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Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.) Moench*

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Summary. Immature embryos of 20 sorghum genotypes were cultured on MS 5 medium containing MS mineral salts supplemented with 2,4-D, zeatin, glycine, niacinamide, Ca-pantothenate, L-asparagine, and vitamins. For regeneration, calli were transferred onto the same medium with the exception that IAA was substituted for 2,4-D. In general, immature embryos obtained 9–12 days after pollination resulted in the best redifferentiation. Ability of calli to regenerate varied among genotypes; cultivars C401-1 and C625 had the highest redifferentiation frequencies. Ability to redifferentiate was heritable and acted as a dominant trait. At least two gene pairs were involved. Regenerated R_0 plants were planted in a greenhouse and their selfed (R_1 and R_2) progenies were planted in the field and examined for morphological and cytological variations. The majority of the phenotypic variations noted in R_0 were not transmitted to later generations. However, variants for plant height, degree of fertility, and midrib color persisted in R_1 and R_2 generations. A variation in tallness was attributable to one dominant mutant gene. Short stature and male sterility variants appeared to be consequences of recessive mutant genes controlling those traits. Minor variations in peroxidase banding patterns were found among R_0 plants.

Key words: Morphological variation – Cytological variation – Peroxidase isozyme patterns – Mutant – Sterility

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Introduction

Development of cell and tissue culture techniques for crop species could provide opportunities for mutant selection for economic traits and also a means to study genetic and physiological aspects of ontogeny. Plants regenerated from callus cultures may furnish useful germplasm for breeding programs. However, implementing a selection system requires effective techniques to initiate and maintain the calli and, subsequently, to regenerate plants from them. Also, heritability of the variations induced from such a system is necessary to document their utility.

Establishing efficient variations in induced tissue culture systems is apparently more difficult in monocotyledonous species than in dicotyledons. Nevertheless, callus formation and plant regeneration have been accomplished in a number of monocots (Gamborg et al. 1977; Gosch-Wackerle et al. 1979; Hanzel et al. 1985; Hu and Zeng 1984; Kaur-Sawhney and Galston 1984; King and Shimamoto 1984; Nabors 1982; Rines and McCoy 1981; Schaeffer et al. 1984; Sears and Deckard 1982; Suenaga et al. 1982; Yamada and Loh 1984). Variations from the regenerated plants were considered as a novel source of genetic variability for plant improvement (Larkin and Scowcroft 1981; Scowcroft and Larkin 1982). The spectrum of variants observed is broad and the variation does not follow the same pattern as that of induced mutations (Evans et al. 1984). The cause of variation is not known, but variants have been produced in many crops (Green 1981; Cummings et al. 1976; Gengenbach 1977; Nabors et al. 1980; Larkin et al. 1984). In sorghum (*Sorghum bicolor* (L.) Moench.), calli have been cultured and selected for tolerance to aluminum, salt, and drought (Smith et al. 1983; Bhasharan et al. 1985; Smith et al. 1985). However, variants that are stable and persistent after sexual cycle(s) have not been reported in sorghum.

The primary goals of our research were to identify genotypes capable of forming calli and regenerating plants, to show the effect of embryo age on callus induction, and to examine heritable variations in R_1 and R_2 generations.

Materials and methods

Genotypes Tx 2759, Tx 2762, Tx 2767, IRSR-1, and F2194 were randomly selected from the Texas Agricultural Experiment Station; C401-1, C433-1, C191, C600-7, C625, and C627 were randomly selected inbred lines from China, and some F_1's were obtained by crossing those inbreds. All material was grown in a greenhouse. Pollination bags were placed over the panicles before blooming to ensure self-fertilization. At 9 to 18 days after anthesis, immature embryos were collected from these plants, sterilized in 60% ethanol for 3 min in a laminar flow hood, and then rinsed twice with sterilized distilled water. Embryos were placed on medium with the plumule-radicle axis side in contact with the medium and the rounded scutellar side exposed.

The callus-including medium was composed of the mineral salts of MS medium, vitamins from B5 (Gamborg et al. 1977) and supplements of 30 g l^{-1} sucrose, 7.7 mg l^{-1} glycine, 200 mg l^{-1} L-asparagine, 2 mg l^{-1} ascorbic acid, 1.3 mg l^{-1} niacinamide, 0.25 mg l^{-1} calcium pantothenate, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.2 mg l^{-1} zeatin. The medium was adjusted to a pH of 5.8 to 6.0, and 6 g l^{-1} difco agar was added. The medium was autoclaved for 20 min at a pressure of 1.5 kg cm^{-2}.

