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Meiotic and isozymic characterization of plants regenerated from euploid and selfed monosomic tall fescue embryos*

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Summary. Tissue culture of tall fescue (*Festuca arundinacea* Schreb., 2n = 6x = 42) would be enhanced by improving the callus induction and plant regeneration efficiency, and evaluating the meiotic and isozymic variation induced by culture. Mature embryos were cultured from four lines of Kenhy tall fescue and from the progeny of three selfed monosomics. Evaluation of six media-auxin combinations showed callus initiation was greatest on SH medium with 2.5 mg/l 2,4,5-T or 7.4 mg/l pCPA, while plant regeneration was greatest on SH medium with 0.5 mg/l 2,4-D. Cytological analyses of 27 plants derived from euploid parents showed a high frequency of aneuploidy (15/27). Chromosome numbers of aneuploids ranged from 36 to 41, with one plant having 80 chromosomes and two plants being asynaptic. Two of ten monosomic-derived plants were euploid, five were monosomic, one was monosomic with a fragment and two were double monosomic. Zymograms of the parents and regenerants were obtained for the enzymes ACPH, ADH, GOT, 6-PGD and PGI. Isozyme variation was observed for two groups of plants derived from the same Kenhy embryos. One group of four monosomic-derived plants differed for the enzymes GOT and ACPH, and all four plants had a PGI pattern different from that of the parental monosomic plant. This indicated loss of a PGI allele, probably as a result of callus culture.

Key words: *Festuca arundinacea* – Tissue culture – Somaclonal variation

Introduction

Tissue culture in tall fescue (*Festuca arundinacea* Schreb., 2n = 6x = 42), an outcrossing pasture and turf grass, has been limited due to poor plant regeneration from callus. Dale (1977) cultured apical meristem tips resulting in an overall 19% regeneration rate. Using embryo culture, Lowe and Conger (1979) found callus induction was close to 100% but shoot formation was poor (3.1%–9.6%). For regeneration, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) was less effective than 2,4-D (2,4-dichlorophenoxyacetic acid), although both gave low regeneration (Conger et al. 1978).

Cytogenetic, morphological, biochemical and molecular changes were observed in plants regenerated from callus culture (Scowcroft 1985). Reed and Conger (1985) found that 40%–50% of their 31 regenerated tall fescue plants had meiotic aberrations. Eizenga (1989) found abnormalities in 100 of 166 regenerants analyzed, with the most predominant abnormality being chromosome loss. These studies suggested that somaclones may provide source materials for a monosomic series in tall fescue similar to that developed for hexaploid wheat (*Triticum aestivum* L.) (McIntosh 1987). Hybridization and genetic studies would be enhanced by the availability of tall fescue aneuploids.

Isozymes have been used in conjunction with aneuploids to determine the chromosomal location of genes for different enzymes in wheat (Hart 1983). Isozyme variation has been reported among individual tall fescue plants (Eizenga and Buckner 1986) and among plants derived from anther-panicle culture (Eizenga 1987). Isozyme variants could be used as chromosome markers with a monosomic series if one were available in tall fescue.

The first objective of this study was to evaluate the callusing and regeneration efficiency of four Kenhy tall

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fescue lines as influenced by media and auxin combinations. The second objective was to characterize the meiotic and isozymic variation in regenerants derived from four Kenhy lines and three selfed monosomic progenies of similar origin. Embryos from selfed monosomics were cultured to determine if nullisomic plants could be obtained through loss of the monosomic chromosome.

Materials and methods

Mature polycrossed seed from four parental lines of the tall fescue variety, 'Kenhy', were used for embryo culture. Two of the lines had high perloline levels (34-27 and 39-27) and two had low perloline levels (84-25 and 86-25). Perloline is the predominant alkaloid in tall fescue and it inhibits digestibility (Buckner et al. 1977). Mature embryos from three selfed monosomic plants (64-11, 67-9 and 65-8) of the same parentage as the Kenhy parental lines were also cultured. Three-year-old embryos from 1981 seed were cultured to ensure that the endophytic fungus in these lines, *Acremonium coenophialum* Morgan-Jones and Gams, was not viable (Siegel et al. 1985). (A large portion of the mycelia of this fungus die after two years.)

