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MEDIA EFFECTS ON PHENOTYPE OF CALLUS CULTURES INITIATED FROM PHOTOPERIOD-INSENSITIVE, ELITE INBRED SORGHUM LINES[1]

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ABSTRACT - Sorghum (*Sorghum bicolor* L. Moench) is a crop of worldwide agronomic importance. Routine production of high quality (friable, embryogenic, fast growing) callus cultures is fundamental to tissue culture based genetic study and improvement of sorghum. Genotype by culture medium interactions for sorghum callus growth and morphology have been previously reported. The objective of this study was to identify tissue culture media that would support high quality callus growth across photoperiod insensitive, relatively elite genotypes. Explants from immature inflorescences of 11 sorghum genotypes were cultured on 6 tissue culture media of differing composition. After 3 and 5 months in culture, calli were rated for pigment and mucilage production, friability, and embryogenesis. Media and genotype effects on callus phenotype were highly significant. Most genotypes produced highest quality callus on MS-based media. Significant genotype by media interactions for callus friability were also documented. Two media formulations, SIM2B5 and N6 were identified as superior for the culture of high quality callus initiated from photoperiod insensitive, elite sorghum lines.

KEY WORDS: Callus culture media; Embryogenic callus; Inflorescence culture; Sorghum culture.

INTRODUCTION

Sorghum ranks fifth worldwide among cereal crops in area of production and is a major source of human food and animal feed (DOGGETT, 1988). Although native to Africa, the economic importance of sorghum has become great in temperate climatic zones such as the Central Great Plains of the U.S. because the crop is well adapted to semi-arid growing conditions. To date, genetic improvement of sorghum for agronomic and quality traits has been carried out by traditional plant breeding methods. However, new biotechnological approaches for genetic study and manipulation of plants have been, and are being developed. Many of these systems depend upon the the routine production of high quality (friable, embryogenic, pigmentless, mucilageless, fast growing) callus cultures for their protocols.

Methods currently available, for example, for transformation of cereal monocots such as sorghum rely on tissue culture during the DNA delivery, selection, and/or regeneration stages of the procedure. Fast growing, type II (friable, embryogenic), low pigment and mucilage-producing, regenerable callus, or cell suspensions derived from such callus, have been the tissue of choice in most successful cereal transformation programs. Establishment of a sorghum tissue culture system from which high quality (fast growing, low pigment and mucilage-producing) type II callus cultures could be routinely initiated and maintained is prerequisite to the use of this and other biotechnological procedures.

Tissue culture studies on *Sorghum* species have been conducted, and media and protocols developed for sorghum callus initiation, maintenance, and plantlet regeneration (GAMBORG *et al.*, 1977; BRETTELL *et al.*, 1980; SMITH *et al.*, 1983; CAI *et al.*, 1990; EAPEN and GEORGE, 1990; LUSARDI and LOPOTTO, 1990; ELHAG and BUTLER, 1992). One study documenting the genetic transformation of sorghum has also been reported (CASAS *et al.*, 1993). A variety of recommended culture media formulations resulted from these studies. However, genotype effects, and genotype by media interactions were noted (BRETTELL *et al.*, 1980; *Cai* and BUT-

Abbreviations: MS, Murashige and Skoog; SAS, statistical analysis system; SIM, sorghum initiation medium; SMM, sorghum maintainance medium.

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TABLE 1 - *Components, formulations, and designations of sorghum tissue culture media.*

Medium components	Sorghum culture media formulations								
	SIM1†	SMM1†	SIM2‡	SMM2‡	SIM2 B5‡	SMM2 B5‡	N6§	N6-P	I6¶
Inorganic salts#	MS	MS	MS	MS	MS	MS	N6	N6	MS
Vitamins/organic##	MS	MS	MS	MS	B5	B5	N6	N6	B5*
Amino acids (mg/L)									
Asparagine	150	150	0	0	0	0	0	0	150
Inositol	0	0	100	100	100	100	100	100	0
Proline	0	0	0	0	0	0	2875	0	0
Casamino acids	0	0	0	0	0	0	100	100	0
Hormones (mg/L)									
2,4-D	2.50	2.50	2.50	1.00	2.50	1.00	1.00	1.00	2.00
Kinetin	0	0.50	0.05	0.05	0.05	0.50	0	0	0
Coconut H_2O (v/v)	0	0	0	0	0	0	0	0	10%
Sucrose (g/L)	20	30	20	20	20	20	20	20	30

