Tropical Agricultural Research & Extension 9,2006

A histological tool to assess the progress of somatic embryogenesis in cotton (*Gossypium hirsutum* L.)

K. N. Ganesan[1*], K. Thiruvengadam[1], D. Sudhakar[2], T. S. Raveendran[1] and P. Balasubramanian[2]
[1]Department of Cotton, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India
[2]Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India

Accepted 01st July, 2006

ABSTRACT

A study was conducted on local elite cultivars of American cotton (*Gossypium hirsutum* L.) *viz.*, MCU7 and TCH 1569 in order to assess their callusing as well as regeneration potential. Although six different media compositions were tested for the callusing, results revealed that the medium MS salts + 0.4 mg/l thiamine HCl + 2.0mg/lNAA+ 1.0 mg/l kinetin+30 g/l glucose + 0.8% agar exhibited successful and faster induction of calli. After the induction of calli, they were placed on different sub-culture media for inducing embryogenic calli. However, significant differentiation for the formation of embryogenic calli was not observed with the media compositions attempted in the study. As the cotton crop is reported to be recalcitrant for regeneration through somatic embryogenesis, to know the progress of tissue culture or development of embryogenic calli, histological studies were carried out using microtomy. The results of the microtome study clearly revealed the clumping and organisation of large number of cells into a definite structure irrespective of the genotypes and explants. Therefore, further manipulation or alteration of hormonal/vitamin combinations of media may induce friable embryogenic calli resulting in mature somatic embryos, which will give raise to a plantlet. This histological tool experienced as an efficient tool to save the time in tissue culture by knowing the progress of somatic embryogenesis on the other hand is also guiding the scientist to switchover to newer media composition in case of non-embryogenesis.

Keywords:*Gossypium hirsutum* L., callus induction, microtomy, histology, cell aggregation, somatic embryogenesis.

INTRODUCTION

For the past few decades, cotton breeders have developed several superior varieties/ hybrids with excellent yield, fibre quality and pest and disease resistance by exploiting the naturally available variability. The resistance level incorporated through classical

* Corresponding author

plant breeding in crops like cotton is not sufficient to overcome the problem of pests like *Helicoverpa* Sp. In order to combat the severity of present day pest situation in cotton, recent advances in molecular biology and biotechnology needs to be combined with the classical plant breeding. Incorporation of foreign gene through genetic transformation will protect the environment polluted by the indiscriminate use of pesticides. In any transformation programme first and foremost requirement is regeneration of fertile plant from single cell through somatic embryogenesis.

Cotton is a notoriously recalcitrant species for regeneration through somatic embryogenesis. The first breakthrough in cotton biotechnology emerged in the mid-1980s with the regeneration of plants from undifferentiated callus cells via somatic embryogenesis after decades of work. Although the successful regeneration of cotton through somatic embryogenesis (Davidonis and Hamilton, 1983; Rangan *et al.*, 1984; Mitten, 1985; Shoemaker *et al.*, 1986 and Trolinder and Goodin, 1987; 1988a; 1988b) ushered cottons entry into the world of biotechnology, most of these works are limited to highly regenerable cotton genotypes viz., Coker and its derivatives. Scientists engaged in cotton transformation can not follow the regeneration protocol standardized for Coker genotypes universally because most of the earlier workers experienced genotype dependent regeneration for cotton crop. Therefore, the scientists have to standardize the regeneration protocol for their own local elite cotton genotypes. While standardizing the regeneration protocol for elite cultivars of the particular region/ country, the scientists have to try with more number of media compositions and they should spent lot of time and energy.

Under this circumstance, in order to understand the progress of somatic embryogenesis and to decide whether to continue with the particular regeneration medium for a particular genotype or to resort for some other media, the histological studies may be a valuable tool to the scientists. With the above background, the present study was designed and attempted to assess the possible development of somatic embryo in the developing callus of local elite cotton genotypes through histological studies.

MATERIALS AND METHODS

Cotton genotypes

In the present study, an elite cotton variety, MCU7 (a released variety of Cotton Breeding Station, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University (TNAU), Coimbatore, India) and a *Gossypium hirsutum* pre-release culture, TCH1569 (Developed at Cotton Breeding Station, Centre for Plant Breeding and Genetics, TNAU, Coimbatore, India) were used as test genotypes in the transformation laboratory of Centre for Plant Molecular Biology, TNAU, Coimbatore, India.

