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The expression and perpetuation of inherent somatic variation in regenerants from embryogenic cultures of *Pennisetum glaucum* (L.) R. Br. (pearl millet)

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Summary. Genetic analysis was conducted on the qualitative and quantitative traits of sexual progeny derived from embryogenic cultures of two inbred lines of *Pennisetum glaucum* (L.) R. Br. (pearl millet). These lines included a genetically stable inbred of Tift 23BE and a genetic marker line, derived from Tift 23BE, which bore qualitative genetic markers for a dominant purple plant trait (P) and two recessive traits, early flowering (e_1) and yellow stripe (ys). Tissue culture regenerant populations (R_0) and progeny populations (R_1) produced from these plants by selfing showed no qualitative genetic variation when derived from the genetically stable inbred Tift 23BE. In contrast, stably inherited qualitative variation for a number of genetic markers was observed in R_0, R_1, and R_2 progeny of the genetic marker line. In a population of 1,911 plants regenerated over a 12-month period, 0.02% of the population lost or showed reduced expression of the purple plant trait and 92% of plants were chlorophyll deficient. Plants showing reduction or loss of anthocyanin synthesis also flowered later. None of the purple plants showed any significant variation in flowering time. The incidence of chlorophyll deficiency increased with time in culture, 51% of the progeny regenerated after 1 month were chlorophyll deficient, while 100% of the plants regnerated after 12 months were chlorophyll deficient. Qualitative variation was also observed in control populations of the genetic marker line where 1 plant in a total of 1,010 lacked purple pigmentation and a total of 6% showed chlorophyll variation in the first generation (S_0). The presence of qualitative variation in controls suggests that the inherent variation present in the original explant was expressed and perpetuated in vitro. Quantitative variation was observed for a number of traits in the first sexual cycle (R_1) of the marker line but did not occur in a subsequent generation, suggesting that this variation was epigenetic.

Key words: Gramineae – Genetic analysis – *Pennisetum glaucum* – Somatic embryogenesis – Tissue culture

Introduction

Experience gained from studies on cultured cells and regenerated plants has demonstrated that the in vitro culture of plant cells can result in the production of epigenetic, physiological, cytological, or genetic variation (Meins 1983; Orton 1984; D'Amato 1986; Gould 1986). This variation is considered a potential source of novel breeding lines (Larkin 1985). Such widespread manifestation of somatic variation, however, creates a major problem if tissue culture is to be used in conjunction with genetic engineering for plant improvement, since the success of this procedure depends on the maintenance of genetic fidelity of the introduced gene or genes. It is, therefore, imperative to establish culture and regeneration procedures that reduce or eliminate variation. Such a system may be established through developing a clearer understanding of the potential sources of somatic variation in cultured cells and the factors that control its expression in regenerated plants. With this knowledge we may be able to minimize the production of somatic variation where genetic fidelity is required or directionally maximize its expression in cases where somatic variation in vitro is desirable as, e.g., in the selection of useful and stable mutants.

Available evidence indicates that the pathway of plant regeneration, genetic architecture of the donor plant material, genotype, and the duration of culture are among the most significant factors affecting both the nature and number of variants produced in tissue culture (e.g., Edallo et al. 1981; Fukui 1983; Oono 1985; McCoy