† SIM1 = Sorghum Induction Medium1; SMM1 = Sorghum Maintenance Medium 1: equivalent to I2 medium and subculture medium, respectively, of CAI and BUTLER (1990).

‡ SIM2 = Sorghum Induction Medium 2; SMM2 = Sorghum Maintenance Medium 2: equivalent to MS-SI medium and MS-SP medium, respectively, of LUSARDI and LUPOTTO (1990).

§ N6 = N6 medium of CHU *et al.* (1975).

¶ I6 = I6 medium of CAI and BUTLER (1990).

MS =inorganic macro- and micronutrients of Murashige and Skoog (1962). N6 = inorganic macro- and micronutrients of CHU *et al.* (1975).

MS = vitamin formulation from MURASHIGE and SKOOG (1962). N6 = vitamin formulation from CHU *et al.* (1975). B5 = vitamin formulation from GAMBORG *et al.* (1968).

B5* = modified B5 vitamins from GAMBORG *et al.* (1977) without the addition of calcium pantothenate.

LER, 1990; LUSARDI and LUPOTTO, 1990; ELHAG and BUTLER, 1992) and most lines tested were nonelite and/or not adapted to temperate climatic growing conditions. Additional research is needed to determine optimum media formulations for elite, photoperiod insensitive sorghum lines to optimize the culture of high quality callus from those genotypes. The following study was conducted to identify from among several culture media formulations the best formulation for supporting high quality type II callus growth across photoperiod insensitive (including elite) genotypes.

MATERIALS AND METHODS

Immature inflorescences of sorghum were used as the explant material to initiate callus cultures. Immature inflorescences were chosen over other explant sources because they are more responsive for callus induction across genotypes (LUSARDI and LUPOTTO, 1990; and CAI, personal communication, 1993). Genotypes (inbred lines) used in this study were: B 35, B KS57, B N122, B Tx623, B Tx630, B Wheatland, Piper Sudangrass, R N71, R N97, R NB9040, and R Tx430. Source plants from which explant materials were collected were grown in replicate under greenhouse and field conditions. Immature inflorescences measuring 1-4 cm were collected from plants at approximately the 9-12 leaf stage of development (genotype dependent). Stem portions were cut from the plants and outer leaves were removed and discarded. The remaining stem portions wrapped in inner leaves were placed in a sterile, laminar flow hood and sprayed with 70% ethanol until runoff. All explant and callus manipulations from this point on were performed aseptically.

Using techniques described by T. Cai (CAI and BUTLER, 1990; and personal communication, 1993), immature inflorescences were dissected out of the stems, cut into 1-3 mm randomly shaped pieces and placed on callus initiation medium (50 ml medium per 100 x 25 mm disposable plastic petri plate). Composition of the culture media tested is given in Table 1. Media were solidified with 2 g/l gellan gum (Phytagel, Sigma Chemical Co.). This gelling agent was chosen after preliminary experiments (data not included) indicated that callus growth and regeneration across genotypes was better on gellan gum-solidified medium than on agar- solidified medium. Explant pieces from each inflorescence were plated on media designated SIM1, SIM2, SIM2B5, N6, N6-P, and I6 (Tab. 1). Approximately five to seven inflorescences were cultured per genotype per culture initiation date. Explants from each genotype were cultured in replicate on three to six different dates. Following explanting, culture plates were sealed with masking tape (3M Brand) and placed in a culture room where they were incubated in the dark at 26 ± 2 °C.