Embryo culture was according to the methods of Lowe and Conger (1979). Six media-auxin combinations were tested using two basal media, Schenk and Hildebrandt (SH) (1976) and Murashige and Skoog (MS) (1962), and three auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) (9 mg/l), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (2.5 mg/l) and para-chlorophenoxyacetic acid (pCPA) (7.4 mg/l). The two media were used in combination with the three auxins. Fifty-six embryos of the four Kenhy lines were plated on each media-auxin combination. After 28 days, calli were transferred to fresh medium containing a lower auxin level (5.0 mg/l 2,4-D, 1.4 mg/l 2,4,5-T or 3.8 mg/l pCPA, respectively), and allowed to grow for another 28 days. Calli were then transferred to still lower auxin levels (2.0 mg/l 2,4-D, 0.56 mg/l 2,4,5-T or 1.9 mg/l pCPA, respectively). At the end of this third 28-day period, callus was transferred to regeneration media containing 0.5 mg/l of either 2,4-D, 2,4,5-T or pCPA. These cultures were incubated at 25 °C with a 16-h photoperiod and transferred to fresh media every 4 weeks. Media and auxin responses were calculated as the mean number of calli or plants produced per plate. Pairwise comparisons of means using a factorial design were conducted to determine differences in response.

Meiotic configurations and pollen stainability of 37 regenerants were determined as described by Eizenga (1987). The isozymes phosphoglucoisomerase (PGI), glutamate-oxaloacetate transaminase (GOT) and acid phosphatase (ACPH) were visualized on starch gels as described by Eizenga (1987). The isozymes alcohol dehydrogenase (ADH) and 6-phosphogluconate dehydrogenase (6-PGD) were visualized on acrylamide gels. The separating gel consisted of 10.0 ml acrylamide stock (30% acrylamide and 0.8% bis-acrylamide), 10.0 ml TRIS-HCl buffer (pH 8.9), 20 ml water, 250 µl ammonium persulfate and 30 µl TEMED. The stacking gel was made of 1.0 ml acrylamide stock, 1.25 ml TRIS-phosphate buffer (pH 6.7), 7.75 ml water, 150 µl ammonium persulfate and 10 µl TEMED. Samples were prepared as for the starch gels, with 0.1 ml of glycerin added before centrifugation. Samples were overlayed with a TRIS-glycine buffer (pH 8.3) with 1.5 ml bromophenol blue. The reservoir was filled with TRIS-glycine buffer without the stain. Gels were run at 30 mA constant current for 4–5 h. Gels were stained and fixed according to Cardy et al. (1981). Allele determinations were based on Ostergaard et al. (1985).

Results and discussion

Embryo culture and regeneration

Four Kenhy lines were evaluated for callus initiation and plant regeneration. Callus initiation began approximately 21 days after the embryos were explanted. The mean number of calli produced per plate was calculated to compare the response to basal media and auxins. Forty percent of the cultured embryos initiated callus. There was no significant difference in callus initiation between the basal media MS and SH, but there were differences in line response and response to different auxins.

The auxins 2,4,5-T (49%) and pCPA (48%) gave a significantly higher callus response than 2,4-D (30%). Kenhy line 86-25 (50%) had a higher callus initiation frequency than the other three lines: 84-25 (35%), 84-27 (34%), and 39-27 (40%). There was no difference in response between high and low perloline lines, which suggests that perloline does not influence callus induction.

Overall plant regeneration was low; 3.7% of the calli produced a total of 144 green plants and 24 albino plants. The low frequency of regeneration could be due to the age of the explanted embryos because older seed has decreased viability (Siegel et al. 1985). There was no difference in regeneration rates (percentage of calli producing plantlets) between the two basal media, MS and SH, or between the four Kenhy lines. The media-auxin combination of SH plus 2,4-D at 0.5 mg/l gave significantly higher plant regeneration (9%) than all other combinations. This combination produced 96 of the 144 green plants regenerated (Fig. 1). The MS plus 2,4-D combination gave 7% regeneration, and the other combinations gave 3% or less regeneration.

Root initiation was more frequent than shoot initiation on all media types. Approximately 25% of calli regenerated roots only. Several pieces of callus contained green spots that could not be induced to regenerate, although other calli that contained green spots regenerated several plants. Heyser and Nabors (1982) reported similar green spots in oat and other cereal cultures. The regeneration rate in this study (3.7%) was low compared to the range of 3.1%–9.6% reported for similarly cultured embryos (Lowe and Conger 1979). It was much lower than the 19% regeneration reported by Dale (1977) after meristem tip culture, presumably reflecting differences in the explant tissues. In the present study, mature embryos were used as opposed to apical meristems, and the embryos were derived from seed that was several years old.

