INDUCTION OF HIGHLY EMBRYOGENIC CALLI AND PLANT REGENERATION IN DIPLOID AND TETRAPLOID COTTONS

H. F. Sakhanokho[1], A. Zipf[1] and G. C. Sharma
Alabama A&M University
Normal, AL
Mehmet Karaca and S. Saha
USDA/ARS/CSRL
Mississippi State, MS
K. Rajasekaran
USDA/ARS, Southern Regional Center
New Orleans, LA

Abstract

Successful plant transformation depends on regeneration of plants from transformed cells. The current commercial transgenic cottons were derived from the tetraploid Coker genotypes. This lack of variability in transgenic cotton could potentially contribute to a narrow genetic base. Therefore, there is a need to broaden the number of regenerable cotton lines. Through a combination of technique, media, and timely manipulations, we have developed a method to produce large numbers of somatic embryos (SEs) in two tetraploid as well as in two diploid accessions. Callus was initiated from hypocotyl or cotyledon explants and transferred, within a critical time frame, to a callus proliferation/maintenance medium. Potential embryogenic calli were then identified and transferred to liquid culture for four weeks, strained through a mesh screen to enrich for embryogenic cells and placed on an embryo development/maturation medium. Large numbers of somatic embryos were reproducibly developed from all these lines. Mature SEs, placed on medium with no hormones, germinated and produced plants. Efforts were undertaken to improve the plant regeneration efficiency by a combination of media composition from cultures of different age. In addition, genetic changes due to the culturing process (somaclonal variation) were also investigated in a preliminary study. Several methods have been used to assess the degree of somaclonal variability both at the macro- (chromosome analysis) and micro- (RAPDs, AFLPs, RFLPs) molecular level. Preliminary micro-molecular analysis, using fluorescently-labeled AFLP primer pairs, indicated little somaclonal variation due to the culturing process. This report offers, for the first time, a reliable and reproducible method to obtain regenerable and highly embryogenic lines in diploid *Gossypium* species as well as tetraploid commercial lines that can be utilized for development of efficient genetic transformation systems. In addition, the methodology followed has the potential for improving embryogenicity of other commercial recalcitrant cotton lines.

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Introduction

The most common methods of gene transfer into plants depend on well-established tissue culture systems to regenerate plants from a single transformed cell. But since the first published report of regeneration in *Gossypium hirsutum* (Davidonis and Hamilton, 1983), efforts to regenerate cotton through embryogenesis have not been particularly successful. Most of the successful reports of regeneration involve the Coker lines (Trolinder and Goodin, 1987; Firoozabady and DeBoer, 1993), which are at the basis of the current generation of commercial transgenic cottons obtained by backcrossing a transgenic Coker plant to an elite cultivar. The recalcitrance of cotton to tissue culture has not only slowed the development of transgenic cottons but has also narrowed their genetic base, leading to a lack of variability that could be a potential source of genetic problems. Therefore, there is a need to develop new and more regenerable cotton lines.

One of the characteristics of cotton tissue culture is the manifold nature of the callus morphologies (Firoozabady et al., 1987; Trolinder and Goodin, 1988; Rajasekaran et al., 1996; Sakhanokho et al., 1998). Recognizing the characteristics of good, pre-embryogenic calli and determining the right time for transfer to the appropriate media are integral components of a good cotton regeneration system.

A phenomenon associated with plant tissue culture, somaclonal variation, may arise from pre-existing or induced variation, and, to some, this variation must be heritable through a sexual cycle (Skirvin, et al., 1994). Somaclonal variation is not a desirable event in transgenic plants as it can affect the expression of the introduced gene (Benedict et al., 1992, 1993, 1996; Sachs et al., 1998).

Cytogenetic and molecular approaches can be used to assess the degree of somaclonal variability at the macro- and micro-molecular level, respectively (Fourré et al., 1997; Piola et al., 1999). At the micro-molecular level, RFLP (restriction fragment length polymorhism) RAPD (random amplified polymorphism DNA), and AFLP (amplified fragment length polymorphism) are tools that can be used for somaclonal studies. However, recent difficulties encountered in the reproducibility of RAPD results among scientific laboratories have raised questions about the reliability of this technique. The RFLP technique is labor intensive, relies on radioactive Southern blotting and yields fewer alleles. On the other hand, the AFLP technique combines both RAPD and RFLP methods, allowing the rapid detection of a large number of polymorphic DNA markers, and is reproducible.