Culture explants were subcultured at 2 to 3-week intervals. After two subcultures, callus growing on initiation media designated SIM1, SIM2 or SIM2B5 was transferred to appropriate maintenance media (SMM1, SMM2, and SMM2B5, respectively, Tab. 1). Relative ratings on callus growth, pigment and mucilage production, friability, and somatic embryogenesis were documented after 3 and 5 months in culture. Rating scales used were:

TABLE 2 - *Analysis of variance for ratings on sorghum callus pigment production, mucilage production, friability, and embryogenesis.*

	Pigment rating			Mucilage rating			Friability rating			Embryogenesis rating		
Source	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F	d.f.	MS	F
Block	1	7.280	17.98***	1	0.758	2.38	1	0.758	4.02*	1	1.704	4.11*
Genotype	10	7.485	18.49***	10	2.930	9.21***	10	1.283	6.81***	10	3.602	8.68***
Medium	5	21.917	54.14***	5	5.267	16.56***	5	5.976	17.98***	5	10.971	26.46***
Genotype x Medium†	50	0.367	0.92	50	0.187	0.45	50	0.332	1.76*	50	0.458	1.20
Error	65	0.419		65	0.419		65	0.188		65	0.381	

† Genotype x Medium mean square pooled with error mean square when Genotype x Medium interaction nonsignificant.
* Significant at $\alpha = 0.05$.
** Significant at $\alpha = 0.01$.
*** Significant at $\alpha = 0.001$.

Pigment/Tannin Production:

1) Callus completely pigmented (black, brown, purple, etc). Culture medium may be heavily darkened by pigment from callus.
2) Callus pigmented, but producing from 5% up to 25% light, nonpigmented sectors.
3) Pigmented callus producing 26-50% light, nonpigmented sectors.
4) 51-75% of callus light and nonpigmented.
5) 76-90% of callus light and nonpigmented.
6) 91-100% of callus light and nonpigmented.

Embryogenesis:

1) Somatic embryos not detectable.
2) Low embryogenicity. 1-15% of callus producing somatic embryos.
3) Medium Low embryogenicity. 16 to 35% of callus producing somatic embryos.
4) Medium embryogenicity. 36-65% of callus producing somatic embryos.
5) Medium High embryogenicity. 66 to 85% of callus producing somatic embryos.
6) High embryogenicity. 86 to 100% of callus producing somatic embryos.

Friability:

1) Nonfriable. Must be subdivided by cutting with scalpel
2) Low friability. Callus composed of large cell aggregates
3) Medium-Low friability. Callus composed of mixture of large and medium sized cell aggregates.
4) Medium friability. Callus composed of mostly medium sized aggregates.
5) Medium High friability. Callus composed of mixture of medium and small sized aggregates.
6) High friability. Callus composed of small aggregates. Easily spread with culturing spatula.

Mucilage Production:

1) Heavy mucilage production. Callus totally covered and mucilage spreading onto culture medium.
2) Medium mucilage production. Callus partially covered, some spread of mucilage onto medium.
3) Medium-Light mucilage production. Callus not covered. Slight spread of mucilage from base of callus onto medium.
4) Light mucilage production. Mucilage not readily visible. Detected between cells aggregates when subculturing. No spread of mucilage onto culture medium.
5) No mucilage produced by callus.

Selection for friability, embryogenic growth, fast growth rate, and low pigment and mucilage production was carried out during each subculture. After four and six months in culture, subportions of calli were placed on R6 medium (CASAS *et al.*, 1993) solidified with 2 g/L gellan gum, placed under long-pass light filters (STASINOPOULOS and HANGARTER, 1990) and maintained at 26 ± 2 °C under a 16 hr photoperiod under 1000 lx florescent, cool white lights to test for regeneration.