Meiotic behavior

Meiotic behavior was analyzed in 27 of the plants regenerated from Kenhy lines by determination of the chromo-

some number, metaphase I (MI) pairing configurations, average number of micronuclei per quartet (M/Q) and percent stainable pollen (Table 1). Seventeen of the plants were derived from the Kenhy line 86-25, 6 from line 84-25, 3 from line 84-27, and 1 from line 39-27. Of the 27 plants analyzed, 11 were euploid with 42 chromosomes and mostly bivalent pairing (Table 1, Fig. 2a and b), however, in some regenerants there was less pairing then in the parents. One plant derived from 84-27 was chimeric and asynaptic with 42 univalents at metaphase I. Another plant derived from 86-25 was also identified as asynaptic with 38 chromosomes (Fig. 2c). The meiotic behavior of both plants was similar to the plants with achiasmic meiosis identified in 'Kenwell' tall fescue somaclones (Eizenga 1989). In addition, the meiotic behavior of seven monosomics and four double monosomic plants regenerated from Kenhy embryos was characterized. Two plants ($2n=40+4f$ and $2n=36+2f$) had chromosome fragments (f). In both plants, the fragments usually did not pair during meiosis, probably due to the terminal deletions, absence of centromere and/or lack of homology. One plant ($2n=80$) had approximately the doubled chromosome number ($2n=12x=84$). It most likely arose from endopolyploidy in the callus.

Fig. 1. Regeneration of a green plant from callus of a mature embryo

Male fertility as determined by pollen stainability was reduced in all plants regenerated from culture compared to the parental checks (Tables 1 and 2). The most noticeable reduction in stainable pollen occurred in the euploid regenerants, which suggested that culture-induced aberrations had occurred. For comparison, only 14 of the 99 (14%) euploid regenerants obtained by Eizenga (1989) using similar methodology also had lowered pollen stainability.

The high frequency of aneuploid regenerants (15/27; 55.6%) produced from explants of euploid plants was not expected. In a previous study (Reed and Conger 1985), only 2 of 31 (6%) plants were aneuploid (trisomic), although other abnormalities, such as laggards and bridges, were found in 41.9% of their regenerants. In a larger study, Eizenga (1989) examined 166

