TISSUE CULTURE POTENTIAL OF DIVERSE DIPLOID AND TETRAPLOID COTTON GENOTYPES

H. F. Sakhanokho, G.C. Sharma and A. Zipf
Department of Plant and Soil Science
Alabama A&M University
Normal, AL
S. Saha
USDA/ARS, Integrated Pest Management Unit
Mississippi State, MS
K. Rajasekaran
USDA/ARS, Southern Regional Research Center
New Orleans, LA

Abstract

Current approaches of cotton improvement include the use of genetic engineering, but progress in this area is limited because of the notoriously recalcitrant tissue culture nature of most elite cotton cultivars. A well-established regeneration system is desirable for cotton improvement through genetic engineering. Our goals were to 1) screen representatives of the four cultivated *Gossypium* species, *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., and *G. herbaceum* L., for regenerability, 2) evaluate callus production on various media used, and 3) characterize calli of the screened genotypes with respect to morphology and other growth parameters. Two initiation media, CIM and MS2NK, and two maintenance media, CMM and L3G, were used. Forty eight lines representing the four cultivated cotton species from diverse geographic areas of the world were screened. In all accessions and media tested, hypocotyl explants were better callus producers than cotyledons. Callus production was higher on CMM than CIM medium, overall. Diploid species produced more callus than the tetraploids on both CIM and CMM, but no significant difference was observed between MS2NK and L3G. Callus morphology varied depending on genotype and hormone levels. Somatic embryos have been obtained for the documented regenerable line Coker 312 cultured either on solid or liquid media. There are no definitive indications of somatic embryos in the remainder of the accessions so far. Monthly selection, transfer, and observations are continuing.

Introduction

Cotton is one of the major economically important crops of the world. In the United States, the retail value of cotton fiber and seed products exceeded 55 billion dollars in 1995 (Anonymous, 1997). Despite market inroads made by synthetic fibers, such as polyester, cotton is still the major textile fiber. Because of this economic importance of cotton and the competition from synthetic fiber industry, several researchers have used the tools offered by genetic engineering to transform and improve cotton for insect or herbicide resistance and to enhance fiber quality. Two major approaches have been used in cotton genetic engineering, transformation through somatic embryogenesis (Firoozabady et al., 1987; Umbeck et al., 1987; Bayley et al., 1992; Cousins et al., 1991; Rajasekaran et al., 1996) and particle bombardment of tissues (Finer and McMullen, 1990; McCabe and Martinell, 1993; John and Keller, 1996). The "gene gun" or meristem particle bombardment method is genotype independent, but it is inefficient because of the low levels of transformed cells. *Agrobacterium*-mediated transformation is a more efficient method, but progress in this area is hampered by the recalcitrant nature of most cotton genotypes to tissue culture. Therefore, the search for more quality cotton lines with good regenerability has taken on added importance. One of the characteristics of cotton in tissue culture is the diverse nature of the callus morphology. This morphology classically included hard green tumorous callus, watery brownish callus, prolifically growing and loose callus with elongated cells, and prolifically growing and loose callus but with smaller cells and very dense cytoplasm. Trolinder and Goodin (1988) reported that callus morphology differed with growth regulator combinations. Embryogenic cotton calli have been described as friable cream-color granular (Firoozabady et al., 1987), mid-friable, creamy granular tissues (Firoozabady and DeBoer,1993), friable, yellowish-green (Rajasekaran et al., 1996). Our objectives were to 1) screen representatives of the four cultivated *Gossypium* species, *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., and *G. herbaceum* L., for regenerability, 2) evaluate callus production on various media used, and 3) characterize calli of the screened genotypes with respect to morphology and other growth parameters.

Materials and Methods

Plant Materials

The plant materials used in this study originated from various geographic regions of the world and were graciously provided by Dr. A.E. Percival, Curator of the National Cotton Germplasm Collection, USDA/ARS, College Station, Texas. Twelve lines from each of the four cultivated species of cotton have been chosen for study.