Explant preparation and callusing

Delinted cotton seeds of MCU7 (an elite cotton variety of India) and TCH1569 (an elite *G. hirsutum* L. culture) were surface sterilized with 70% ethanol for 5 min, followed by 0.1% mercuric chloride for 20 min and washed with sterile distilled water thrice. The sterilized seeds were plated onto a half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for germination under aseptic condition. Hypocotyl and

cotyledon explants were sourced from those seedlings (5-7 day-old) and collected aseptically under laminar air flow chamber. Size of the explant in case of hypocotyl was 2 mm whereas it was 5-mm squares in case of cotyledons. The explants of each genotype were plated on the following MS salts-based compositions in four replications (25 explants / replication / genotype) (Table 1) and subculturing on same medium was done once in two weeks. The observations were recorded in each replication for both genotypes and a percentage of callusing was worked out. The percentage of callusing was converted in to transformed values by the method developed by Finney (1952). Standard error and critical differences were calculated for genotypes, explants, media and their interactions for assessing the statistical significance among them.

After the proliferation of calli, they were transferred onto different sub-culture media (Table 2) with a view to observing the influence of media compositions on differentiation of calli to form embryogenic calli. The media compositions were selected with slight modifications in hormonal, carbon source, gelling agent and vitamin combinations of the published reports.

Table 1. List of media compositions used for the induction of callus

Sl. No.	Media composition
1.	MS salts + 0.4 mg/l thiamine HCl+2.0 mg/l NAA+1.0 mg/l kinetin+30.0 g/l glucose+0.8% agar
2.	MS salts + B5 vitamins (Gamborg *et al.*, 1968) + 0.1 mg/l zeatin + 30 g/l glucosa + 0.8% agar
3.	MS salts + B5 vitamins + 0.2 mg/l zeatin + 30.0 g/l glucosa + 0.8% agar
4.	MS salts + B5 vitamins + 0.3 mg/l zeatin + 30.0 g/l glucosa + 0.8% agar
5.	MS salts + B5 vitamins + 0.3 mg/l zeatin + 100 ml/l coconut water + 30.0 g/l glucose+0.8% agar
6.	MS salts + B5 vitamins + 0.1 mg/l 2,4-D + 0.5 mg/l kinetin + 30.0 g/l glucose + 0.8% agar

Histological studies on calli

With a view to understand the differentiation of calli into embryogenic calli on different media compositions, histological studies were carried out. A piece of calli was taken from each genotype/explant after two to three sub-culturing on regeneration media. Thin sections of the calli specimens were obtained by using the microtomy by following the method developed by Johanson (1940). The sections were observed under microscope and photographed.

RESULTS AND DISCUSSION

Among the media compositions tried for callus induction, a medium containing MS salts + 0.4 mg/l thiamine HCl + 2 mg/l NAA + 1 mg/l kinetin + 30 g/l glucose + 0.8% agar significantly improved the per-

centage of callusing (88.32%) as compared to other media tested (Table 3). On the same medium, MCU7 hypocotyls induced 91.70% callusing while it was 75.76% in MCU7 cotyledons. A high degree of genotype-dependent callus induction in cotton was reported by Davidonis and Hamilton (1983), Shoemaker *et al.* (1986), Umbeck *et al.* (1987), Trolinder and Goodin (1987, 1988a and 1988b) Trolinder and Xixian (1989), Firoozabady and Deboer (1993), Koonce *et al.* (1996), Sakhanakho *et al.* (2000 and 2001), Suresh kumar *et al.* (2002) and Suresh kumar *et al.* (2003). Highly regenerable lines of upland cotton identified by these groups were sister lines of Coker varieties sharing the same ancestry derived from a cross between Deltapine 15 and Coker 100W. The lowest percentage of callus induction of 40.37% was observed in cotyledon explants of TCH1569 on a medium containing MS salts + B5 vitamins + 0.1 mg/l 2,4-D + 0.5 mg/l kinetin + 30 g/l glucose + 0.8% agar besides producing rhizogenic calli.