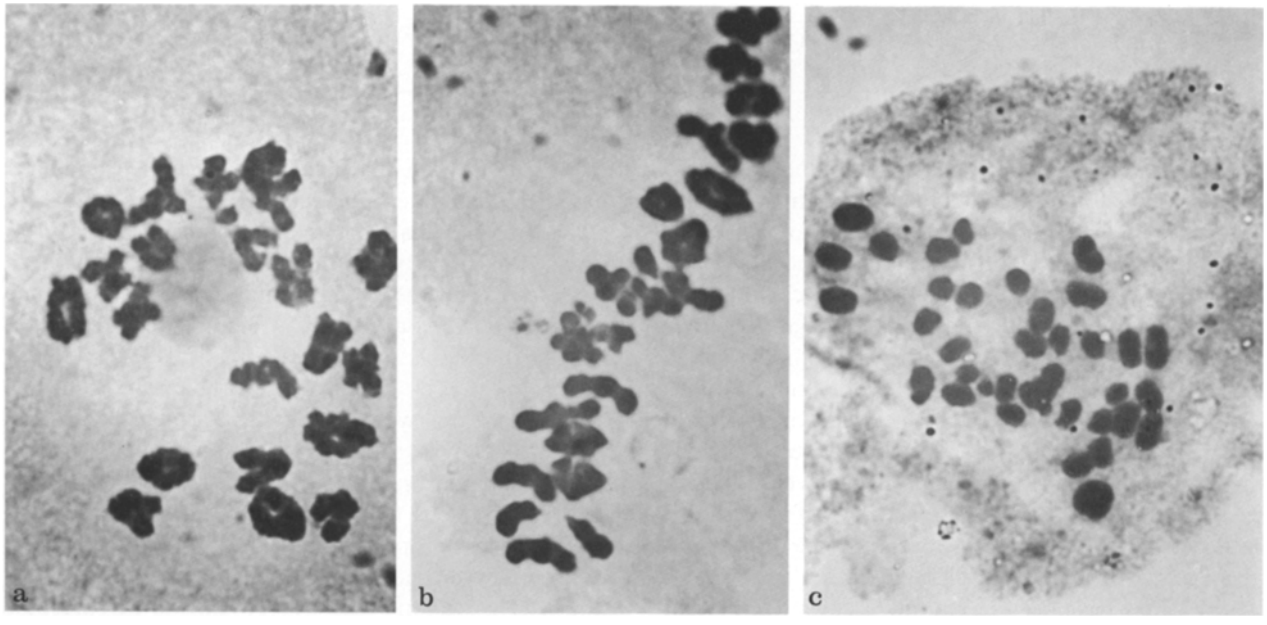

Fig. 2a–c. Comparison of chromosome pairing in euploid regenerant ($2n=42$) with 21 bivalents at **a** diakinesis, **b** metaphase I, and **c** lack of pairing in an asynaptic aneuploid regenerant ($2n=38$)

Table 1. Meiotic analyses of plants regenerated from 'Kenhy' tall fescue embryo callus. Means are calculated based on chromosome number. Ranges are given in parentheses

Chromosome no.	No. plants	Methaphase I configuration				M/Q[a]	% pollen stainability
		I	II	III	IV		
Parents (42)	4	0.14 (0.00–0.24)	20.89 (20.80–21.00)	0.00	0.02 (0.00–0.04)	0.1 (0.1–0.2)	93.1 (77.8–98.6)
42	11	0.55 (0.00–1.84)	20.69 (19.96–21.00)	0.02 (0.00–0.12)	0.00 (0.30–4.40)	2.1 (0.3–4.4)	38.2 (8.0–68.0)
41	7	1.50 (0.92–2.36)	19.56 (19.18–19.92)	0.13 (0.00–0.28)	0.01 (0.00–0.08)	1.9 (1.1–2.7)	56.3 (31.0–74.4)
40	4	2.77 (2.32–3.93)	18.33 (17.40–18.76)	0.15 (0.08–0.32)	0.03 (0.00–0.08)	1.5 (1.2–1.7)	25.3 (3.4–66.2)
40+4f[b]	1	2.88	20.52	0.00	0.00	1.7	5.3
36+2f	1	4.96	16.48	0.00	0.00	2.3	23.6
80	1	7.60	33.32	1.08	0.60	6.0	0.8
42	1	42.00	0.00	0.00	0.00	0.6	–[c]
38	1	38.00	0.00	0.00	0.00	–[d]	–[d]

[a] M/Q = Micronuclei per quartet
[b] Fragments included in counts
[c] Plant was asynaptic, pollen not formed
[d] Plant was asynaptic, micronuclei or pollen not formed

Table 2. Meiotic analysis of plants regenerated from callus of selfed seed of monosomic tall fescue lines. Means are calculated based on chromosome number. Ranges are given in parenthesis

Chromosome no.	No. plants	Metaphase I configuration				M/Q[a]	% pollen stainability
		I	II	III	IV		
Parents (41)	3	1.13 (1.08–1.24)	19.93 (19.88–19.96)	0.00	0.00	0.8 (0.7–0.9)	92.9 (89.8–95.6)
42	2	0.82 (0.44–1.20)	20.54 (20.40–20.68)	0.02 (0.00–0.04)	0.00	0.5 (0.2–0.9)	11.2 (2.2–20.2)
41	5	1.17 (0.88–1.96)	19.88 (19.52–20.00)	0.03 (0.00–0.13)	0.00	1.1 (0.2–2.6)	78.8 (54.0–88.6)
40	2	1.80 (1.52–2.08)	18.72 (18.52–18.92)	0.24 (0.00–0.48)	0.00	2.0 (1.1–2.8)	86.2 (73.0–99.4)
41+f	1	1.12	19.64	0.20	0.04	1.8	32.2