et al. 1982; Vasil 1987; Zehr et al. 1987). In contrast to these reports, the majority of plants regenerated from both short- and long-term embryogenic cultures is cytologically and phenotypically similar to the donor plant (Hanna et al. 1984; Swedlund and Vasil 1985; Kobayashi 1987; Rajasekaran et al. 1987). This stability is believed to be a result of the single cell origin of somatic embryos (Vasil and Vasil 1982), the relatively low level of cytological variation produced in cultured embryogenic cells (Karlsson and Vasil 1986; Swedlund and Vasil 1985), and stringent selection in favor of cytologically normal cells during embryo differentiation (Swedlund and Vasil 1985; Cavallini et al. 1987; Cavallini and Natali 1989). Because of this stability it has been suggested that regeneration from embryogenic cultures could be used to maintain genetic fidelity in genetically engineered plant cells (Vasil 1987). In order to exploit this potential, however, plants regenerated from embryogenic cultures must be evaluated for the absence of mutations and epigenetic variation through comparative genetic studies on sexually propagated tissue culture regenerants and control progeny. A factor that also requires testing is the influence of plant genotype on the level of variation produced from embryogenic cultures. In the past, studies on tissue culture regenerants in *Pennisetum* have been conducted using male-sterile lines. This has prevented an evaluation of any putative genetic variation that may have arisen in tissue culture. The present study, therefore, was conducted with sexually fertile *Pennisetum glaucum* (L.) R. Br. [formerly *P. americanum* (L.) K. Schum.] lines. The main objectives were (i) to compare the level of genetic variation produced in both a genetically stable inbred and a genetic marker line of Tift 23BE, (ii) to evaluate the inheritance of any variation produced, and (iii) to assess the effects of long-term culture on the production of genetic variants in the marker line.

Materials and methods

Callus culture, plant regeneration, and maturation

The two *Pennisetum glaucum* (L.) R. Br. lines used in this study consisted of a marker line Tift 23BE containing the dominant genetic marker purple plant (P) and recessive markers for early flowering (e_1) and yellow stripe (ys), and a genetically stable green inbred Tift 23BE. The marker line was derived from a cross of a purple plant and yellow stripe line of *P. glaucum* (Pys-7) with inbred Tift 23BE. The plant phenotype was characterized by purple pigmentation in stems, leaves, and inflorescences and by the presence of narrow stripes of chlorophyll-deficient tissue in the leaves.

In March, 1986, young immature inflorescences (10–20 mm in length) were collected from plants of the marker line grown in the glasshouse. The inflorescences were surface-sterilized and cultured according to procedures outlined by Vasil and Vasil (1981). The floral spikes on tillers of plants used as a source of inflorescences were allowed to mature, and were selfed by enclosing in paper bags before the emergence of stigmas and until all the anthers dehisced. This selfed seed (S_0) was used as control material. All inflorescences placed in culture were pedigreed, thus ensuring that regenerated plants were compared with seedling controls derived from the original donor plant. Similar procedures were used for the genetically stable inbred Tift 23BE, except that the inflorescences were derived from field-grown material in September, 1987.

Embryogenic callus was maintained by culturing at 2-week intervals on MS medium (Murashige and Skoog 1962) containing 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose, and 5% coconut milk. Plants were regenerated directly from inflorescence explants after 1 month of culture and from subcultured callus after 6 and 12 months. Plant regeneration occurred through the formation of somatic embryos. Embryo germination was achieved by transfer of embryos or embryogenic callus to MS medium containing 0.25 mg/l 2,4-D, 0.25 mg/l 6-benzylaminopurine (BAP), 0.25 mg/l kinetin, 3% sucrose, and 5% coconut milk (EG medium). Cultures were incubated in an illuminated growth chamber (160 $\mu E \cdot m^{-2} s^{-1}$, 27 °C). Shoots developed on transfer of callus to EG medium with 0.13 mg/l 2,4-D. The shoots were rooted in test tubes or Magenta boxes (Magenta Corp., Chicago) on MS medium containing 3% sucrose and 0.2 mg/l naphthaleneacetic acid. Control seed was planted at this stage. After 1 month, rooted tissue culture plantlets (R_0) and seedling controls were transferred to soil (Metro Mix 300, Grace Horticultural Products, Cambridge/MA) in plastic tubes ('Conetainers', Ray Leach Conetainer Nursery, Cambridge/OR). Plantlets were then grown in the glasshouse (26°–28 °C) under sunlight in Florida for 1 month before transfer to Tifton/GA. Both populations were grown to maturity in the glasshouse in Tifton and all plants were selfed by bagging before anthesis to produce R_1 or S_1 seed.

