Theor Appl Genet (1986) 73:4–10

Characterization of biomass production, cytology and phenotypes of plants regenerated from embryogenic callus cultures of *Pennisetum americanum* × *P. purpureum* (hybrid triploid napiergrass)

K. Rajasekaran, S. C. Schank [1] and I. K. Vasil *

Department of Botany and [1] Department of Agronomy, University of Florida, Gainesville, FL 32611, USA

Received March 19, 1986; Accepted June 28, 1986
Communicated by P. L. Pfahler

Summary. Five hundred and twenty-four plants of a triploid, sexually sterile hybrid napiergrass (*Pennisetum americanum* × *P. purpureum;* 3x = 21) were regenerated from embryogenic callus cultures obtained from segments of young inflorescences. Replicated field trials were conducted for two consecutive years to compare the biomass yield, phenotype and cytology of tissue culture regenerants (TC) and vegetatively propagated (V) plants. In the first year total biomass yield of TC plants was significantly greater than V plants but there was no significant difference in the second year. TC plants had more tillers compared to V plants. V plants did not show any morphological variability. The TC population also exhibited a high degree of phenotypic stability (96%). There were 23 phenotypic variants in the TC population of 524, most of them being more dwarf and late-flowering. Detailed morphological analysis of the TC-variant plants suggests that they very likely arose from only a few variant cell lines. Cytological analysis indicated stability of the triploid status in randomly selected regenerants. Two of the morphological variants were hexaploids (6x = 42). It is concluded that embryogenic callus cultures can provide useful alternative for the rapid propagation of hybrid napiergrass which is commonly propagated by cuttings.

Key words: Biomass – Gramineae – Genetic variability – Hybrid triploid napiergrass – *Pennisetum* – Plant tissue culture – Somatic embryogenesis

* To whom correspondence and reprint requests should be addressed

Introduction

Plant tissue cultures, including those of gramineous species, are prone to chromosomal (Orton 1980; McCoy et al. 1982; Nakamura and Keller 1982), genetic (Edallo et al. 1981; Larkin et al. 1984) and molecular (Gengenbach et al. 1981; McNay et al. 1984) changes. In addition to commonly observed transient and epigenetic changes, plants regenerated from such cultures show heritable variation for morphological and physiological traits (Larkin et al. 1984). In all the above studies, plant regeneration was achieved by organogenesis.

More recently, plants regenerated from embryogenic callus cultures of some grass species have been shown to be relatively uniform and cytogenetically stable. Examples include *Panicum maximum* (Hanna et al. 1984), *Pennisetum americanum* (Swedlund and Vasil 1985), *Zea mays* (Armstrong and Green 1985) and others (see Vasil 1985). These observations have led to the suggestion that plants regenerated from embryogenic callus cultures are cytologically normal and stable because somatic embryos are derived from single cells and because embryogenic cells appear to have a selective advantage in morphogenesis (Vasil 1983, 1985; Swedlund and Vasil 1985).

Regeneration through somatic embryogenesis of *Pennisetum* spp, including pearl millet and napiergrass has been demonstrated previously using a variety of explants (Vasil and Vasil 1981; Haydu and Vasil 1981; Wang and Vasil 1982; Chandler and Vasil 1984; Chandler et al. 1984). Napiergrass and pearl millet-napiergrass hybrids are both efficient biomass species, and currently are undergoing extensive testing for use as animal feed as well as an important, renewable source of energy through production of methane by fermentation (Smith and Frank 1985).

In this paper we report biomass yields and cytological and morphological analyses of a large number

of plants regenerated from inflorescence derived embryogenic callus cultures of a semi-dwarf, sexually sterile, triploid napiergrass hybrid.