Statistical analysis

The experimental design was a randomized complete block with 2 replications (3 and 5 mo. rating dates) over time. The main effects, genotype and medium, were considered random and fixed, respectively. Analyses were done using the PROC ANOVA procedure of SAS (SAS Institute, 1990). A pooled error term was used when genotype x medium interactions were nonsignificant. Where significant differences were detected (probability of F value ≤ 0.05), mean comparisons were made using Duncan's new multiple range test or a Least Significant Difference (LSD) value calculated.

RESULTS AND DISCUSSION

Effects of genotype and medium were highly significant for all traits rated in this study. Results from statistical analysis of ratings for callus friability, pigment production, mucilage production, and embryogenesis are presented in Table 2. Mean trait ratings across genotypes for the different sorghum tissue culture media tested are presented in Table 3.

A general trend was observed in which callus cultures tended to be more embryogenic, produce less pigments and mucilage, and exhibit faster growth rates on MS-based media than on N6-based media. There was variation in the size of this effect, however, depending on callus genotype. For example, R Tx430 and B NB9040 produced little to no pigment or mucilage across media, while B Tx630 and B Tx623 produced medium to high amounts of pigment on most of the media. Most remaining genotypes produced

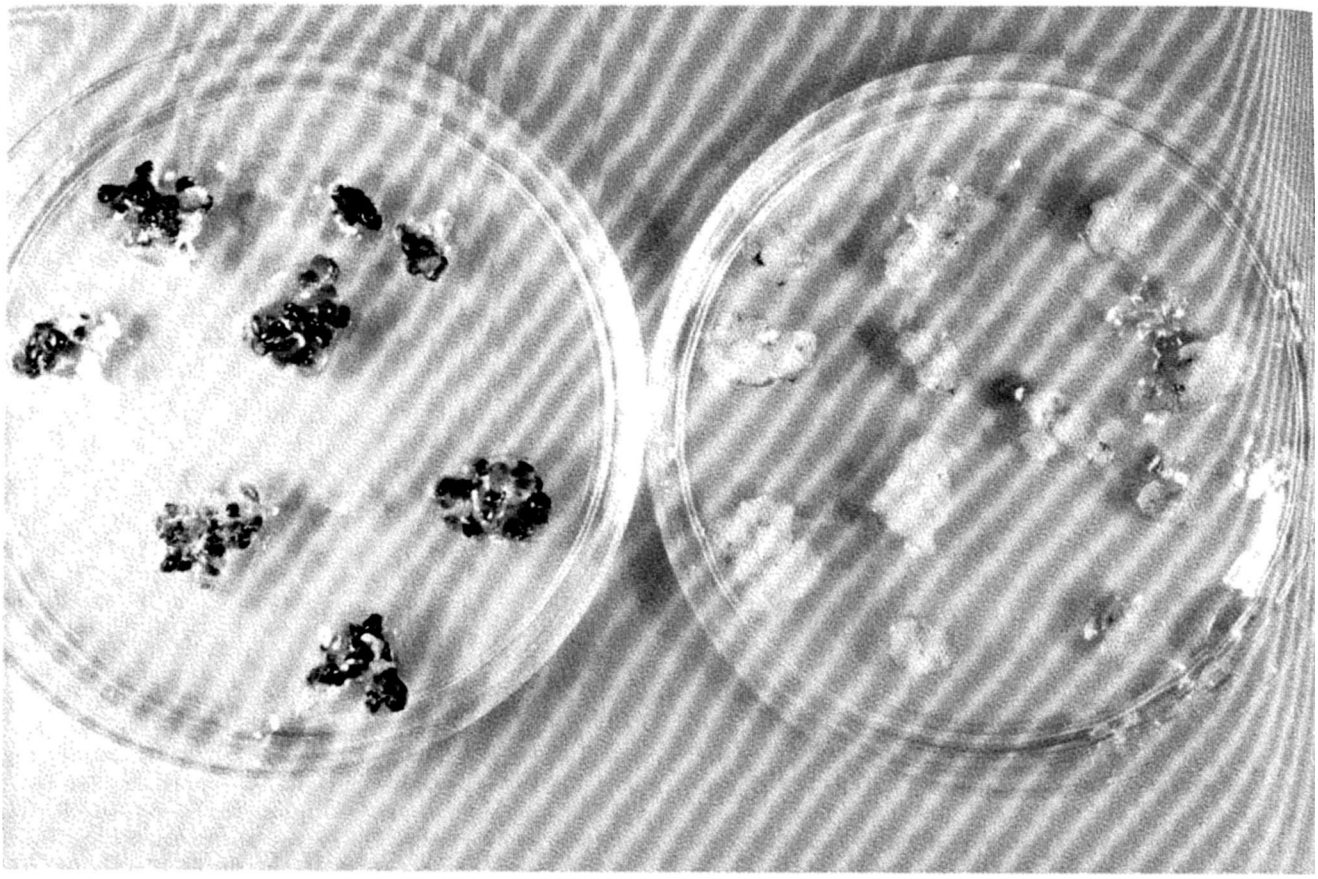

FIGURE 1 - Callus of sorghum inbred line B35 growing on N6 medium (left) and SMM2B5 medium (right).