The callus-redifferention medium was the same as the callus-inducing medium, except that indole-3-acetic acid (IAA) at a concentration of 0.1752 mg l^{-1} was substituted for 2,4-D.

The inoculated embryos were cultured in an incubation chamber at 20 °C ± 1 °C with a photoperiod of 18 h day^{-1}. When the regenerated plants reached a height of 10 cm, they were transplanted to soil in pots but kept under moist conditions in a growth chamber. Once the regenerated plants had recovered and resumed vigorous growth, they were transferred to a greenhouse.

Morphological variations of fertility, height, midrib color, panicle shape, chlorophyll deficiency, kernel weight, flowering time, growth rate, and pollen staining ability were recorded from the R_0 plants. All 215 R_0 plants were selfed, including 158 from C401-1. Among the latter, 7 albinos died and 151 head rows were planted at the Agronomy Farm at Kansas State University with C401-1 controls planted in every 10th row. R_1 plants were examined morphologically and selfed. R_2 plants were planted in an experiment farm at Shenyang Agricultural College, China for additional observation.

Peroxidase extraction was made following the procedure of Liang et al. (1977). The supernatant fractions of the extractions from the C401-1 control and four C401-1 R_0 plants were concentrated by increasing the ammonium sulfate to near saturation (0.4 g $(NH_4)_2SO_4$ added to each ml of extract with continuous stirring). After 8 h of refrigeration, the samples were centrifuged at 20,000 × g (3 °C) for 10 min. The precipitates were dissolved in 1 ml of 1% (w/v) glycine and dialyzed against that buffer for 12 h at 4 °C with constant stirring. The samples were centrifuged again for 10 min at 20,000 × g (3 °C) before use.

Isoelectric focusing was carried out on LKB 8 × 11 cm Ampholine PAG plates at pH 3.5–9.5. Each of the five samples (5 to 10 µl) was applied to paper wicks in the center of the gel. The focusing was made on a Bio-Rad model 1405 electrophoresis apparatus for 3 h, 4–6 W maximum. Platinum electrodes contacting the gel through paper strips were soaked in 1M H_3PO_4 (anode) and 2% diethylamine (cathode).

Chromosome number and behavior of 12 slow growing and low-seed-setting R_0 plants were examined from microsporocytes. The panicles were fixed in Carnoy A solution (3 95% ETOH : 1 glacial acetic acid, v/v) for 24 h and then stored in 70% ETOH. Chromosomes were stained with carbol fuschin. Pollen from R_0 plants was stained with 1% KI_2 and examined for its staining ability.

Results and discussion

Genotypic response to in vivtro culture

Number of immature embryos inoculated, age of the embryos, frequency of callus formation, and plant regeneration for the 20 genotypes are shown in Table 1. Five of the inbreds, C401-1, C625, C627, F2194, and IRSR-1, demonstrated a higher frequency of plant regeneration than the others. When C401-1 and C625 were used as parents in hybrid combinations, their F_1 progeny also showed a high frequency of plant regeneration, occasionally higher than that of the high parent. However, the F_1 hybrid derived from two non-regenerable parents, Tx2759A and C600-7, had a 5.2% regeneration, suggesting that at least two complementary genes controlled the ability to regenerate.

Ability for immature embryos to regenerate appeared to vary, even among plants within an inbred. With C401-1, for example, embryo-derived calli were regenerable from six of the 12 plants sampled. Considering this ability to regenerate, the inbred line apparently was composed of plants individually homozygous but collectively heterogeneous. Heterogeneity of inbreds appears to be possible because none of the inbreds had been selected for responses to embryo culture. Thus, for tissue culture experiments, materials from a single head or plant selection should be used.

Many genotypes did not produce regenerable calli, and for those whose calli were regenerable, frequencies tended to be low, indicating that embryo culture may be medium-dependent. Refined media with higher regenerability for many genotypes are indeed desirable.