Materials and methods

Callus initiation, maintenance and plant regeneration

Six young inflorescences (10–30 mm long) were collected from field-grown plants of a triploid (3x=21), sterile *Pennisetum* F_1 hybrid: dwarf *P. americanum* cv. 'Tift 23-DA' (2x=14) ×dwarf *P. purpureum* cv. 'N-75' (4x=28); selection No. 3. Callus cultures were initiated from inflorescence explants according to the methods of Chandler et al. (1984) on agar-solidified (8.0 g l^{-1} Bactoagar, Difco) medium (Murashige and Skoog 1962; MS) supplemented with 0.5 mg l^{-1} benzyladenine, 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 5% (v/v) coconut milk and incubated at 27 °C in the dark. Embryogenic callus cultures were maintained by subculture at 3–4 week intervals. Germination of somatic embryos was accomplished by growing in the light (160 µmol m^{-2} s^{-1}; 27 °C) on MS medium devoid of growth regulators. Plantlets were first grown on semi-solid agar (6.0 g l^{-1}) medium in test tubes containing half strength MS inorganic nutrients for 7–10 days to promote root proliferation and erect growth. After transfer to soil (MetroMix® 300, Grace Horticultural Products, Cambridge, MA) in plastic tubes ("Conetainers", Ray Leach Conetainer Nursery, Canby, OR), plantlets were maintained in a growth cabinet illuminated with fluorescent and incandescent light (300 µmol m^{-2} s^{-1}; 27 °C) for 7–10 days.

Field experiments

Individual plantlets in "Conetainers" were transferred to a greenhouse (26±2 °C) and were grown under sun light for 30–40 days before planting in the field. An equal number of control plants were grown from single node cuttings of vegetatively propagated clones of Selection No. 3. Care was taken to keep the age of the tissue culture-derived (TC) and vegetatively propagated control (V) plants uniform. Both TC and V plants were repeatedly trimmed at 50 mm above soil level to promote uniform growth in the greenhouse. The first planting was made on 13 June 1984 with 224 each of TC and V plants in a randomized, replicated field trial (Field 1; 8 replications; 28 plants per replication). The second planting was on 17 July 1984 with 300 each of TC and V plants (Field 2; 12 replications of 25 plants each).

Morphological characters including height of the plants at flowering, length and width at the broadest point of uppermost expanded leaves and number of tillers were recorded from a minimum of 80 plants of TC and V populations at the end of the first growing season in 1984 and the second growing season in 1985. Stem diameter was measured at the 5th internode from soil level. Biomass yield was recorded from each harvest and expressed in kg dry matter ha^{-1}. Two harvests were made in 1984; 80 days after planting and 75 days after regrowth. In 1985 the first and the second harvests were made 108 and 102 days after regrowth, respectively. Percentage dry matter was determined from samples of biomass after drying at 60 °C for 72 h.

Data were subjected to analysis of variance using Statistical Analysis System (SAS Inc., Cary, NC). Duncan's Multiple Range Test ($P=0.05$) was used to study the significance of morphological variation among the TC and V populations.

Cytological studies

Root tips were collected from a minimum of 40 randomly selected plants in each of TC and V population for chromosome staining according to the procedures of Swedlund and Vasil (1985). All the plants showing one or more variant characters, as compared to the normal TC regenerants, were also included for cytological analyses. At least 3 root tips were examined from each plant and a minimum of 25 cells were counted in each root tip. Meiotic chromosomes were also counted by staining pollen mother cells with aceto-orcein.

Results

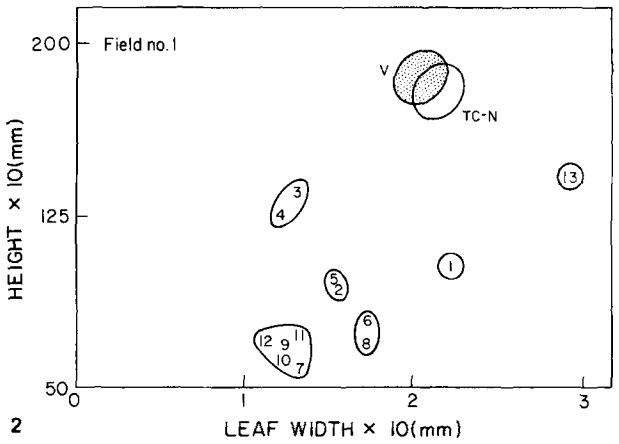
More than 80% of the inflorescence segments produced embryogenic callus within two weeks of culture initiation. Five hundred and twenty-four plants were regenerated in two batches of 224 and 300 plants after six subcultures. All the regenerated plants survived the transfer from aseptic conditions to soil.