low amounts of pigment and mucilage on MS-based media and medium to high amounts on N6-based media. Slower, less friable callus growth was observed with increased pigment and mucilage production. This observation agrees with what of ELKONIN *et al.* (1995) who noted a negative correlation between pigment production and formation of friable embryogenic callus in sorghum. Typical phenotype differences observed when calli originating from identical sources were plated on MS-based versus N6-based media are shown in Fig. 1. Callus friability and somatic embryo production were higher for most genotypes on MS-based media (Table 3).

TABLE 3 - *Mean ratings of sorghum tissue culture media for effects on sorghum callus pigment and mucilage production, friability, and embryogenesis.*

Medium/ Media Pair	Mean Rating† Across Genotypes			
	Pigment	Mucilage	Friability	Embryogenesis
SIM2B5/SMM2B	5.14 a	4.18 a	4.68 a	4.64 a
SIM2/SMM2	4.91 a	4.00 a	4.13 b	4.23 b
SIM1/SMM1	3.91 b	3.50 b	3.82 c	3.68 c
N6	3.32 c	3.32 b	3.72 c	3.50 c
I6	3.27 c	3.18 bc	3.36 d	3.09 d
N6 minus proline	2.59 d	2.91 c	3.27 d	2.73 d

† Duncan's Multiple Range test at $\alpha = 0.05$. Means followed by the same letters are not significantly different.

While callus growth and morphology of the majority of genotypes was superior on MS-based media, significant genotype by media interactions were observed for friability (Table 2). Although not statistically significant, embryogenesis also appeared to display genotype by media interactions for a few genotypes. In contrast with other genotypes, friability and embryogenicity for callus of genotypes B Wheatland and Piper Sudangrass were actually highest on N6 medium. Highly embryogenic, very friable and fast growing callus of B Wheatland obtained on N6 medium is shown in Fig. 2. Pigments and mucilage were produced by cultures of B Wheatland and Piper Sudangrass when first initiated on N6 medium and through the next several subcultures. However, se-