Relationship between age of immature embryo and plant regeneration

Immature embryos obtained 9 to 12 days after pollination (DAP) showed a higher frequency of plant regeneration than those of 13- to 18-day-old embryos (Table 2). For the 9- to 12-DAP embryos, scutellar tissue grew rapidly and the plumule appeared from the embryos 10 to 15 days after inoculation. The scutellum reached a diameter of 3 to 4 mm with a light green color, and the plumule reached 5 mm in length before the scutellum expanded in size promptly and callus was formed, about 30 days after inoculation. When several leafy shoots were formed on each callus, the plumule stopped growing and died. After calli with shoots were transferred to the regeneration medium to develop leaves and roots, some regenerated plants reached about 100 mm in height within 8 weeks. Several plants grew from a single section of callus (Fig. 1).

Table 1. Response of 20 sorghum genotypes to in vitro culture of immature sorghum embryos

Genotype	No. embryos innoculated	Age of embryos (DAP)	Embryos producing callus		Embryos producing plants	
			No.	%	No.	%
C401-1	301	9–18	138	45.8	22	7.3
C625	5	9	5	100.0	2	40.0
C600-7	128	11–15	127	46.5	3	0
C191	55	11–13	40	72.2	0	0
C433-1	28	10	15	53.6	0	0
C627	33	9–18	33	100.0	1	4.3
T×2767	7	9–11	6	85.0	0	0
T×2771	12	9–11	12	100.0	0	0
T×2772	22	11–12	5	22.7	0	0
T×2776	12	11–13	3	25.0	0	0
IRSR1	18	10–12	16	88.8	1	5.5
F2194	273	11–15	127	46.5	3	1.1
T×2759A×2759B	22	11–13	15	68.0	0	0
T×2759A×C600-7	19	11–14	19	100	1	5.2
F219×C40-1	24	12–14	12	50	4	16.7
F2194×C625	9	11–15	9	100	2	22.2
F2194×C600-7	33	9–15	30	90.0	0	0
F2194×C191	28	9–14	20	71.0	0	0
C600-7×C401-1	42	12–17	19	45.2	13	31.0
C191×F2194	25	9–13	20	80	0	0

DAP = days after pollination

Table 2. Effect of sorghum embryo age on regeneration and general morphology

Cultivar	Age of embryo DAP	Color of embryos	Diameter of embryos (mm)	No. of embryos inoculated	Source of callus	Fate of scutellum	Plant regeneration	
							No.	%
C401-1	9–12	Semitranslucent	0.7–1.0	27	Scutellum	Forming callus	14	51.9
	13–18	White	1.3–2.2	184	Plumule-radicle	Dead	6	3.2
F2419	11–12	Semitranslucent	0.9–1.0	15	Scutellum	Forming callus	1	6.6
	14–15	White	1.4–1.6	103	Plumule-radicle	Dead	2	1.9

DAP = days after pollination

Embryos obtained 13 days (or more) after pollination usually had a diameter of 1.5 to 2.5 mm. Ten days after the embryos were placed on the callus-inducing medium, the plumule and radicle grew rapidly and varied from 20 to 10 mm in length, whereas growth of the scutellum was inhibited. However, growth of the plumule and radicle was suppressed shortly afterwards, presumably by 2,4-D in the medium, and calli started to form from them. Although calli originating from the plumules and radicles reached 7 to 10 mm after 7 weeks, they rarely formed shoots on the regeneration medium. For example, among 184 embryos from C401-1, 53 had calli originating from the plumules and radicles but only one regenerated plant was noted.

Morphological and peroxidase isozyme variations

Most of the R_0 plants were fertile and normal in appearance. Variations among R_0 plants included albinos, onion-leaf, satellited panicle branches, chimera, slow growth, poor seed set, tall plant, mixoploid, and waxy midrib (Table 3). However, most of the variations were epigenetic and did not reappear in R_1 plants. Mutants for tallness and waxy midrib did reappear, indicating that dominant heritable changes occurred in embryo culture. The appearance of male- and female-sterile plants and dwarfs in the R_1 (but not in R_0) generation suggests that recessive mutations occurred in R_0 plants.

The sterility occurred on the panicles of the main stalks and of the tillers. The sterile plants did not

Fig. 1. Plant regeneration from immature-embryo-derived callus of a sorghum inbred C401-1

Table 3. Types and frequencies of variations occurring in R_0 and R_1 generations from culture of immature sorghum embryos for C-401-1

Type of variation	R_0 plants showing variation	R_1 plant rows showing variation
Albino	7	0
Onion-leaf	2	0
Satellite branch	2	0
Chimera	1	0
Slow growth & poor seed set	11	0
Mixploid	1	0
Tall stature	11	11
Dwarf	0	6
Waxy midrib	9	9
Male & female sterility	0	6
Total	44	32
Total number examined	158 plants	151 rows

produce stainable pollen, and they never set seeds regardless of whether they were bagged or free. The average length and width of 100 anthers were 2.793 mm and 0.787 mm, respectively, and differences between sterile and fertile plants were not statistically significant. The color of anthers on sterile plants was pale yellow instead of bright yellow as in fertile plants.