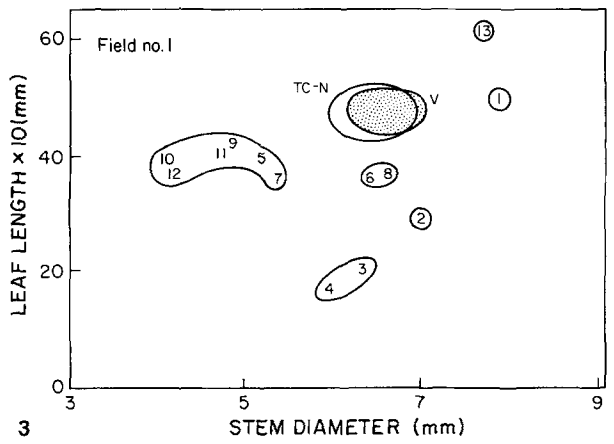
Morphology of tissue culture regenerated (TC) and vegetatively propagated (V) plants

Several morphological characters including leaf length, stem diameter, height of plants at flowering and leaf width were measured from 80 randomly selected plants in each of the TC and V populations. V plants were morphologically uniform. TC regenerants, when compared to V plants, exhibited a high degree of morphological uniformity except that they had significantly more tillers (50% in the first field trial; 115% in the second field trial; Tables 1, 3). Among the TC regenerants there were 13 morphological variants in the first field (out of 224 plants) and 10 in the second (out of 300 plants). Two of the variants in the first field were hexaploid. Most of the variants were dwarf with either narrow and erect or broad leaves and were late-flowering (Fig. 1) as compared to normal TC and V plants. The two hexaploid plants were also shorter and one of them had broader leaves and thick internodes compared to the normal TC and V plants (Figs. 2 and 3). The leaves of this hexaploid were longitudinally split near the midrib. A few basal tillers of one regenerant in the first field trial had variegated leaves in the second regrowth in 1985.

Morphological characters recorded from the variants and from randomly selected normal plants of TC origin were plotted on a graph using two parameters at a time (for example: plant height and leaf width or leaf length and stem diameter; Figs. 2 and 3). Variant plants from the first field trial fell into six significantly distinct morphological groups (according to Duncan's Multiple Range Test, $P=0.05$; Figs. 2 and 3), and there were three groups from the second field trial (data not shown), irrespective of the parameters

Fig. 1. A dwarf, late-flowering variant (*right*). This variant is identified as 11 in Figs. 2 and 3. Compare its narrow and erect leaves with that of normal plant (*left*)





Figs. 2 and 3. Morphological groups among the 13 variants (shown as 1 to 13) from Field 1. Approximate values for the normal TC regenerants (TC-N) and vegetatively propagated control clones (V) are also given. Note the grouping pattern of variants is similar (except for plant 5) irrespective of the parameters used for plotting. Plants 8 and 13 are hexaploids

used for plotting. There was no significant difference between V and TC normal plants (Table 1).

Biomass yield and percent dry matter content

The biomass yield of TC plants was significantly greater (41 to 85%) than V plants at first harvests, made 80–120 d after planting or regrowth, from the first field trial in both 1984 and 1985 (Table 2). Similar results were also obtained from the second field in 1984 (Table 3). However, there was no significant difference between biomass yields of TC and V plants at second harvest. Although cumulative annual biomass yield was significantly higher from TC derived plants than from V plants in the first year, the difference was not significant in the second year (Table 2). The method of propagation, either tissue culture or vegetative, did not affect the percentage dry matter content (Tables 1 and 3).