Seedling and mature plant analysis

To assess for any visible variants at the seedling stage in S_1, R_1 and S_2, R_2 populations, seeds from both control and tissue culture populations were planted in flats in the glasshouse. Seeds were planted for 78 S_0, 100 R_0, and 70 parental (parents of the control) first-generation plants and 50 R_1 and S_1 second-generation plants. The seed was planted in 50–100 seed rows and after 3–4 days the number of plants segregating for evident phenotypic variation was counted. The genetic marker line as a stripe variant of *P. glaucum* gives non-Mendelian segregation ratios for yellow, albino, striped, and green plants (Appa Rao and Mengesha 1984; Reddy and Subrahmanyam 1988). To determine the possible influence of tissue culture on the incidence of these chlorophyll variants in seedling segregation, counts were made of the total number of each variant produced and these were compared as total percentage of variants produced per population.

For the analysis of mature plants, seed from control, tissue culture, and parental material was planted in the field in Tifton/GA in early May, 1987 (R_1, S_1) and June, 1988 (R_2, S_2) in individual plots, each representing the progeny of a single tiller. The limited amount of seed available prevented replication of plots for R_1, S_1 populations but three replicate plots were planted in the R_2 and S_2 populations. In the R_1, S_1 and R_2, S_2 field trials, three random plants from each plot were bagged before anthesis to provide selfed seed and data on panicle length and seed weight.

Phenotypic and cytological analysis

Only qualitative characters were assessed in the R_0 generation, as the germination rate of plants is not synchronous in culture and thereby invalidates any quantitative analysis of morpholog-

ical traits or flowering date. The two characters assessed were plant color and male sterility. Color variants were classified according to the pigmentation in their leaves, stems, and inflorescences. Plants that were green or showed a significant reduction in pigmentation were classified as purple variants (P). Plants that had significant chlorophyll-deficient sectors on their leaves were classified as highly variegated (HV). In general these plants had no purple pigmentation. Male sterility was evaluated by ascertaining the presence or absence of pollen-shedding anthers. Cytological analysis was also conducted on a random population of 30 plants from both control and tissue culture populations. Stages of meiosis examined included diakinesis, anaphase, and metaphase; ten cells per plant were examined for each stage.

Both quantitative and qualitative traits were assessed in R_1, S_1 and R_2, S_2 populations planted at Tifton/GA. These consisted of measurements of plant height, panicle length, and seed weight and an analysis of plant color, tiller number, male sterility, and flowering date. Field data were subjected to analysis of variance using the Statistical Analysis System (SAS Inc., Cary/NC). The Waller-Duncan *K*-ratio *T*-test was used to study the significance of variation in quantitative characters between S_1, parental, and R_1 populations. R_2 and S_2 generation progeny were assessed for variation using Duncan's Multiple Range Test. Statistical methods for rates and proportions (Fleiss 1981) were used to test for the significance of variation in quantitative traits. Cytological analysis was also conducted on a random population of 50 plants from R_1, R_2 and S_1, S_2 populations and, where possible, on any variants observed.

Nomenclature

The nomenclature system proposed by Chaleff (1981) was used to describe the various generations of tissue-culture-derived plants in this study:
R_0 plants – primary regenerants from tissue culture
R_1 plants – plants grown from R_1 seed produced by selfing of R_0 plants
R_2 plants – plants grown from R_2 seed produced by selfing of R_1 plants
Controls (S_0, S_1, and S_2) – plants that have not been produced in tissue culture.

Results

Phenotypic and cytological analysis of R_0 and S_0 plants of the marker line Tift 23BE

Over a 12-month period, a total of 1,911 plants was regenerated from tissue cultures of the marker line of Tift 23BE. These regenerants were compared with appropriate seedling controls to evaluate the level of variation expressed and induced in tissue culture. Such a comparison is illustrated in Table 1.