Our objectives were to 1) develop a tissue culture protocol that can be used to regenerate plants from diverse cotton accessions and 2) use fluorescently-labeled AFLPs to assess

tissue culture-induced somaclonal variation among various callus types and regenerated plants.

Materials and Methods

Plant Materials and Tissue Culture Protocol

Results presented here are a part of a larger study involving the screening of about 50 cotton accessions (including both diploid and tetraploid species) originating from various geographic regions of the world. These plant materials were graciously provided by Dr. A. E. Percival, Curator of the National Cotton Germplasm Collection, USDA/ARS, College Station, TX.

Steps indicated in Figure 1 were followed to conduct the tissue culture experiment. Seed sterilization and germination, explant dissection, and callus initiation, selection and maintenance followed protocols earlier described (Rajasekeran et al., 1996; Sakhanokho et al., 1998). The liquid medium used as somatic embryo initiation medium (EIML) is a modification of a protocol described by Zhang et al. (1991) using a modified MS medium in which NH^{+}_{4} was removed and the amount of KNO_3 was doubled.

Potential embryogenic calli were identified and transferred to liquid culture for four weeks, strained through various sized mesh screens to enrich for embryogenic cells and placed on various development/maturation media ($EMMS_0$, $EMMS_1$, $EMMS_2$, and $EMMS_3$), which were similar to the suspension medium but containing various NAA and kinetin combinations. $EMMS_0$ (0mg/L NAA+0mg/L kinetin), $EMMS_1$(1mg/L NAA+0.1mg/L kinetin), $EMMS_2$ (0.5mg/L NAA+0.05mg/L kinetin), and $EMMS_3$ (0.1mg/L NAA+0.01mg/L) were tested for their ability to develop and mature somatic embryos. After development, embryos were transferred onto various modified MS germination/rooting media containing different levels of IAA: MS0 (MS+0mg/L IAA), MS1IAA (MS+1mg/L IAA), and MS2IAA (MS+2mg/L IAA). Ungerminated embryos on MS2IAA callused and were put back into liquid for a second cycle.

After two to three weeks on liquid culture, these plant materials generated hundreds of SEs and tulip-shaped embryos when placed on $EMMS_2$ and $EMMS_3$. The question raised was whether the MS2IAA step was indispensable in the regeneration of so many embryos. An experiment is currently underway to elucidate the question.