Cytology of the regenerated plants

Cytological analyses of root tip cells from 40 randomly selected normal plants of TC origin indicated triploid status (3x=21) similar to that of the donor plant (Fig. 4). Similar analysis of 23 morphological variants revealed chromosome numbers consistent with 21 in all the plants except two variants in the first field trial which were hexaploids (6x=42; Fig. 5). The two hexaploid plants have been found to be fertile. When compared with controls, gross chromosomal abnormalities were not observed both in mitotic and meiotic analyses of TC regenerants.

Table 1. Morphological characters and percent dry matter content of hybrid triploid napiergrass (Field 1)

Method of Propagation	Mean no. of tillers	% Dry matter	Height of plants (cm)	Leaf width (cm)	Leaf length (cm)	Stem diameter (mm)
Tissue culture	48*	19.3 ns	173 ns	2.2 ns	49 ns	6.3 ns
Vegetative	32	20.0	177	2.1	46	6.5

* Significant at $P = 0.05$
ns = not significant

Table 2. Biomass yield of hybrid triploid napiergrass (Field 1). Transplanted 13 June 1984 into 8 field replications, each containing 28 plants. Days to each harvest are given in materials and methods

Method of propagation	Biomass yield in kg ha^{-1}					
	1984			1985		
	Harvest 1	Harvest 2	Total	Harvest 1	Harvest 2	Total
Tissue culture	5,426*	4,808 ns	10,234*	8,207*	8,859 ns	17,066 ns
Vegetative	3,288	4,506	7,794	5,813	8,669	14,482

* Significant at $P = 0.05$
ns = not significant

Table 3. Biomass yield of hybrid triploid napiergrass (Field 2). Transplanted 14 July 1984 into 8 field replications, each containing 25 plants. Due to late planting only one harvest was made 120 days after planting. All the plants in this field were killed in 1984 due to freezing temperatures

Method of propagation	Biomass yield kg ha^{-1}	Number of tillers produced	% dry matter
Tissue culture	7,616*	43*	20.8 ns
Vegetative	4,106	20	20.1

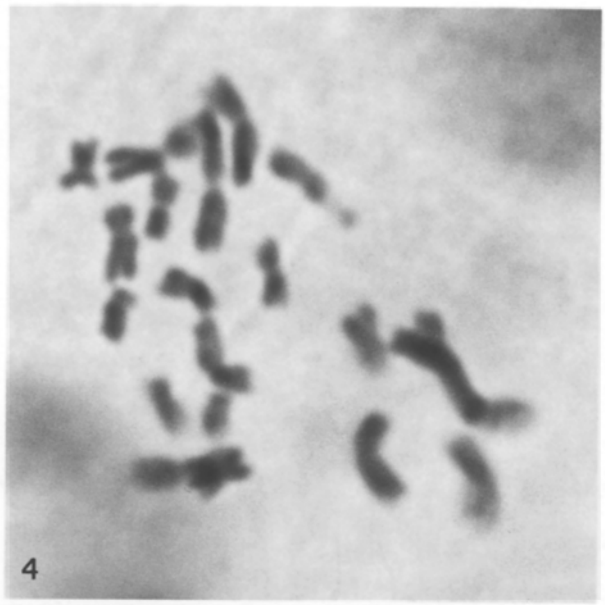
* Significant at $P = 0.05$
ns = not significant