A medium containing MS salts + B5 vitamins + 0.1 mg/l kinetin + 30 g/l sucrose + 0.8% agar did not support callusing in any of the genotypes or explant types (data not shown). Callusing percentages of media compositions MS salts + B5 vitamins + 0.1 mg/l zeatin + 30 g/l glucose + 0.8% agar, MS salts + B5 vitamins + 0.2 mg/l zeatin + 30 g/l glucose + 0.8% agar and MS salts + B5 vitamins + 0.3 mg/l zeatin + 30 g/l glucose + 0.8% agar were 77.47%, 85.93% and 75.61% respectively and were not significantly different from each other.

Of the two explant types used in callus induction, hypocotyls recorded higher callusing percentage than cotyledons. Such a high callusing in hypocotyls was also reported by Katageri and Khadi (1998), Sakhanokho *et al.* (1998 and 2001), Suresh Kumar *et al.* (2002) and Suresh kumar *et al.* (2003). Present study indicated that glucose appeared to play a major role in callus induction. In contrary, Beasley and Ting (1973), Sandstedt (1975), Smith *et al.* (1977), Rangan (1993), Rangan and Rajasekaran (1996) and Rajasekaran *et al.* (2001) reported the use of glucose prevented browning of explants due to phenolic oxidation. Present study further indicated that the use of sucrose as a carbon source did not favour callus induction.

Sub-culturing of calli derived from different genotypes/explants on different regeneration media did not differentiate to friable calli instead differences were noticed in morphology of callus in respect of growth, colour, friability and survival (Table 2). Katageri and Khadi (1998) and Suresh Kumar *et al.*, (2002) reported recalcitrance to regeneration through somatic embryogenesis in many Indian cotton varieties.

With a view to know the progress of callus differentiation and embryogenic calli formation, microtome sectioning of calli induced from different genotypes/ explants. The sections observed under microscope revealed the aggregation of large number of cells to form cell clumps irrespective of genotypes/explants (Plate 1). The cell clumps were found to be distinct from surrounding cells by forming definite structures of uniform size (Plate 2). Apart from this, cell aggregates observed in both the genotypes also showed different shapes (Plates 3 and 4). A closer view of cell clumps revealed the aggregation of several thousands of cells (Plates 5 & 6). Aggregation of cell is an indication of the development of embryogenic calli subsequently the development of somatic embryo.