[a] M/Q = Micronuclei per quartet

regenerants. Fifty-nine (36%) were aneuploid (2n = 38–41) and 33 (20%) of the euploid plants had meiotic aberrations. These increases in numbers of aneuploids recovered may be due to the longer culture period. Reed and Conger (1985) recovered plants 11–12 weeks after the initial plating of the embryos, while Eizenga (1989) regenerated plants 10–24 weeks after plating. In this study, plants were regenerated 16–40 weeks after culture initiation. Increased time in culture has been shown to increase chromosome abnormalities in polyploid species such as wheat and oats (Scowcroft 1985). It has been hypothesized that tissue culture delays replication of heterochromatic regions of DNA, causing chromosome bridges and breakage events during anaphase (Johnson et al. 1987). This also may have occurred in tall fescue in the present experiment, resulting in aneuploids and plants with fragments. The occurrence of heterochromatic blocks in tall fescue has not been documented, but heterochromatin is present in the related *Lolium* species (Thomas 1981).

Ten regenerants from selfed monosomic seed were analyzed meiotically and the meiotic analyses was compared to that for the parents (Table 2). Four regenerants were from 64-11, four from 67-9 and two were from plant 65-8. Of these ten, two were euploid (21 II), five were monosomic (20 II + 1 I), two were double monosomic (19 II + 2 I) and one was monosomic with a chromosome fragment (20 II + 1 I + f). It was thought that chromosome loss might result in the elimination of the chromosome homologous to the monosome if the parent was monosomic. Unfortunately, plants nullisomic for the monosomic chromosome in the parent were not obtained. The meiotic analysis of the 40-chromosome plants indicated the regenerants were double monosomic.

The percentage of aneuploid regenerants from selfed monosomic embryos (80%) was higher than from euploid embryos (53%), as was expected. This frequency (80%) was comparable to that found on selfing monosomic wheat plants (76% aneuploids) (McIntosh 1987). Monosomics and double monosomics from both groups had similar metaphase I configurations (Tables 1 and 2) and similar frequencies of micronuclei in microspores as expected, but the aneuploids from monosomic parentage had higher levels of pollen stainability. This suggests that chromosome loss in aneuploids regenerated from euploid embryos was culture-induced, while aneuploids regenerated from embryos of selfed-monosomic plants resulted from lack of monosome transmission in the gametes.

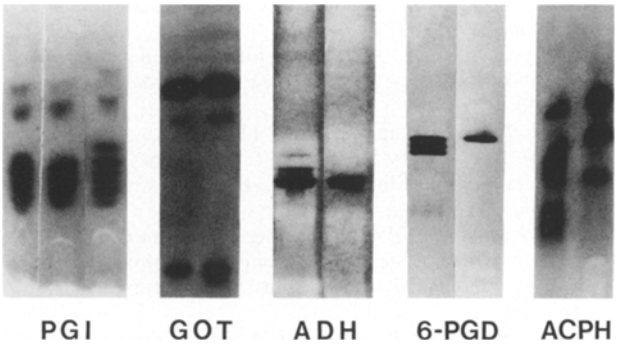

Fig. 3. Variation in isozyme banding pattern generated through tissue culture of Kenhy and selfed monosomic embryos for the enzymes PGI, GOT, ADH, 6-PGD and ACPH

This is supported by the similarity in meiotic behavior among the monosomic parents and the monosomic regenerants (Table 2). It is likely that loss of whole chromosomes in euploid-derived regenerants was accompanied by other changes that caused the reduction in pollen stainability.

Isozyme analysis

Isozyme banding patterns for five enzymes, PGI, ADH, 6-PGD, ACPH and GOT, were examined for the same 37 regenerated plants. Variation was observed for all of the enzymes, but only certain changes could be attributed to tissue culture because of possible heterogeneity due to the polycrossed origin. Differences were observed in the banding patterns of several groups of plants each regenerated from the same embryo. Plants 6 and 7, regenerated from a single embryo, had a PGI-2 banding pattern different from that of their 86-25 parent in that the 'a' allele was not expressed (Figs. 3 and 4). This difference was not related to chromosome number, as plant 6 was monosomic and plant 7 was euploid, but plant 7 may have a culture-induced deletion or modification of the 'a' allele. Plants 29 and 30, regenerated from an 84-27 embryo, differed in banding patterns for the isozymes ADH-1 and 6-PGD-1 (Figs. 3 and 4); their pattern represented loss of an allele for each of these enzymes.