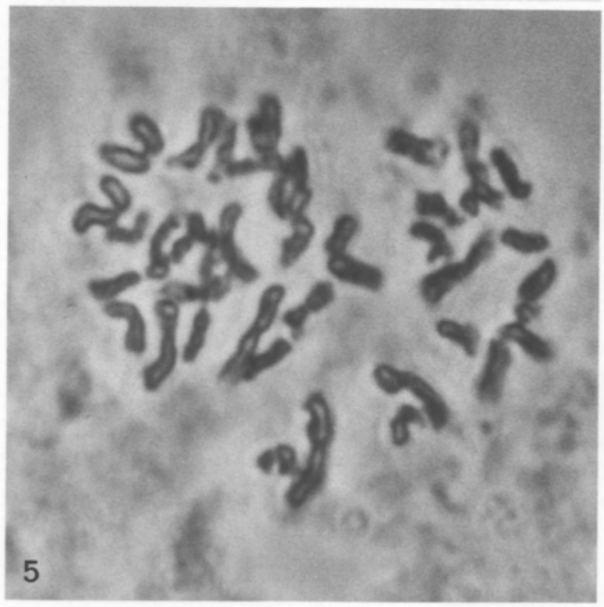
Morphology of the vegetative progeny of the variant plants

Changes in phenotypes including dwarfness, erect leaves and narrow blades in the variant plants were found to be persistent in the regrowths that appeared after harvests in 1984 (one regrowth) and 1985 (two regrowths). Single node cuttings of all the variant plants (10 to 12 per plant) were taken and transplanted to an adjacent field to study if variant characters are stable in the vegetative progeny. Variant characters were present in the progeny from two successive vegetative propagation cycles and resembled that of the parent plant from which the cuttings were originally made. Similarly, stability of morphological characters in regrowths and also in vegetative progenies of 20 randomly selected normal TC plants was also observed.

Discussion

In the first year, TC derived plants yielded more biomass than V plants but there was no significant difference between them at later harvests. Several studies have pointed out that plants of TC origin tend to be shorter or less vigorous in comparison to seed or vegetatively propagated plants (Damiani et al. 1985; Bruneau 1985; Ahloowalia and Sherington 1985). In the present study, TC plants were observed to be more vigorous than V plants in the first growing season (data not shown) but the differences in vigor and growth were not found at later stages. Such time-related disappearance of phenotypic variation has been observed among regenerated plants of *Lotus corniculatus* (Pezzoti et al. 1985). It would appear that the increased tiller number in the TC plants contributed to the initial increase in biomass yield observed in this study. Environmental and nutritional factors as well as levels of endogenous phytohormones affect tiller production in grass species (Langer 1963; Jung 1984). It is likely that the increased tiller production by TC plants is due to the effects, either direct or indirect, of possible changes in endogenous growth regulator levels caused by tissue culture conditions. Furthermore, the TC plants have a well developed primary root system in good vascular contact with the shoot system as compared to adventitious roots in the vegetatively propagated single node cuttings. Gradual disappearance of differences in biomass yield between TC and V plants may be due to less vigorous growth of tillers in TC plants caused by





Figs. 4 and 5. Chromosome numbers in TC root tip cells. Normal- and most of the phenotypically variant-TC plants were triploid (3x=21; Fig. 4) except two variants which were hexaploids (6x=42; Fig. 5). Stained with Feulgen (both ×4000)

competition for available nutrients in the second growing season.

Cytological analysis of randomly selected TC regenerants indicates the stability of the normal chromosome complement (3x=21; Fig. 4). Only two plants were found to be hexaploid (6x=42; Fig. 5) and the origin of these plants is not known.

Previous studies with tissue cultures of *P. americanum* (Swedlund and Vasil 1985) have shown that although a slight shift toward polyploidy does occur in embryogenic callus cultures, there is a strong selection during morphogenesis in favor of cytogenetically normal cells. Neither variation in chromosome numbers nor any structural rearrangements were observed in plants regenerated from embryogenic callus cultures of *Panicum maximum* (Hanna et al. 1984) and *Pennisetum americanum* (Swedlund and Vasil 1985). Several studies have described variations of chromosome number in cells of the original explants as a possible source of chromosomal variation in callus cultures and regenerated plants (Nakamura and Keller 1982; Swedlund and Vasil 1985). In instances where regeneration of plants occurs via both embryogenesis and organogenesis in the same culture, chromosomally abnormal plants may arise from non-embryogenic cells (Karp and Maddock 1984; Maddock 1985).