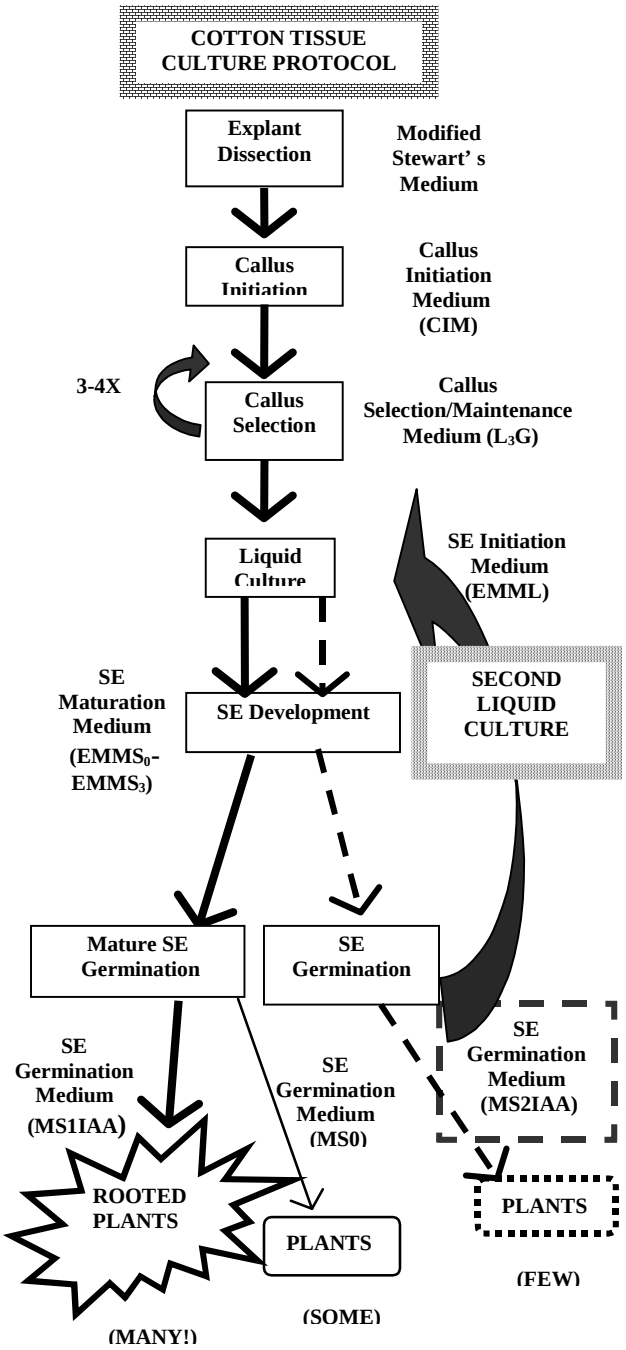

Figure 1. Tissue Culture Protocol.

Somaclonal Variation Study

DNA was extracted from several plant sources, including various embryogenic and non-embryogenic callus types and regenerated plantlets from tissue culture. DNA extraction was performed following the protocol described in a commercial kit. (Dneasy™ Plant Kit, Qiagen, Valencia, CA). Genomic DNAs were digested with *EcoR I* and *Mse I* restriction enzymes, the resulting fragments ligated to *EcoR I* and *Mse*

I adaptors with T4 DNA ligase and pre-selective and selective AFLP amplifications were performed according to the manufacturer's recommendations (AFLP Plant Mapping Kit, PE Applied Biosystems, Foster City, CA). Amplified products were separated by capillary electrophoresis using the ABI 310 Genetic Analyzer (Applied Biosystems/Perkin Elmer) and data analysis was performed using the bundled GeneScan software.

Results and Discussion

The procedure used to produce somatic embryos as well as to generate plants followed the protocol summarized in Figure 1.

Callus Initiation (Figure 2)

A callus initiation medium (Rajasekaran et al., 1996; Sakhanokho et al., 1998) was used for callus induction for both hypocotyl and cotyledon explants. This initiation medium improved callus production from not only hypocotyl but also cotyledon explants, which produced mostly roots when other published callus initiation media were used. Hypocotyl callus production was optimized by longitudinally dissecting hypocotyls, allowing more cut surface to be in contact with the initiation media. This medium easily generated callus in all the 50 or so accessions studied and in both hypocotyl and cotyledon explants. Hypocotyls produced more callus than cotyledons and the diploid species generally gave rise to more callus than the tetraploid species.

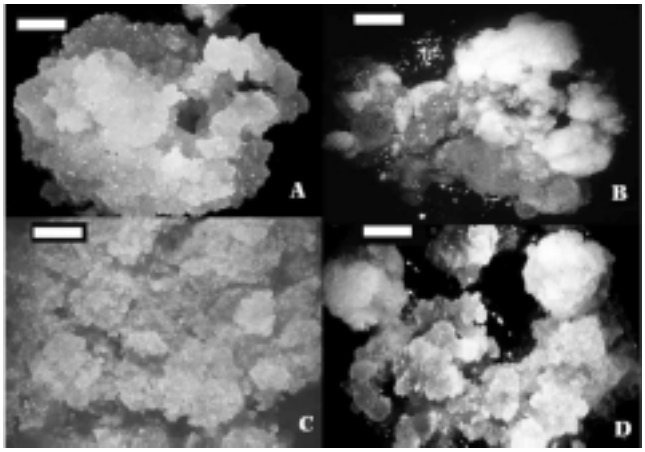

Figure 2. Callus Initiation. **(A)** *Gossypium barbadense* cv. V135. **(B)** *Gossypium hirsutum* cv. MD51. **(C)** *Gossypium arboreum* cv. A_2-9. **(D)** *Gossypium herbaceum* cv. A_1-25. Note the differing callus morphologies. Scale bar is 2mm for (**A**),(**C**) and 10mm for (**B**),(**D**).