Table 4. Effect of gradual withdrawal of 2,4-D on calli regenerated in a sorghum inbred line C-401-1

Treatment	No. of calli	No. of plants regenerated	*t*-test
Stepwise withdrawal of 2,4-D	68	50 (73.5%)	
			3.11**
Direct transfer	51	33 (64.7%)	

Difference in ovary size between sterile and fertile plants also was nonsignificant. Nevertheless, ovaries on fertile plants showed a yellow-green color and those on sterile plants had pale yellow color. No differences were noted for stigma size or color.

Eleven fertile R_1 plants were randomly selected from segregating rows, and seven of the 11 corresponding R_2 rows showed sterile plants. Among a total of 153 R_2 plants in the seven rows, there were 117 fertile and 36 sterile plants or a 3 : 1 ratio ($\chi^2 = 0.176$). Thus, the results indicate that the seven normal-appearing R_1 plants were heterozygous for sterility, which was controlled by a recessive mutant gene.

Which of the three dominant height genes mutated is unknown, as is the stability of the mutant gene. Because the dw_3 allele is unstable and tends to revert to DW_3 at a frequency of 1 in 800, additional studies are necessary to determine the identity of the mutant allele and its stability.

Banding patterns of peroxidase isozymes for a C401 control and for four embryo-regenerated plants suggest that minor variations occurred among three of the four randomly selected regenerates (Fig. 2). The significance of the variation in banding patterns is not clear, but peroxidase activity is inversely related to internodal length (Liang et al. 1977; Schertz et al. 1971).

Plant height variation in R_2 and R_3 generations

C-401 is a 1-dwarf type ($Dw_1Dw_2Dw_3dw_4$). When randomly selected, normal-height R_1 plants were selfed, some of the head-rows showed short plants (dwarfs) in the R_2 generation. Thirty normal-height plants in the segregating rows had an average height of 216 cm (ranging from 190 to 237 cm, s.d. = 14.4 cm), whereas 89 dwarf mutants had an average height of 157 cm (ranging from 152 to 178 cm, s.d. = 15.7). The height difference between the normal and the dwarf plants was highly significant (t = 19.1**), indicating that a recessive height mutation had occurred in the process of embryo culture.

All the R_2 plants in the head rows derived from the 6 dwarf R_1 mutants remained dwarf. However, among