In the first regeneration cycle, 262 plants were produced. These plants were dervied from embryogenic callus or from somatic embryos arising directly from the cultured inflorescence. In this population 57% of the plants produced were phenotypically different from the original donor plant. The majority of these (51%) were chlorophyll variants with yellow or variegated phenotypes, while the remaining 6% were putative anthocyanin mutants with a green phenotype. There were evident dif-

Table 1. Tissue culture (TC) and control (C) plants and their associated variation after 1 and 6 months of subculture

Original line	Variation[b]		R_0 variants	R_1[a] variants	R_2[a] variants
	No. of plants	%			
1 Month					
TC5-1	7	42	2S, 1Y	0	0
TC17-1	4	25	1Y	0	0
TC23-2	6	0	0	0	0
TC25-1	150	63	15S, 71Y, 6P, 2HV	7P, 2HV	7P
TC27-1	22	68	9S, 1P, 3HV	2P	0
TC31-1	3	33	1Y	0	0
TC33-1	6	16	1HV	0	0
TC33-2	64	60	13S, 17Y, 7P	9P	9P
	262		150 (57%)*		
C5-1	50	8	2Y	0	2P
C23-2	50	12	4Y, 1HV	1HV	1P
C25-1	45	22	7Y, 1HV	0	2P
C27-1	52	7	2S, 2Y	0	0
C31-1	47	10	2S, 1Y, 1HV	1HV	1P
C33-2	40	30	12S	0	0
	280		35 (12%)		
6 Months					
TC4-1	23	100	23Y	0	0
TC5-2	158	99	156Y, 1P	1P	1P
TC11-1	194	100	194Y	0	0
TC-20-2	408	100	408Y	0	0
TC22-2	165	99	149Y, 14P, 1HV	14P	14P
TC25-2	147	100	147Y	0	0
TC26-1	12	100	12Y	0	0
TC28-2	20	100	20Y	0	0
TC43-2	22	100	22Y	0	0
	1,149		(1,147 (99.8%)*		
C4-1	70	4	3HV	0	0
C5-2	121	3	3Y, 1HV	0	0
C11-1	57	5	1Y, 1HV, 1P	1P	1P
C20-2	65	3	2HV	0	0
C25-2	105	0	0	0	0
C26-1	13	15	2Y	0	0
C28-1	160	2	3Y, 1HV	0	0
C43-2	69	13	5Y, 4HV	0	0
	730		27 (3.7%)		

* $P<0.0001$
[a] – Variants emerging de novo in sexual generations
[b] – Variation in R_0 generation
Y – Yellow plants
S – Striped plants
HV – Highly variegated plants
P – Plants lacking or showing varying intensities of purple pigmentation

ferences in the level of variation produced in tissue culture progeny as compared with seedling controls derived from the same plant. For example, line 33-2 showed 60% variation in progeny dervied from tissue culture (TC), while only 30% of the control (C) progeny were variants. It is also evident from this analysis that different plants had inherently different levels of stability both in vivo and in vitro. In contrast to line 33-2, line 27-1 showed a difference of 61% in the level of variation produced in tissue culture as compared with controls. When all the progeny from tissue culture and controls were compared on a population basis, this variation was highly significant ($P<0.0001$).

On subculture, the number of putative chlorophyll-deficient variants increased and again the difference between controls and tissue culture plants was highly significant ($P<0.0001$) (Table 1). After 6 months, 99.8% of the 1,149 plants regenerated were yellow and all of the 500 plants regenerated from callus after 12 months had a yellow phenotype. Yellow phenotypes were lethal and hence could not be grown to maturity. The nonlethal phenotypes produced in cultures subcultured for 6 months consisted of variegated plants and putative anthocyanin mutants. Only 2 plants showing the parental phenotype were produced.

In the controls, putative chlorophyll mutants were also produced, albeit at a lower frequency (1%–30%). Like the tissue culture regenerants, the level of variation produced varied between plants. No correlation was evident between the relative level of variation present in controls as compared to their related tissue culture regenerants. Only one putative anthocyanin mutant was produced from a total of 1,101 control seedlings.

At maturity, 4% of the tissue culture and 3% of control plants were male sterile. This difference was not significant ($P>0.05$), hence the incidence of plant sterility did not appear to have increased in tissue culture regenerants.