Embryogenic Callus Selection and SE Development and Germination

The capability of a plant genotype to produce huge amounts of callus is not indicative of its regenerability capacity (Sakhanokho et al., 1998). Very often, only a small portion of a massive callus, formed from either hypocotyl or cotyledon explants, showed embyrogenic potential. Identifying and selecting potential embryogenic calli is a crucial step for obtaining highly regenerable cultures. Several authors, Shoemaker et al. (1986), Firoozabady et al. (1987), and Rajasekaran et al. (1996), have characterized and described embryogenic cotton calli. An embryogenic callus is generally friable, granular, and yellowish-green (Figure 3). After initiation, the embryogenic callus was selected, proliferated, and transferred three to four times after every two to four weeks on L_3G medium.

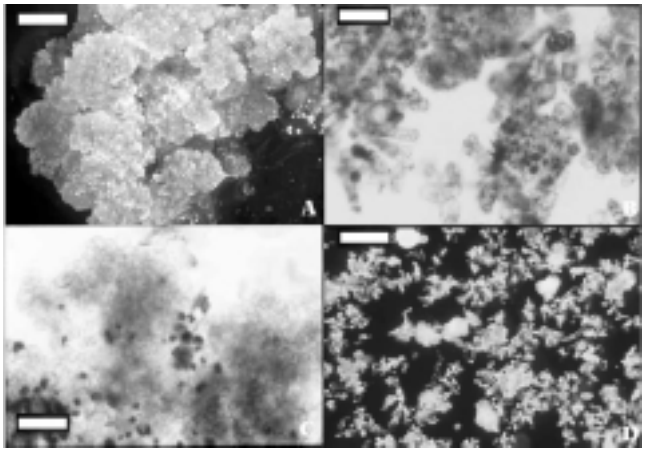

Figure 3. Selection of Embryogenic Callus. **(A)** *Gossypium arboreum* cv. A_2-9. **(B)** *Gossypium hirsutum* cv. DPL 90. Stained with toluidine blue. **(C)** *Gossypium barbadense* cv. GB35B126. Stained with toluidine blue. **(D)** *Gossypium hirsutum* cv. Coker 312. Note the numerous organized = pre-embryogenic clusters of cells. Scale bar is 1mm for (**A**), 100mm for (**B**),(**C**) and 300mm for (**D**).

The embryogenic callus was then transferred to the somatic embryo (SE) initiation medium (liquid medium, EIML). As on solid media, callus morphology and callus color in liquid culture varied drastically; callus colors were yellowish-green, yellow, whitish, and black. The liquid medium consistently initiated and produced more SEs than the solid media used for this purpose. After transfer on SE development media, more embryos developed on $EMMS_2$ and $EMMS_3$ than on $EMMS_0$ and $EMMS_1$. Hundreds of tulip-shaped embryos (Figure 4) per plate developed on both $EMMS_2$ and $EMMS_3$ (Table 1).

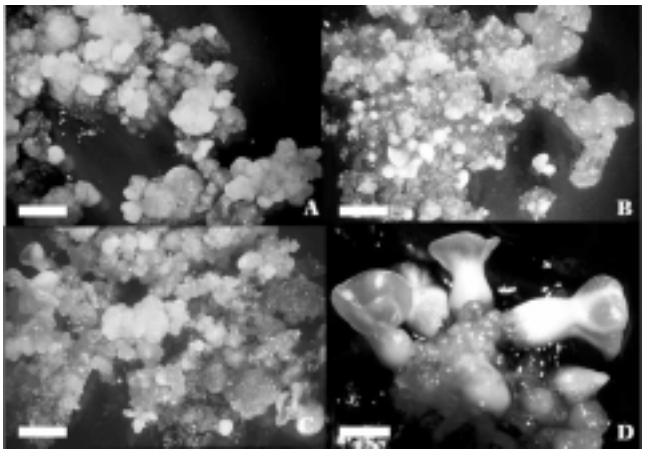

Figure 4. Somatic Embryogenesis. **(A)** *Gossypium arboreum* cv. A_2-9 **(B)** *Gossypium barbadense* cv. GB35B126. **(C)** *Gossypium hirsutum* cv. DPL90 **(D)** Mature somatic embryos of *Gossypium hirsutum* cv. DPL90. Note the large numbers of immature and mature somatic embryos. Scale bar is 7.5mm for (**A**),(**C**), 10mm for (**B**), and 3 mm for (**D**).