Isozyme comparisons can be made between a group of four regenerants derived from one selfed 67-9 embryo. These plants (41, 42, 43, 44) were all monosomic, similar

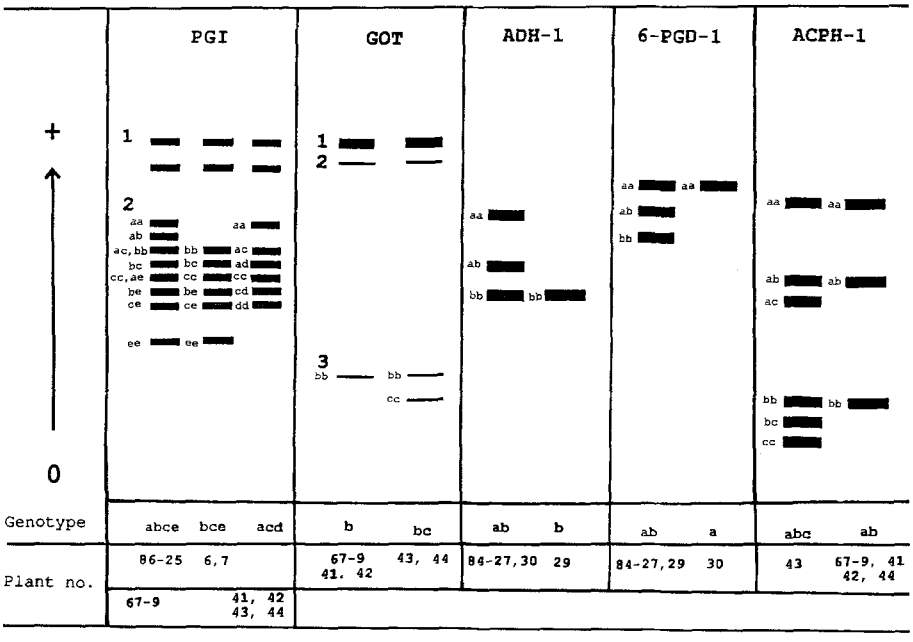

Fig. 4. Diagram of the isozyme variation shown in Fig. 3. The genotype of the isozyme and parents (86-25, 67-9, 84-27) and regenerants (6, 7, 29, 30, 41, 42, 43, 44) exhibiting differences are below the diagram. Genotypes are given as alleles (a, b, c, d and/or e) present for the isozymes PGI-2, GOT-3, ADH-1, 6-PGD-1 and ACPH-1 based on Ostergaard et al. (1985)

to the parent, and had differences in their GOT-3 and ACPH-1 loci. Plant 43 had an additional ACPH-1 allele that was absent from the rest of this group (Figs. 3 and 4). The PGI banding patterns indicated that all four regenerants had lost a PGI-2 allele found in the parent plant. This loss resulted from either culture or genetic segregation.

Differences in storage protein patterns were reported in regenerants of barley (Breiman et al. 1987), wheat (Cooper et al. 1986) and triticale (Jordan and Larter 1985), and differences in β-amylase were observed among regenerants of wheat (Ryan and Scowcroft 1987). Variation in ADH-1 was attributed to molecular changes in the gene in maize (Brettell et al. 1986), and to aneuploidy and a translocation in wheat regenerants (Davies et al. 1986). Because the isozyme variation in this study could not be correlated with chromosome loss, variation may be from (a) mutation that altered or inactivated an allele or enzyme locus, (b) deletion of a small portion of the chromosome which occurred as a result of tissue culture, or (c) genetic segregation which occurred in culture. Also, there is the possibility that these changes were epigenetic. Progeny of regenerants have not been analyzed for their isozyme banding patterns. For monosomic-derived-plants it was also possible that univalent shift (McIntosh 1987) took place; thus, the univalent chromosome in the regenerant may not always have been the same chromosome as in the parent.

In conclusion, this study showed that the auxin 2,4-D gave significantly lower callus response than 2,4,5-T or pCPA but was the best auxin tested for plant regeneration. Chromosome loss was the most notable response to embryo culture; thus, this appeared to be a useful method for obtaining monosomics. Unfortunately, nullisomic plants were not found among regenerants from selfed monosomics. The variation in isozyme patterns suggested that a mutation or deletion had occurred, since there was loss of the expression of an allele. Future studies should be focused on the characterization of the monosomic chromosomes, so that these aneuploids can be used in tall fescue improvement.

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