Cytological analysis indicates that all plants examined were diploid. Seven bivalents were observed in diakinesis. Metaphase was normal and 14 chromosomes were present in late anaphase. A low incidence of anaphase bridges was observed in both control and tissue culture populations. Twenty-six percent of the tissue culture and 20% of the controls examined showed this abnormality and in these plants bridges occurred in 2% of cells. There were no other meiotic abnormalities.

Seedling analysis of R_1, R_2 and S_1, S_2 of the marker line Tift 23BE

In the R_1 and S_1 generation 94% of the tissue culture, 100% of the control, and 84% of the parental plots segregated for the production of chlorophyll-deficient variants in seedling populations. These differences in segregation were not significant (data not shown). In no case was the segregation ratio found to be Mendelian and individual plants gave different segregation ratios. Similar results were observed in analysis of R_2 and S_2 populations.

Table 2. Quantitative measurements of phenotypic traits in tissue culture, parental (P), and control populations in the marker and in a genetically stable line of Tift 23BE

Population	Height (cm)	Tiller no.	Seed weight (g)	Panicle length (cm)
R_1[a]	80.7**	4.9**	2.4**	18.3
S_1[a]	84.0	4.2	3.3	18.5
R_1P[a]	84.8	5.0**	3.5	19.3**
R_2[a]	73.8	6.7	2.1	18.3
S_2[a]	74.5	6.4*	1.6**	18.0*
R_2[b]	77.9	6.8	1.9	16.4
S_2[b]	75.6*	6.6	1.5	16.7
R_1[c]	60.2**	14.9	6.3**	20.9**
S_1[c]	65.9**	14.4	7.7	21.9
R_1P[c]	67.0	15.5	8.4	21.7

* $P<0.05$
** $P<0.001$
[a] – Progeny of marker line
[b] – Progeny of 6-month subcultures
[c] – Progeny of Tift 23BE

Phenotypic analysis of R_1, S_1 and R_2, S_2 progeny of the marker line Tift 23BE

In comparing R_1 and S_1 populations, the quantitative characters assessed included plant height, tiller number, panicle length, and seed weight. An overview of the results is presented in Table 2. A number of qualitative characters was also evaluated including plant color, flowering date, chimerism, and male sterility.

Color

In the S_1 generation all control plots bred true and were the same color as the parental material, with the exception of one plot that had one green plant and four plots with highly variegated plants (data not shown). Two of the latter were the progeny of highly variegated S_0 plants. The remaining variegated plots were the progeny of normal plants. Control S_2 plots were predominantly similar to the parental material with the exception of six green plots, all of which had been derived from normal S_1 plants. Pedigree data indicate that these plants were derived from four original parental plants (Table 3).

Putative anthocyanin mutants produced in tissue culture were stable in both R_1 and R_2 generations. As indicated in Table 3, a number of new color variants arose in the R_1 generation. These variants were present in segregating plots of plants that showed differences in the intensity and distribution of anthocyanin and consisted of

Table 3. Pedigree and inheritance data for anthocyanin variants produced from tissue culture and control populations

Original lineage	R_0	R_1	R_2
TC25-117	1	stable	stable
TC25-125	1	stable	stable
TC25-126	1	stable	stable
TC25-127	1	stable	stable
TC25-128	1	stable	stable
TC25-132	0	segregant	stable
TC25-134	1	stable	stable
TC27-114	0	segregant	stable
TC27-117	1	stable	stable
TC33-23	0	segregant	stable
TC33-24	1	stable	stable
TC33-215	1	stable	stable
TC33-216	1	stable	stable
TC33-217	1	stable	stable
TC33-218	1	stable	stable
TC33-225	0	stable	stable
TC33-238	1	stable	stable
TC33-312	1	stable	stable
C5-1	0	0	2
C23-2	0	0	1
C25-1	0	0	2
C31-1	0	0	1

0–No anthocyanin variants produced from this line
1–One anthocyanin variant produced from this line
2–Two anthocyanin variants produced from this line
Segregant–progeny segregation for plants with varying quantities of purple pigmentation
Stable–progeny that maintained the variant phenotype produced in R_0

green, rose, and dark green plants. These plots also contained putative chimeral plants with different color tillers. Pedigree data indicate that these new variants originated from three of the original inflorescence explants which had given rise to color variants in the R_0 generation (see 27-1, 33-2, and 25-1 in Table 1). In the R_2 generation solid plots of one color phenotype were produced and the segregation for color variation was discontinued. The R_2 progeny plots of different chimeral tillers produced in the R_1 generation maintained the phenotype of the original tiller.