Table 1. Average somatic embryo number (per gram of starting material) from GB35B126 (*G. barbadense*) and A_2-9 (*G. arboreum*) cultured on various maturation media.

	Medium			
Genotype	**EMMS$_2$**	**EMMS$_3$**	**EMMS$_4$**	**EMM.2IAA**
GB35B126	120	75	44	99
A_2-9	66	72	21	59

The results summarized in Table 1 are lower than those obtained with Coker 312 (Trolinder and Goodin, 1988), but are close to results reported with Coker 201 (Zhang et al., 1991) and are much higher than the results obtained with another species, *G. klotszchianum (*Price and Smith, 1979). In addition, only the material that did not pass through the mesh screen was taken into consideration; smaller embryos that passed through the filter were not cultured and thus, were not included in this count.

However, not all SEs developed into normal plants with meristems and roots when plated on MS0. To help promote germination and rooting efficiency, SEs were transferred onto MS2IAA, which promoted rooted development and the germination of a few plants, but a large proportion of SEs began to degenerate into callus. This observation led us to decrease the IAA concentration and test MS0 (MS+0mg/L IAA), MS0.01IAA (MS+0.01mg/L IAA), MS1IAA (MS+1mg/L IAA). Preliminary results suggest that MS1IAA has a better germination efficiency. Work is currently underway on improving the regeneration frequency.

Fluorescence-Based AFLP Analysis for Somaclonal Variation

Cultures in a tissue culture environment sometimes undergo genetic changes referred to as somaclonal variation. Somaclonal variation is influenced by several factors, including the degree of departure from organized meristematic growth, the genetic constitution of the starting material, the growth regulators in the medium, the tissue source (Karp, 1995) as well as the length of culture time. Somaclonal variation may or may not be desirable depending on the objectives of the researcher, but it became necessary to assess the amount of variation induced by our tissue culture protocol, particularly because of the liquid culture step which has been associated with elevated levels of genetic variation.

The AFLP method requires good quality DNA. Extracted DNA is often contaminated with high levels of polysaccharides, which are often elevated during tissue culture. However, this extraction protocol allowed for quality DNA from various cultured cotton sources, including various callus types (Figure 5).

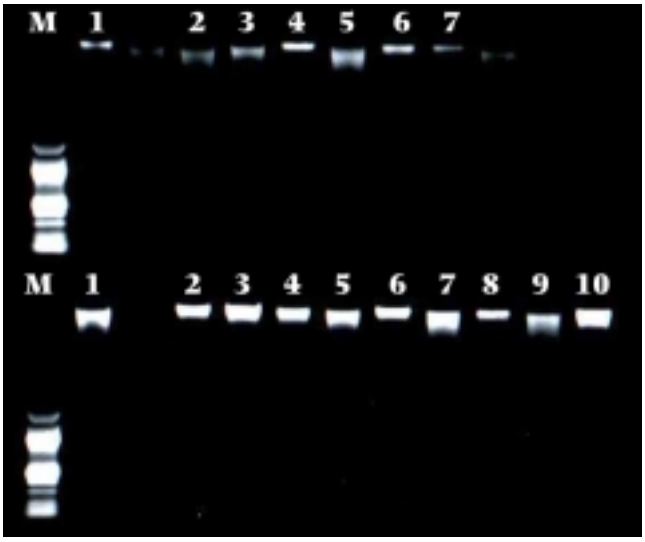

Figure 5. DNA Extraction. DNA was extracted from somatic embryos and regenerated plants using a commercial kit and analysed by electrophoresis through a 1% agarose gel. Note the good quality of the DNA samples.