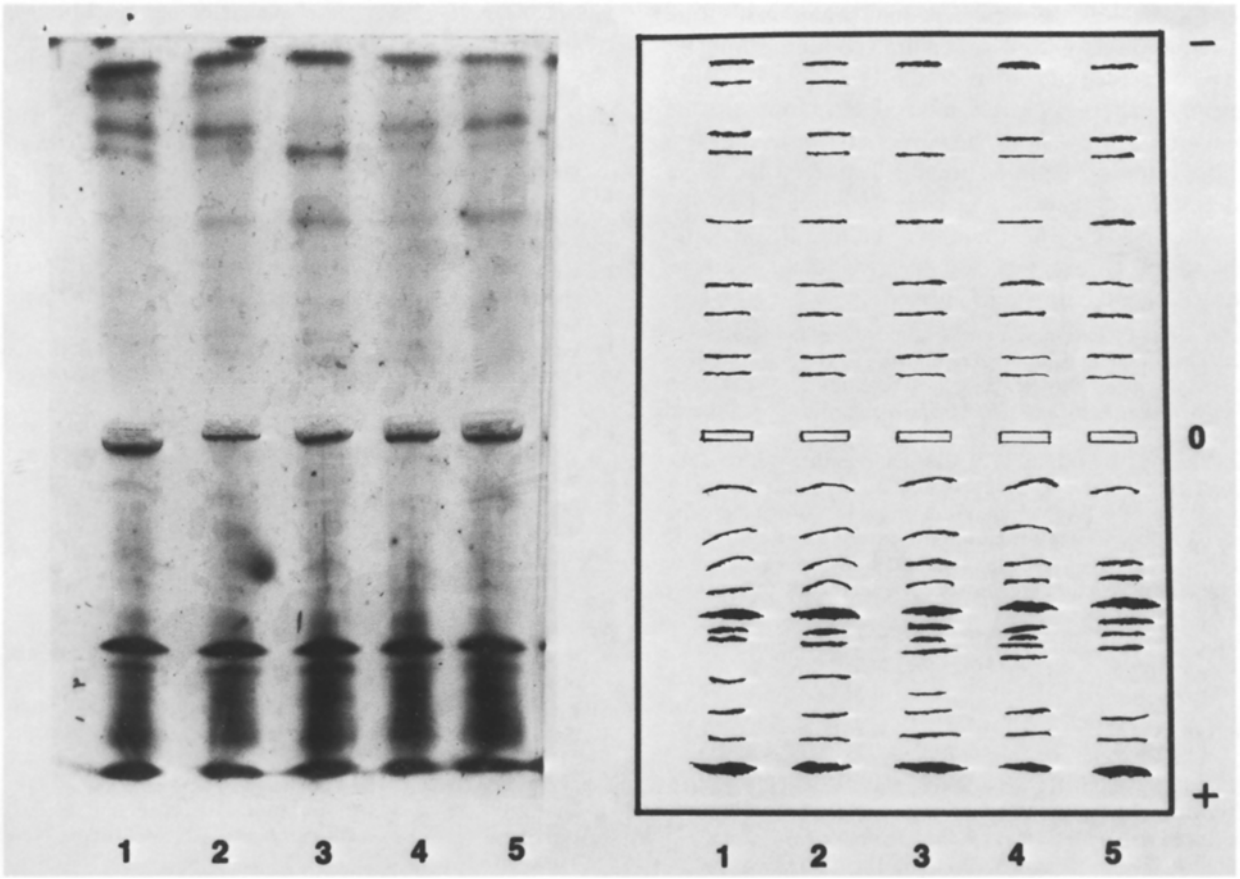

Fig. 2. Peroxidase isozyme patterns for control (1) and four regenerated (2 to 5) plants of a sorghum inbred C401-1

Table 5. Effect of temperature or season on plant regeneration for calli induced from sorghum immature embryos

Genotypes	Age of embryos (DAP)	Months of inoculation	No. of embryos	Plant regeneration	
				No.	%
C401-1	1) 9–18	April, late September, and October	21	20	9.5
	2) 9–12	June 25 to Aug 31	90	2	2.2
F2194	1) 12–15	April 22, September 28 to October 28	118	3	2.5
	2) 11–13	May 6 to Aug 22	34	0	0

DAP = days after pollination

the 11 R_2 rows derived from normal-height plants that occurred in the segregating R_1 rows, four showed segregating in height. Among a total of 88 plants in the four R_2 rows, there were 62 normal and 26 dwarf plants, exhibiting a segregation of 3 : 1 with a chi-square value of 0.96. This confirmed that a recessive height mutant gene had occurred in R_0 plants. The 26 R_2 dwarf plants had an average height of 171.4 cm (s.d. = 13.6), whereas the normal plants in the same rows had an average height of 207.5 cm. However, both height groups had an average of 21 leaves per culm.

Effects of gradual withdrawal of 2,4-D from the medium and temperature on plant regeneration

When calli possessing shoots were transferred in sequence to intermediate media containing 2,4-D at 1 mg l^{-1} and 0.5 mg l^{-1}, respectively, the frequency of

regeneration was 10% higher than when calli were transferred directly from a medium containing 2 mg l^{-1} 2,4-D to a medium free of 2,4-D (Table 4). Thus, stepwise transfers appeared to be effective in increasing regeneration frequency. However, stepwise transfers require extra time and material. Transfer of calli to media with gradually decreasing levels of 2,4-D was also effective in wheat embryo culture (Sears and Deckard 1982), but was not effective in rice embryo culture (Gu and Liang, unpublished).

An effect of temperature was noted when immature embryos were obtained from greenhouse-grown plants during different seasons. The frequency of regeneration was much higher with embryos harvested from plants during April and late September to October than from those harvested from June through August (Table 5). The temperature during June through early September reached 30 to 40 °C inside the greenhouses. The effect of temperature may be associated with levels of endogenous hormones in the embryos or with activity of receptor sites of growth regulators. Seasonal variation affecting callus regeneration also occurred in potato (Murashige 1974).

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