Quantitative analysis in R_1, S_1 and R_2, S_2 generations of the marker line Tift 23BE

A total of 489 tissue culture, 250 control, and 281 parental plant progeny was quantitatively evaluated in the R_1 generation. As indicated in Table 2, there were highly significant differences between tissue culture and control or parental populations for height ($P=0.0001$) and seed weight ($P=0.001$), with tissue culture progeny having lower mean values. Mean values for number of tillers in controls were significantly lower than those of both tissue culture and parental material ($P=0.0001$). Mean differences in panicle length between tissue culture and control populations were not significant, but parental material had a significantly greater panicle length compared to tissue culture or control populations ($P=0.0001$). Only tissue culture and control populations were evaluated in the second sexual cycle. In this generation differences between mean values in control and tissue culture populations were not significant for height ($P=0.06$). However, there were significant differences between seed weight means ($P=0.0001$), panicle length ($P=0.02$), and the mean number of tillers ($P=0.02$), with controls having lower means in all cases.

Due to late planting and the small size of the populations, no quantitative evaluation was made on the R_1 generation of progeny from plants regenerated after 6 months of subculture. In the second generation, 105 tissue culture and 33 control progeny were evaluated. In these populations, there were no significant differences between means in three of the quantitative characters assessed. These included means for number of tillers ($P=0.7$), panicle length ($P=0.7$), and seed weight ($P=0.1$). However, there was a significant difference in height ($P=0.05$).

Flowering in R_1 and R_2 generations of the marker line of Tift 23BE

Normal phenotypes of R_1 and R_2 generation showed no variation in flowering date when compared with the controls. However, all plots in the R_1 generation with highly variegated plants (HV) or color variants flowered later. Many of the HV plants failed to flower or were male sterile.

Male sterility occurred in both tissue culture and control populations. Its incidence in tissue culture progeny in the R_1 generation was significant ($P<0.05$), with 1% male sterile plants as compared to 0.5% for controls. This trend did not transmit to the R_2 generation, as both populations showed nearly equal numbers of male-sterile plants.

Cytological analysis in R_1 and R_2 generations of the marker line of Tift 23BE

Meiotic analysis of 15 color variants and a random sample of 50 plants from both control and tissue culture populations indicates that these plants were diploid. In all cases there were seven bivalents in diakinesis and 14 chromosomes at metaphase. Both populations also showed a low incidence of anaphase bridges as had been found in the R_0 and S_0 population.

Phenotypic and cytological analysis of R_0 and S_0 generations of normal green Tift 23BE

A total of 66 plants was regenerated from embryogenic callus produced directly on inflorescences of the geneti-

cally stable inbred of Tift 23BE explants after 1 month of culture. At maturity these plants showed no noticeable phenotypic variation when compared with controls. However, a significant number (70%) of the tissue culture regenerants was male sterile. Cytological analysis of fertile plants indicated that regenerants had the normal complement of chromosomes. Both control and tissue culture populations showed anaphase bridges at a low frequency but no other meiotic abnormalities were observed.

Quantitative, qualitative, and cytological evaluation of phenotypes of the R_1 progeny of normal green Tift 23BE

A total of 200 tissue culture, 222 controls, and 72 parental progeny were evaluated in the R_1 generation. Highly significant differences were evident between means of tissue culture and control or parental material for height ($P=0.0001$) and seed weight ($P=0.0001$) (Table 2). Differences between means of controls and tissue culture populations for panicle length were not significant; however, tissue culture populations were significantly different from the parents according to the Waller-Duncan *K*-ratio *T*-test ($P=0.0001$). No significant difference was evident between population means for numbers of tillers ($P=0.3$).