Preliminary micro-molecular analysis, using fluorescently-labeled AFLP primer pairs, indicated little somaclonal variation due to the culturing process (Table 2). Polymorphisms (Figure 6) were detected in both callus and plantlets of the *G. barbadense* accession GB35B126 at 0.07% and 0.37% variability, respectively. These results are comparable to the results reported by Chen et al. (1999) who reported a 0.3% to 0.7% variability in some tissue-cultured vandaceous orchids. On the other hand, the cultivar DPL90 (*G. hirsutum*) showed no fragment differences (Figure 7).

Table 2. Percentage of polymorphism detected in various cotton tissue culture-derived plant materials using AFLP.

	Genotype			
	GB35B126		DPL90	
% Polymorphism	0.12 (1636)		0 (1324)	
	Tissue Type		Tissue Type	
	Callus	Plantlets	Callus	Plantlets
% Polymorphism	0.07 (1364)	0.37 (272)	0 (604)	0 (720)

The numbers in parentheses represent the total number of peaks.

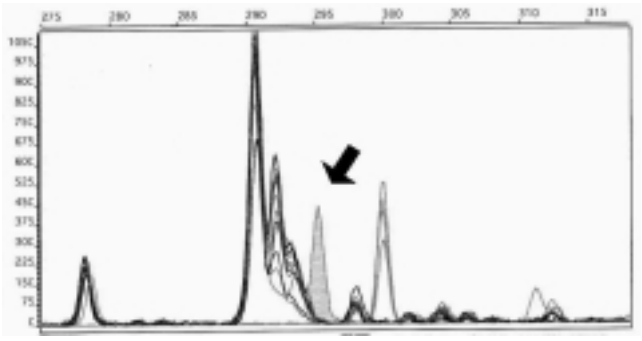

Figure 6. AFLP polymorphism. An electropherogram showing banding profiles of amplified products of DNAs from somatic embryos and plants of *Gossypium barbadense* cv. GB35B126. The X axis represents the DNA fragment size and the Y axis indicates the amount of the amplified products. Each color represented a single sample. The red curves (MW 299) are from the internal size standard. The cross-hatched peak (arrow) represents a unique peak = a polymorphism.

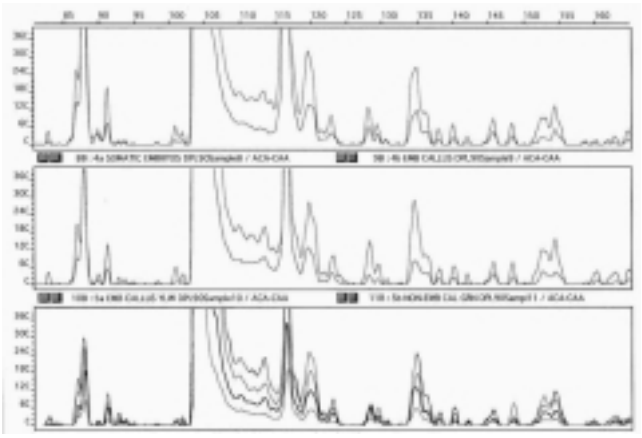

Figure 7. AFLP Analysis. Three electropherograms showing banding profiles of amplified products of DNAs from somatic embryos and plants of *Gossypium hirsutum* cv. DPL90. Each color represented a single individual sample. The X axis represents the DNA fragment size and the Y axis indicates the amount of the amplified products. Note the lack of peak differences within and between curves.

Expanded AFLP screening along with chromosomal analysis of regenerated plants will be necessary before it can be suggested that the protocol developed will be appropriate for the regeneration of genetically stable plants.

Summary

The tissue culture protocol developed has generated large amounts of embryogenic calli and copious numbers of somatic embryos in both tetraploid (*G. hirsutum and G. barbadense*) and diploid accessions. Although the numbers for regenerated plants were low (mostly for the diploid species), the protocol has the potential to be used for the regeneration of other commercial cultivars. A minimal amount of genetic change is associated with the protocol as the somatic embryos and regenerated plants were mostly genetically uniform.

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Disclaimer: Allusion to a product or a company does not constitute in any way an endorsement of that product or company.

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