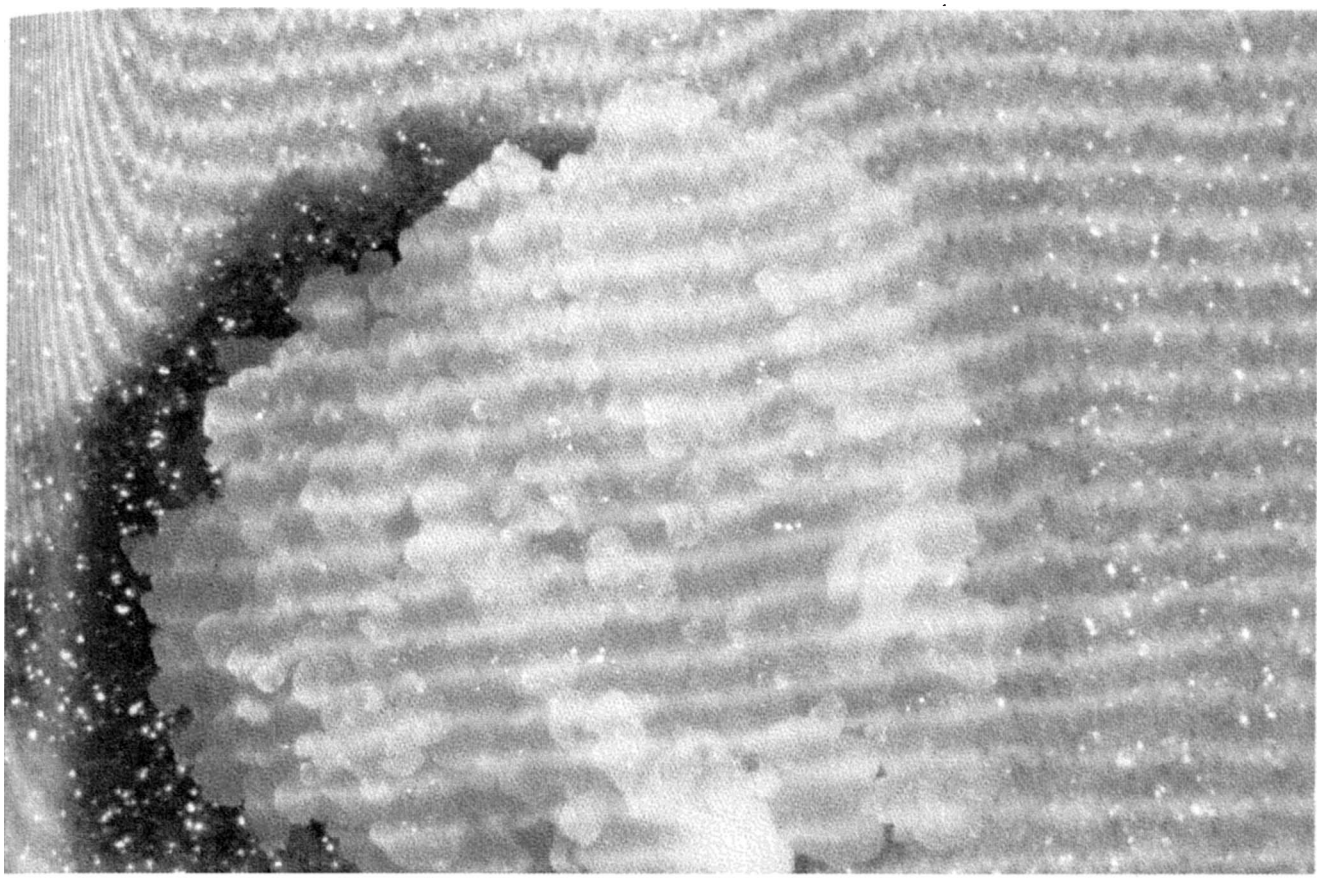

FIGURE 2 - Friable, highly embryogenic callus of B Wheatland cultured on N6 medium.

lection for low pigment-producing, friable, embryogenic sectors resulted in the establishment of relatively fast growing cultures that have remained friable, almost pigmentless, highly embryogenic and regenerable for over 1.5 years in culture. Selection for this type of high quality callus from these genotypes was not successful on other culture media. In contrast, selection for high quality callus of other genotypes was much more successful on the MS-based media. Traits most improved by plating callus of B Wheatland and Piper Sudangrass on N6 medium (versus all other media) were friability and somatic embryo production. Removal of proline from the N6 medium generally resulted in slower, less friable, and more pigmented callus growth across all genotypes. These results concur with findings of ELKONIN *et al.* (1995) that addition of proline and asparagine in high (1-3g/l) concentrations increased sorghum callus friability and decreased pigment production.