No qualitative variation for color was observed in R_1 populations of the inbred Tift 23BE and male sterility was observed at a low level (0.1%) in both populations. Cytological analysis indicated that no meiotic variation was present in tissue culture as compared to control R_1 populations.

Discussion

The present study suggests that genetic fidelity in the progeny of embryogenic tissue cultures is dependent on the genotype of the explant introduced into culture. Phenotypic variants for anthocyanin pigmentation and chlorophyll deficiency were produced exclusively in tissue culture regenerants from a Tift 23BE line with the dominant genetic marker purple plant (P), and recessive genetic markers for early flowering (e_1) and yellow stripe (ys). In contrast, none of the regenerants from a genetically stable inbred line of Tift 23BE showed any color variation, nor were there any color variants produced in the sexual progeny of these tissue culture regenerants. Hence, *P. glaucum* shows a genotypic effect on stability in culture similar to that found in other gramineous species (e.g., McCoy et al. 1982; Zehr et al. 1987). Such a difference in the generation of variation between genotypes is generally attributed to the genetic architecture of the plant and the effects of environmental stress on genome stability (Walbot and Cullis 1985; D'Amato 1986). In the present study, the presence of chlorophyll and anthocyanin variants in the control population of the marker line suggests that tissue culture allowed the increased expression and perpetuation of inherent variation present in the explant material. This is evident from the fact that when compared with control populations of the same plant lineage, tissue culture populations derived from 1-month-old cultures produced up to 30% more chlorophyll variants than their controls, while 6-month-old cultures produced 100% chlorophyll variants. The increased incidence of variants found in primary tissue culture regenerants may be due to de novo mutations induced in unstable genes because of the destabilizing effect of tissue culture on the plant genome. It is also possible, however, that tissue culture resulted in an increase in the expression of inherent variation in somatic cells by bypassing the selection that occurs in favor of normal cells during gamete formation, fertilization, and germination in plants with chloroplast mutator genes (Coe et al. 1988). Furthermore, the increased incidence of variants found in regenerants from subcultured callus may also be a consequence of selection or de novo induction of chloroplast mutations resulting in the predominance of variant cell genotypes in subculture populations. The emergence of chlorophyll variants from somatic tissue cultures of the Gramineae has been reported for *Lolium* (e.g., Dale and Dalton 1983; Jackson and Dale 1988) and also for *Hordeum* (Thomas and Scott 1985), *Pennisetum purpureum* (Chandler and Vasil 1984), and a number of forage grasses (Dale and Dalton 1983). Most workers found that only a minor proportion of young callus cultures formed albino shoots, the frequency depending on genotype and media. In general, as found in the present study, the frequency rapidly increased with culture age (e.g., Chandler and Vasil 1984).

Other color variants produced included plants that lacked purple pigmentation in all organs. The majority of these arose directly from tissue culture and were similar in phenotype to color variants that arose at a low frequency in control populations. Inheritance studies indicate that these variants are stable and heritable. Pedigree data furthermore indicate that these variants are clonally related and derived from three inflorescence explants. The appearance of similar phenotypes in control populations indicates that these variants are most probably the result of residual heterozygosity in the marker line, which was derived from a cross of a purple plant and yellow stripe line of *P. glaucum* (Pys-7) with inbred Tift 23BE. The frequency and early expression of this variation in tissue culture may be the result of regeneration of plants from somatic cells which, unlike gametophytic cells, do not routinely undergo recombination. Variant phenotypes were produced in the R_1 generation that showed variation in the intensity and distribution of purple pig-

ment. These plants segregated in progeny populations of plants that did not show a deviant phenotype in the R_0 generation, but were derived from plants that were clonally related to purple R_0 variants. This phenotype was stable and inherited in the R_2 generation. The lack of such variation in the control population suggests that tissue culture had some unique influence on the control of the expression of the purple plant trait. Changes in plant pigmentation as a result of tissue culture have also been reported in *Medicago*, and Groose and Bingham (1986) attribute this variation to the potential induction of transposable elements. The production of anthocyanin is known to be influenced by the presence of modifier genes or inhibitory elements (Harrison and Fincham 1968), and it is possible that such genes were activated in tissue culture resulting in the reduction or elimination of anthocyanin production.