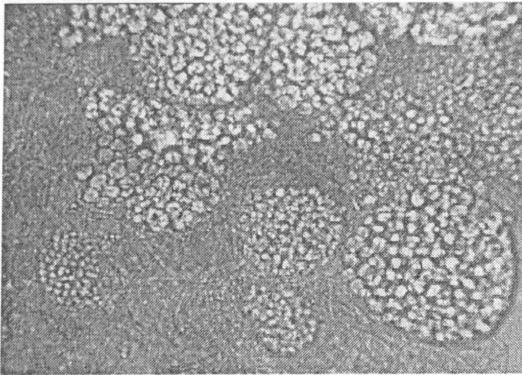

Plate 1. Clustering of cells in MCU7 calli to form cell clumps

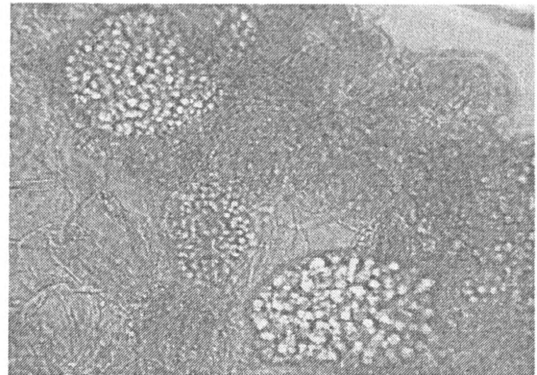

Plate 2. Isolation of clumped cells to form definite structures

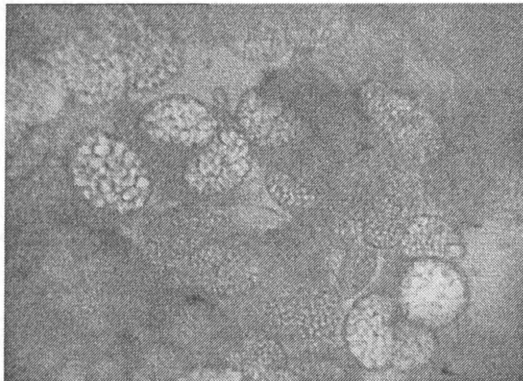

Plate 3. Appearance of different shapes of cell clumps in MCU7 calli

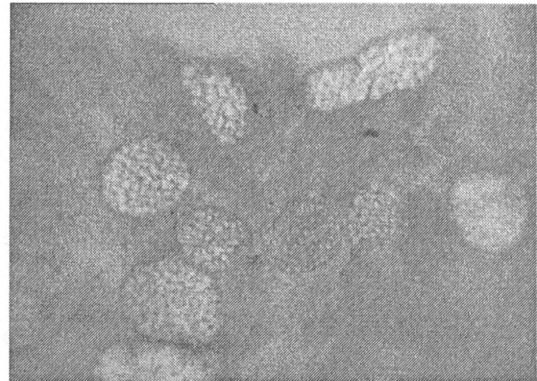

Plate 4. Appearance of cell aggregates in TCH1569 calli

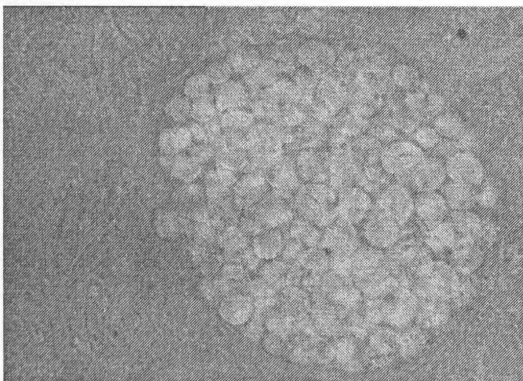

Plate 5. Appearance of a cell aggregate in MCU7 calli

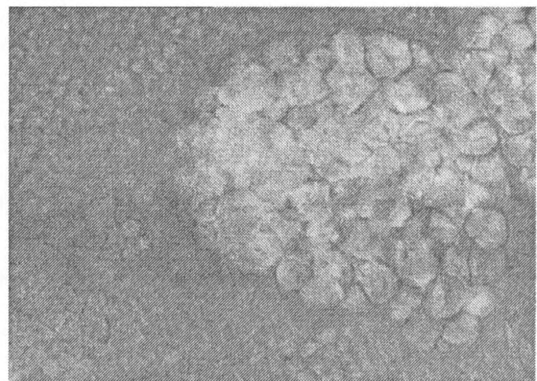

Plate 6. Appearance of a cell clump in TCH1569 calli

Table 2. List of different sub-culture media used and nature of calli

S. No.	Media composition	Remarks
1	MS salts +B5 vitamins+0.1mg/l TDZ +1 mg/l BAP+30 g/l sucrose+0.8% agar	Friable calli after sub-culturing
2	MS salts +B5 vitamins+0.05 mg/l TDZ +1 mg/l BAP + 30 g/l sucrose + 0.8% agar	Friable calli after sub-culturing
3	MS salts +B5 vitamins+0.05 mg/l TDZ +1 mg/l BAP+100 ml/l coconut water+30 g/l sucrose+0.8% agar	Friable calli after sub-culturing
4	MS salts +B5 vitamins+0.01 mg/l TDZ +0.5 mg/l BAP+30 g/l glucose+3 g/l Phytagel	Friable calli after sub-culturing
5	MS salts +B5 vitamins+0.01mg/l TDZ +0.25 mg/l BAP+30 g/l glucose+3 g/l Phytagel	Friable calli after sub-culturing
6	MS salts +B5 vitamins+0.01 mg/l TDZ +1 mg/l BAP+30 g/l glucose+3 g/l Phytagel	Friable calli after sub-culturing
7	MS salts +B5 vitamins+0.2 mg/l zeatin+1 mg/l BAP+0.5 mg/l 2,4-D+30 g/l sucrose+1% agar	Browing and drying of calli 15 d after sub-culturing
8	MS salts +B5 vitamins+0.1 mg/l zeatin+1 g/l charcoal+30 g/l glucose+ 0.8% agar	Dark green calli
9	MS salts +B5 vitamins+2 mg/l zeatin+30 g/l glucose+3g/l Phytagel	Dark green calli
10	MS salts +B5 vitamins+0.4 mg/l thiamine HCl+0.1 mg/l IAA+1 mg/l kinetin +15 g/l sorbitol+15 g/l mannitol+30 g/l glucose+3g/l Phytagel	Drying of calli within week after sub-culturing
11	MS salts +B5 vitamins+0.01 mg/l TDZ+1 mg/l kinetin+200 mg/l sodium thiosulphate+50 g/l KNO3 +30 g/l glucose+3g/l Phytagel	Drying of calli within week after sub-culturing