A high level of phenotypic uniformity (96%) was observed among the regenerated plants of *Pennisetum* in the present study, and this is in agreement with earlier accounts of relative uniformity of plants recovered from somatic embryos in cereals and grass species (see Vasil 1983, 1985). Twenty-three plants out of 524 (4.0%) were identified as variants. From a breeder's point of view 4% variation is high. Most of the phenotypic variants were dwarf with narrow or broad leaves. However, on detailed morphological analysis, similarities in characters among the 23 dwarf variants were observed indicating that they may have arisen from no more than nine variant cell lines (six from the first batch and three from the second batch). Variants from the two field trials were analysed separately because of the differences in planting date and soil fertility. It is likely that a combined analysis of variants from the two fields would have yielded six to nine variant groups and this would correspond to far less than 4% variation in the embryogenic cultures. This further confirms earlier observations that variations in embryogenic callus cultures are minimal (Swedlund and Vasil 1985; Hanna et al. 1984). It also highlights the fact that the total number of variant plants observed among a regenerated population does not necessarily correspond to the number of independent "mutational" events which may have occurred in the callus cultures. The origin of the variant characters observed among the regenerants in this study is not clear. It is possible that they arose as a result of mosaicism in the original explants (Barbier and Dulieu 1980; Swedlund and Vasil 1985) and/or as a result of tissue culture procedures (Larkin and Scowcroft 1981).

The origin of the plant with variegated leaves is also not clear. It is generally acknowledged that the possibility of occurrence of chimeras is high in adventitious shoot buds which are of multicellular origin (see Vasil 1983, 1985). Appearance of variegation in the second regrowth in the second year of the trial indicates that it may be due to point mutation in vivo rather than its possible origin from adventitious shoot buds in the highly embryogenic cultures. Vegetative progeny of this variegated plant are being evaluated at present.

Our results showing a high degree of morphological and chromosomal stability of regenerated plants of *Pennisetum* are at variance with other reports where extensive variability has been reported (see Orton 1984). Cytological and morphological stability among regenerants of other species has been attributed, at least in part, to rapid regeneration systems (Hayashi and Nakajima 1984; Wheeler et al. 1985) and the use of embryogenic cultures (Hanna et al. 1984; Swedlund and Vasil 1985).

Morphological characters of the variant plants were stable in several regrowths and in at least two successive vegetative progenies suggesting that they are not transient and may have epigenetic/genetic basis. Both permanent phenotypic changes and remissions of some qualitative markers have been observed in vegetative progenies of sugarcane plants of tissue culture origin (Irvine 1984). Further characterization of the observed phenotypic variation, by analysis of sexual progeny, is not possible with the hybrid napiergrass used in this study because of its sexual sterility and triploid status. Attempts are being made to study the selfed-progeny of the fertile hexaploid plants.

In summary, plants regenerated from embryogenic callus cultures of hybrid napiergrass exhibit degrees of both chromosomal and morphological uniformity. These results, along with our previous demonstration of the potential for rapid multiplication of this species (at the rate of 25,000 green plants from a single explant in seven months; Chandler and Vasil 1984; Chandler et al. 1984), indicate that regeneration of plants from somatic embryos is a very reliable and attractive procedure for clonal propagation of hybrid napiergrass, an important biomass species which normally must be vegetatively propagated.

Acknowledgements. We thank Doug Manning and Cathleen Burns for assistance in the field experiments. The research work reported in this paper was supported by a cooperative project between the Institute of Food and Agricultural Sciences, University of Florida and the Gas Research Institute, Chicago, Illinois. Florida Agriculture Experiment Station Journal Series No. 7340.