Six putative chimeras for stem color were found in the R_1, seven in the R_2, and one in the S_0 populations of the marker line of *P. glaucum*. In other cereal tissue culture regenerants, the presence of chimeras is generally believed to be a result of the multicellular origin of shoots. These have been identified as chimeras because of mixoploidy (Bennici and D'Amato 1978) or through segregation in the R_2 generation because of disparity between the pollen and egg cells (McCoy and Phillips 1982; Lee and Phillips 1987; Armstrong and Phillips 1988). A multicellular origin is not a tenable explanation for the presence of putative chimeras in the present population, as somatic embryos in *P. glaucum* are of single cell origin (Vasil and Vasil 1982). There is a greater likelihood that these plants may have arisen as a result of mutation occurring during development, or from a genetic change occurring during the first embryonic cell division, which would give plants with genotypically and phenotypically distinct halves. It is also possible that these putative chimeras may have arisen from the fusion or twining of somatic embryos. Both twin and triplet embryos have been reported in *P. glaucum* (Powell and Burton 1968). If these embryos bore different mutations for anthocyanin formation on germination, they would produce tillers with different phenotypes giving the appearance of chimeras.

Data from the R_1 generation suggest that there was no real influence on flowering date in tissue culture R_1 progeny with the exception of color variants, which flowered 2 weeks later than the rest of the population. This trait was heritable and similar late flowering was observed in color variants in the R_2 generation. This related change suggests that the genes for early flowering and purple pigmentation may be linked.

A high level of male-sterile plants (70%) was produced in regenerants from the genetically stable inbred Tift 23BE line. This sterility may have been caused by temperature fluctuations occurring in the growth chamber during plant regeneration. In the marker line, a 0.5% increase in the incidence of sterile plants in R_1 regenerants was not transmitted to the R_2 generation, and this suggests that tissue culture had no long-term influence on the incidence of male-sterile plants.

Analysis of quantitative traits of the marker line in both the R_1 and R_2 generation indicates that tissue culture had a transient effect on reducing the overall mean height and yield and increasing the numbers of tillers present in the tissue culture population. The highly significant differences found for these traits in the R_1 generation were not present in the R_2 generation. This suggests that tissue culture had a carryover effect on some quantitative characters, which was transferred through one but not a second sexual cycle. Such time-related disappearance of quantitative phenotypic variation has also been observed among regenerants of *Pennisetum purpureum* (Rajasekaran et al. 1987). The reversion or remission of certain traits after sexual propagation is regarded as being a result of their epigenetic nature (Meins 1983). Results of the present study suggest that in some cases it may require two sexual cycles before epigenetic effects are uncovered. The absence of significant quantitative variation after a second sexual cycle, furthermore, indicates that in this instance regeneration via somatic embryogenesis had no long-term effects on quantitative characters.

Significant variation in quantitative traits was found in the R_1 tissue culture population of the genetically stable inbred line Tift 23BE. In the absence of R_2 data the nature of this variation cannot be confirmed; however, judging from the results of R_2 studies on the marker line it is possible that these effects could be epigenetic and may not carry over to the R_2 generation.

The diploid nature of all plants examined and the absence of major cytological change demonstrates that all plants regenerated from tissue culture were derived from cytologically normal cells. This corroborates previous evidence for the cytological stability of regenerants from embryogenic cultures (Hanna et al. 1984; Swedlund and Vasil 1985). Reports of cytological instability in regenerants from embryogenic cultures of maize, however, suggest that this stability may be related to genotype or to the growth rate and level of organization present in the embryogenic callus (Armstrong and Phillips 1988).

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