Seed Sterlization and Germination

Seeds were surface sterilized in consecutive washings of 70% ethanol and 23% commercial bleach, rinsed with and then placed in distilled sterile water overnight. The next day, the seed coats were removed and the seeds placed on a modified MS (Murashige and Skoog, 1962) medium containing 0.49 mg/L nicotinic acid, 0.82 mg/L pyridoxine, 1.35 mg/L thiamine, 20 g/L glucose, 2 g/L Gelrite™, and 0.75 mg/L $MgCl_2$ (pH 6.8). Germination took place in the dark.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:590-593 (1998)
National Cotton Council, Memphis TN

Callus Initiation and Fresh Weight Measurements

Hypocotyl sections from seven to ten day old seedlings were aseptically transferred to Petri dishes containing callus induction medium (CIM), which is made up of MS salts supplemented with 0.4 mg/L thiamine, 2.0 mg/L NAA, 1.0 mg/L kinetin, 100 mg/L myo-inositol, 30 g/L glucose, 2 g/L Gelrite™, and 0.75 mg/L $MgCl_2$ (pH 5.8). This medium is identical to that of Rajasekaran et al. (1996), except that Gelrite™ was used as gelling agent instead of agar. After five weeks on this medium, callus fresh weight was measured before the transfer to callus maintenance medium (CMM), which is the same as the CIM except that sucrose is the carbon source. Calli from some of the screened genotypes were characterized for callus production and callus types. Callus was also induced on MS2NK medium consisting of MS salts supplemented with 2 mg/L NAA, 0.1 mg/L kinetin (Dr. N. Trolinder, personal communication, 1995). Different callus types were subcultured on MS2NK medium and characterized. In addition, 4-5 day old seedlings of sixteen accessions were aseptically transferred to the callus induction medium MS2NK for a four week period. The calli were then transferred to L3G medium for a period of four weeks. L3G medium consists of MS salts supplemented with 0.4 mg/L thiamine, 0.5 mg/L NAA, 0.1 mg/L kinetin, 100 mg/L myo-inositol, 30 g/L glucose, 2 g/L Gelrite™, and 0.75 mg/L $MgCl_2$ (pH 5.8). Callus initiation and maintenance took place at 28 $\pm$ 2^0C under conditions of 16 h light and 8 h dark, with light intensity of 70uE $m^{-2}s^{-1}$ in all cases. Selected calli were also placed into suspension culture using liquid MS2NK according to Trolinder and Goodin (1987), and the suspension was plated on solid MSK media. MSK consists of MS salts, 1.9 g/L KNO_3, 30 g/L glucose, 2 g/L Gelrite™, and 0.75 mg/L $MgCl_2$ (pH 5.8).

Statistical Analysis

Statistical analysis of the data was done using the General Linear Model (GLM) procedure of SAS (SAS Institute, Inc., Cary, NC). A Student's t-test was performed to compare the effects of CIM and CMM and MS2NK and L3G media, respectively, on callus production.

Results and Discussion

Seed Germination

Uniform germination in cotton tissue culture is a desirable goal because this would allow submission of a uniform set of seedlings to a treatment but is often difficult to realize because of the variability in seed coat strength. Poor and non uniform germination rate was a problem at the beginning of the experiment, but soaking the seeds in distilled sterile water overnight and removing the seed coats before transferring to the germination medium dramatically improved the germination rate, especially for the diploid species, which increased from about 50% to over 90%.

Genotypes

Diploid species produced often more callus than the tetraploid species for both CIM and CMM (Table1), but this was not the case for MS2NK and L3G media (Table 5). The *G. herbaceum* accessions tended to produce readily callus on either MS2NK or CIM initiation media. It was common to observe that the entire hypocotyl explants were transformed into callus on these media after 5-6 weeks. This was particularly true for the *G. herbaceum* accessions A_{1-70} and A_{1-77}. Calli of these same accessions tended to be whitish or brownish and watery by the time they were transferred onto maintenance media CMM and L3G.

Effects of Explant Source

Hypocotyl explants were significantly better callus producers than cotyledons for both diploid and tetraploid species. These results are in agreement with those obtained by Smith et al. (1977) for *G. arboreum*. Cutting the hypocotyl sections longitudinally and placing the cut surface on the media greatly increased callus production compared to intact explants. The age of the explants was critical as older (10-15 d) cotyledons generated primarily roots and little callus on CIM medium. Cotyledons of even younger seedlings (4-5 d) produced very little callus on the MS2NK initiation medium; instead, root proliferation was the norm here for all accessions tested, even after 5-6 weeks on MS2NK medium. On the other hand, the cotyledon explants produced callus on CIM medium but not to the same degree as the hypocotyl explants (Table 2).

Media Effects

Callus production was significantly higher on CMM than CIM (Table 3). The only difference between the two media is the carbon source, sucrose and glucose, respectively. This increase may be related to the ease of transport of sucrose in the plant.

Callus Types

Depending on genotype and hormone levels, cotton callus generally falls into a diversity of morphological types, including hard green tumorous callus, watery brownish callus, and prolifically growing and loose callus but with smaller cells and very dense cytoplasm. The same callus may produce one or more callus types and this trend may continue even after selection and subculture (Table 4).