References

Ahloowalia BS, Sherington J (1985) Transmission of somaclonal variation in wheat. Euphytica 34:525–537

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Barbier M, Dulieu HL (1980) Effets génétiques observés sur des plantes de Tabac régénérées à partir de cotylédons par culture in vitro. Ann Amélior Plant 30:321–344

Bruneau R (1985) Régénération in vitro à partir de cals d'embryons immatures de maïs. Aspects quantitatifs et transmission héréditaire. Agronomie 5:591–596

Chandler SF, Vasil IK (1984) Optimization of plant regeneration from long term embryogenic callus cultures of *Pennisetum purpureum* Schum. (napier grass). J Plant Physiol 117:147–156

Chandler SF, Rajasekaran K, Vasil IK (1984) Large scale propagation of napiergrass and giant napiergrass by tissue culture. In: Proc 1984 Int Gas Res Conf. Gas Res Inst, Chicago, pp 561–566

Damiani F, Mariotti D, Pezzotti M, Arcioni S (1985) Variation among plants regenerated from tissue culture of *Lotus corniculatus* L. Z Pflanzenzücht 94:332–339

Edallo S, Zucchinali C, Perenzin M, Salamini F (1981) Chromosomal variation and frequency of spontaneous mutation associated with in vitro culture and plant regeneration in maize. Maydica 26:39–56

Gengenbach BG, Connelly JA, Pring DR, Conde MF (1981) Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. Theor Appl Genet 59: 161–167

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

Hayashi M, Nakajima T (1984) Genetic stability in regenerated plants derived from tobacco mesophyll protoplasts through three-step culture. Jpn J Breed 34:409–415

Haydu Z, Vasil IK (1981) Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum.* Theor Appl Genet 59:269–273

Irvine JE (1984) The frequency of marker changes in sugarcane plants regenerated from callus culture. Plant Cell Organ Tissue Culture 3:201–209

Jung J (1984) Plant bioregulators in cereal crops: action and use. In: Ory RL, Rittig FR (eds) Bioregulators – chemistry and uses. American Chemical Society, Washington, pp 29–43

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

Langer RHM (1963) Tillering in herbage grasses. Herb Abstr 33:142–148

Larkin PJ, Ryan SA, Brettell RIS, Scowcroft WR (1984) Heritable somaclonal variation in wheat. Theor Appl Genet 67:443–455

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

Maddock SE (1985) Cell culture, somatic embryogenesis and plant regeneration in wheat, barley, oats, rye and triticale. In: Bright SWJ, Jones, MGK (eds) Cereal tissue and cell culture. Martinus Nijhoff/Junk Publ, Dordrecht, pp 131–174

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

McNay JW, Chourey PS, Pring DR (1984) Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of maize. Theor Appl Genet 67:433–437

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

Nakamura C, Keller WA (1982) Callus proliferation and plant regeneration from immature embryos of hexaploid triticale. Z Pflanzenzücht 88:137–160

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

Orton TJ (1984) Somaclonal variation: Theoretical and practical considerations. In: Gustafson JP (ed) Genetic manipulation in plant improvement – 16th Stadler Genet Symp. Plenum, New York London, pp 427–468

Pezzoti M, Arcioni S, Damiani F, Mariotti D (1985) Time-related behaviour of phenotypic variation in *Lotus corniculatus* regenerants under field conditions. Euphytica 34: 619–623

Smith WH, Frank JR (1985) Comparative biomass yields of energy crops. In: Palz W, Coombs J, Hall DO (eds) Energy from biomass. Elsevier, New York, pp 323–329

Swedlund B, Vasil IK (1985) Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum. Theor Appl Genet 69:575–581

Vasil IK (1983) 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 (1985) Somatic embryogenesis and its consequences in the gramineae. In: Henke RR, Hughes KW, Constantin MP, Hollaender A (eds) Tissue culture in forestry and agriculture. Plenum, New York, pp 31–47

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

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

Wheeler VA, Evans NE, Foulger D, Webbs KJ, Karp A, Franklin J, Bright SWJ (1985) Shoot formation from explant cultures of fourteen potato cultivars and studies of the cytology and morphology of regenerated plants. Ann Bot 55:309–320