Of the MS-based media, the initiation/maintenance combination of media designated as SIM2B5/SMM2B5 produced the best callus growth and morphology across the genotypes tested (Table 3). Calli produced on this media combination tended to be yellowish in color, grow quickly, produce less pigment and mucilage, and produce a higher percentage of embryogenic sectors. Calli produced on MS-based medium I-6 and media combination SIM1/SMM1 grew slower and produced more pigment and mucilage than calli produced on the other MS-based media.

Significant genotypic effects on callus quality were observed in this study (Table 4). Across media, the genotypes producing the highest quality callus were R Tx430, B Wheatland and Piper Sudangrass. The genotype producing the lowest quality callus across media was B Tx630. Examination of morphological characters (such as plant/seed pigmentation, growth habit, maturity class, and amount of tillering) of plants and seeds of the genotypes tested did not reveal any clear associations among the morphological traits and the callus quality traits. The number of genotypes examined in this study was not large, however. A screen of a greater number of sorghum genotypes using selected media might reveal such associations if they exist.

TABLE 4 - *Mean ratings across media for pigment production, mucilage production, friability, and embryogenesis of callus cultures initiated from 11 sorghum genotypes.*

Genotype	Mean Rating Across Media			
	Pigment	Mucilage	Friability	Embryogenesis
B 35	4.2	2.9	4.0	3.7
B KS57	3.5	3.5	3.4	3.1
B N122	3.2	3.2	3.6	3.3
B Tx623	3.2	4.2	3.7	3.3
B Tx630	2.2	2.9	3.8	2.8
B Wheatland	4.6	3.5	4.6	4.5
Piper Sudangrass	4.4	4.2	4.5	4.5
R NB9040	4.0	3.0	3.7	4.0
R N71	3.9	3.5	3.6	3.4
R N97	4.1	3.5	3.8	3.6
R Tx430	5.1	4.2	3.9	3.8
LSD[1]	1.3	1.3	1.2	0.9

[1] Least Significant Difference at $\alpha = 0.05$.

Quantification of regeneration capacities was not originally planned as part of this study. However, as the study progressed, plants were regenerated from some of the genotype x medium combinations for use in other research. Notes on regenerative capacity were taken, but the data were incomplete and unbalanced, and therefore not subjected to statistical analysis. Observations documented that overall mean callus quality rating of a culture was generally predictive of the regenerative capacity of that culture. Cultures receiving highest overall quality ratings, such as B Wheatland and Piper Sudangrass, readily regenerated hundreds of fertile plants, even after 1.5 years in culture. Cultures assigned lowest quality ratings failed to regenerate any plants. Medium to medium-high rated cultures, such as those initiated from R Tx430 and RN97, tended to regenerate fertile plants numbering in the tens relative to the higher rated cultures. Plant regeneration was associated with embryogenic growth and higher for cultures grown on MS-based media.

In summary, 6 tissue culture media formulations were tested for effects on growth, embryogenesis, friability, pigment and mucilage production of callus cultures initiated from 11 photoperiod insensitive, relatively elite, inbred sorghum genotypes. Highly significant media and genotype effects on callus quality traits were observed in these materials. Significant genotype by media interactions were also observed for callus friability. Regeneration capacity of the cultures could generally be predicted based on the overall mean quality rating of the callus. Cultures receiving the highest quality ratings were the most regenerable.

Media combination SIM2B5/SMM2B5 supported the highest quality callus growth across the majority of genotypes tested. Callus growth and morphology of B Wheatland and Piper Sudangrass was superior on N6 medium. Presence of this genotype-by-medium interaction effect suggests that future testing of additional genotypes for production of high quality callus should be conducted using both N6 medium and the SIM2B5/SMM2B5 media combination. While superior media supporting high quality callus growth were identified within the group of genotypes tested, further optimization of the media formulations is desirable because large differences in callus quality were still observed among genotypes cultured on even the "best" media. The ability to obtain high quality callus cultures of any genotype is needed in a crop improvement-oriented program so that genotype choices for tissue culture-based genetic study and improvement are not limited.

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