Sequential Callus Production

There was a highly significant difference ($P = 0.01$) among genotypes for both MS2NK and L3G media, but a significant difference was only observed among species for the MS2NK medium (Table 5). These results can be contrasted with those obtained on CIM medium where callus production of the diploid species readily separated from the tetraploids. Overall, callus production did not differ significantly between the two media based on Student's t-test (Table 5) unlike the situation when calli were grown on CIM and CMM media (Table 3).

Somatic Embryogenesis

Somatic embryos have been obtained for hypocotyl explants of Coker 312 cultured either on solid or liquid media. There are no definitive indications of somatic embryos in the remainder of the accessions so far, even though some accessions, such as $A_{2\text{-}38,}$ look promising. Monthly selection, transfer, and observations are continuing.

Summary

The diploid genotypes were better callus producers than tetraploid species on both CIM and CMM media, but this was not the case for MS2NK and L3G. Hypocotyl explants were better than cotyledons for all the accessions tested with respect to callus production. Cotyledons placed on MS2NK media produced primarily roots, so MS2NK was not a good initiation medium for cotyledons. Callus morphology varied according to genotype and was also variable in morphology even after selection, highlighting the importance of continued selection.

Acknowledgements

We would like particularly to thank Dr. A. E. Percival, USDA/ARS, College Station, Texas for his assistance in obtaining the germplasms used in this study. Portions of this work were financially supported by grants from Cotton Incorporated and USAID.

References

Anonymous, 1997. Cotton Up Close. http.//nola.srr.usda.gov/cotton/ cotupcl.html.

Bayley, C., N. Trolinder, C. Ray, M. Morgan, J.E. Quisenberry, and D.W. Ow. 1992. Engineering 2,4-D resistance into cotton. Theor. Appl. Genet. 83:645-649.

Cousins, Y.L., B.R. Lyons, and D.J. Llewellyn. 1991. Transformation of an Australian cotton cultivar: prospects for cotton improvement through genetic engineering. Aust. J. Plant Physiol. 18:481-494.

Finer, J. and M.D. McMullen. 1990. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Reports 8:586-589.

Firoozabady, E.and D.L. DeBoer. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). *In Vitro* Cell Dev. Biol. 299:166-173.

Firoozabady, E., D.L. DeBoer, D.J. Meslo, E.L. Halk, L.N. Amerson, K.E. Rashka, and E.E. Murray. 1987. Transformation of cotton (*Gossypium hirsutum* L) *by Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Molecular Biology 10:105-116.

John, M.E. and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. U.S.A. 93:12768-12773.

McCabe, D.E. and B.J. Martinell. 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. Biotechnology 11:596-598.

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

Rajasekaran, K., J.W. Grula, R.L. Hudspeth, S. Pofelis, and D.M. Anderson. 1996. Herbicide-resistant Acala and Coker transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. Molecular Breeding 2:307-319.

SAS Institute, 1991. SAS systems for linear models. 3rd ed. SAS Institute, Inc., Cary, NC.

Smith, R.H., H.J. Price, and J.B. Thaxton. 1977. Defined conditions for the initiation and growth of cotton callus *in vitro*. I. *Gossypium arboreum*. *In Vitro* 13:329-334.

Trolinder, N. and J.R. Goodin. 1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Reports 6:231-234.

Trolinder, N. and J.R. Goodin. 1988. Somatic embryogenesis in cotton (*Gossypium*). II. Requirements for embryo development and plant regeneration. Plant Cell, Tissue, and Organ Culture 12:43-53.

Umbeck, P., G. Johnson, K. Barton, and W. Swain. 1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Biotechnology 5:263-266.

Table1a. Analysis of variance of callus weight on CIM* medium after a five week period.

Source	Degrees of freedom	Mean squares
Genotypes	31	5.015**
Species	3	21.849**
Explants	1	26.174**
Error	160	

* CIM = Callus induction medium by Rajasekaran et al. (1996).
** Significant at 0.01 probability level.

Table 1b. Mean fresh weight values of calli cultured on CIM medium from the four cultured species of cotton.

Species[1]	Mean (g)	Duncan grouping
G. *herbaceum*	3.28	a*
G. *arboreum*	2.87	a
G. *barbadense*	2.07	b
G. *hirsutum*	1.88	b

[1]Notice the grouping of diploid and tetraploid species.
*Mean followed by the same letter are not significantly different (P = 0.05) according to the Duncan multiple range test.

Table 2a. Analysis of variance for callus weight of nine genotypes initiated from to explants grown on CIM for five weeks.

