natl. Acad. Sci. USA. **91**: 5222–5226.

Ramulu, K. S., Dijkhuis, P., Rutgers, E., Blaas, J., Krens, F. A., Dons, J. J. M., Colijn-Hooymans, C. M. and Verhoeven, H. A. 1996. Micro protoplast-mediated transfer of single specific chromosomes between sexually incompatible plants. Genome **39**: 921–933

Rhodes, C. A., Phillips, R. L. and Green, C. E. 1986. Cytogenetic stability of aneuploid maize tissue cultures. Can. J. Genet. Cytol. **28**: 374–384.

Sabir, A., Newbury, H. J., Todd, G., Catty, J. and Ford-Lloyd, B. V. 1992. Determination of genetic stability using isozymes and RFLP's in beet plants regenerated *in vitro*. Theor. App. Genet. **84**: 113–117.

Saghai-Maroof, M. A., Soliman, K. M., Jorgenson, R. A. and Allard, R. S. 1984. Ribosomal DNA space-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Nat. Acad. Sci. USA **81**: 8014–8018.

Sairam, R. V., Seetharama, N., Devi, P. S., Verma, A., Murthi, U. R. and Potrykus, I. 1999. Culture and regeneration of mesophyll derived protoplasts of sorghum, *Sorghum vulgare* (L.). Plant Cell Rep. **18**: 972–977.

Seetharama, N., Sairam, R. V. and Rani, T. S. 2000. Regeneration of sorghum shoot tip cultures and field performance of the progeny. Plant Cell Tiss Org Cult. **61**: 169–173.

Shimron-Abarbanell, D. and Breiman, A. 1991. Comprehensive molecular characterization of tissue culture derived *Hordeum marinum* plants. Theor. Appl. Genet. **83**: 71–80.

Taylor, P. W., Geijskes, J. R., Ko, H. L., Fraser, T. A., Henry, R. J. and Birch, R. G. 1995. Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. Theor. Appl. Genet. **90**: 1169–1173.

Valles, M. P., Wang, Z. Y., Montavon, P., Potrykus, I. and Spangenberg, G. 1993. Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds.). Plant Cell Rep. **12**: 101–106.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalsk, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. **18**: 6531–6535.

Wang, Z. Y., Nagel, J., Potrykus, I. and Spangenberg, G. 1993. Plants from cell suspension derived protoplasts of *Lolium* species. Plant Sci. **94**: 179–193.