Source	Degrees of freedom	Mean squares
Genotype	8	5.70***
Species	3	19.28***
Explants	1	31.56***
Error	83	

***Significant at (P = 0.001).

Table 2b. Mean fresh weight values of callus initiated from two different explants.

Explant	Mean (g)	Duncan grouping
Hypocotyl	3.01	a*
Cotyledon	1.84	b

* Means followed by the same letter are not significantly different (P = 0.05) according to the Duncan multiple range test.

Table 3. Fresh weights of diploid cotton calli grown on two different media.

Genotype	Species	CIM (g)	CMM (g)	Difference (g)
Al-50	G. *herbaceum*	5.6	9.8	4.2
Al-50	G. *herbaceum*	1.9	3.8	1.9
Al-50	G. *herbaceum*	5.5	7.8	2.3
Al-50	G. *herbaceum*	1.5	3.4	1.9
A2-57	G.*arboreum*	5.2	9.2	4.0
A2-57	G.*arboreum*	2.3	8.1	5.8
A2-57	G.*arboreum*	3.8	10.4	6.6
A2-57	G.*arboreum*	3.0	6.2	3.2
A2-57	G.*arboreum*	2.2	9.7	7.5
A2-57	G.*arboreum*	2.6	6.0	3.4
A2-57	G.*arboreum*	2.7	6.5	3.8
A2-58	G.*arboreum*	4.6	7.2	2.6
A2-58	G.*arboreum*	4.0	8.5	4.5
A2-58	G.*arboreum*	6.6	10.4	3.8
A2-58	G.*arboreum*	6.4	9.4	3.0
Mean *		**3.9**	**7.9**	
Standard deviation		**1.7**	**2.1**	

*Means were significantly different (P < 0.01) according to Student's t-test.

Table 4. Callus production from hypocotyl explants of diverse *Gossypium* genotypes after selection and subculture on MS2NK media.

Line	**Species**	**Ploidy**	**W_1^2 (g)**	**Callus types[2]**	**W_2^2 (g)**	**Callus types[3]**
C312	*Gossypium*	T[1]	1.06	Yellow green	0.98	Yellow green
			2.22	Green	1.08	Yellow brown
			1.80	Brown	0.95	Brown
Total			**5.08**		**3.01**	
Satara	*Gossypium hirsutum*	T	2.64	Green	0.44	Yellow green
448	*Gossypium hirsutum*	T	4.28	Brown	0.32	Yellow
			4.32	Yellow	0.35	Yellow
Total			**8.60**		**0.67**	
Giza70	*Gossypium*	T	1.42	Brown	0.13	Brown
			2.40	Yellow green	0.35	Brown
Total			**3.82**		**0.48**	
A1-77	*Gossypium herbaceum*	D[1]	3.06	Brown	0.94	Brown
			1.35	Yellow green	0.71	Yellow
Total			**4.41**		**1.65**	
A2-67	*Gossypium arboreum*	D	5.06		1.06	

[1] T = tetraploid; D = diploid.
[2] Callus production is much reduced after first subculture.
[3] After initial selection, callus types do not always remain true.

Table 5a. Analysis of variance for callus weight of sixteen diploid and tetraploid cotton accessions initiated from hypocotyl explant grown on MS2NK and L3G media for four weeks each.

		Mean squares	
Source	Degrees of freedom	MS2NK	L3G
Genotype	15	4.61**	3.81**
Species	3	2.04*	3.99^{NS}
Error	32		

*, ** Significant at (P = 0.05) and (P = 0.01) probability levels, respectively.
NS = Not significant at the 5% level.

Table 5b. Mean fresh weight values of cotton calli cultured on MS2NK and L3G media.

	[1]Mean fresh weight values (g)	
Genotype	MS2NK	L3G
A1-23	6.4a	4.6abcde
A2-5	6.0a	4.9abcde
GB149	5.8a	6.3a
GB35-B126	5.3ab	3.6cde
Komati	4.0bc	3.8abcde
A1-26	3.9c	4.7abcde
A2-39	3.7c	5.3abcd
Sabie	3.7c	4.7abcde
A1-25	3.5c	4.9abcde
Coker312	3.4c	2.9de
Giza70	3.4c	4.0abcde
GB30-V61	3.2c	2.9de
A2-11	2.8c	2.8e
Limpopo	2.7c	3.7bcde
A1-70	2.6c	6.2ab
A2-38	2.5c	5.9abc
Mean[2]	**3.9**	**4.5**
Standard deviation	**1.4**	**1.5**

[1]Means followed by the same letter within the same medium type are not significantly different (P = 0.05) according the Duncan multiple range test.
[2]There was no difference between the two media means according to the Student's t-test (P = 0.05).