12	MS salts +B5 vitamins+200 mg/l malt extract+250 mg/l casein hydrolysate +0.1 mg/l TDZ+30 g/l glucose+4g/l Phytagel	No further differentiation/ growth on sub-culturing
13	MS salts +B5 vitamins+0.4 mg/l thiamine HCl+1 mg/l 2 ip+0.1 mg/l NAA +30 g/l glucose+3 g/l Phytagel	No further differentiation/ growth on sub-culturing
14	MS salts +B5 vitamins+0.4 mg/l thiamine HCl+1g/l KNO3 +1 g/l MgCl2 +30 g/l glucose+3 g/l Phytagel	No further differentiation/ growth on sub-culturing
15	MS salts + B5 vitamins+0.4 mg/l thiamine HCl+6.5 mg/l TDZ+1 mg/l AgNO3+30 g/l sucrose+3 g/l Phytagel	No further differentiation/ growth on sub-culturing
16	MS salts + B5 vitamins+0.4 mg/l thiamine HCl+0.1 mg/l NAA+2 mg/l kinetin+30 g/l glucose+3 g/l Phytagel	No further differentiation/ growth on sub-culturing
17	MS salts + MS vitamins+0.4 mg/l thiamine HCl+0.5 mg/l NAA+0.1 mg/l kinetin+0.75 mg/l MgCl2+30 g/l glucose+2 g/l Phytagel	No further differentiation/ growth on sub-culturing
18	MS salts + B5 vitamins+1.9 g/l KNO3+30 g/l glucose+2 g/l Phytagel	No further differentiation/ growth on sub-culturing
19	Basal MS medium	No further differentiation/ growth on sub-culturing

Table 3. Callusing response (%) of explants and genotypes for different media compositions in cotton

Media	MCU7		TCH1569		Mean
	Percentage of callusing				
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
MS salts +0.4 mg/l thiamine HCl+2 mg/l NAA + 1 mg/! kinetin + 30 g/l glucose + 0.8% agar	91.70 (1.16)	75.76 (0.86)	85.64 (1.03)	80.65 (0.94)	88.32[a] (1.09)
MS salts +B5 vitamins+0.1 mg/l zeatin+ 30 g/l glucose + 0.8% agar	89.14 (1.10)	78.52 (0.90)	88.46 (1.09)	73.08 (0.82)	77.47[b] (0.89)
MS salts +B5 vitamins+0.2 mg/l zeatin+ 30 g/l glucose + 0.8% agar	85.25 (1.02)	77.88 (0.89)	89.02 (1.10)	78.63 (0.91)	85.93[b] (1.04)
MS salts +B5 vitamins+0.3 mg/l zeatin+30 g/l glucose+ 0.8% agar	88.67 (1.09)	85.81 (1.03)	84.17 (1.00)	72.82 (0.82)	75.61[b] (0.86)
MS salts +B5 vitamins+0.3 mg/l zeatin+ 100 ml/l coconut water+30 g/l glucose + 0.8% agar	90.83 (1.14)	75.81 (0.86)	84.44 (1.01)	75.21 (0.85)	85.43[c] (1.04)
MS salts +B5 vitamins+0.1 mg/l 2,4-D+ 0.5mg/l kinetin+30 g/l glucose + 0.8% agar	88.66 (1.09)	71.08 (0.79)	72.73 (0.82)	40.37 (0.42)	68.33[d] (0.77)
Mean	89.04 (1.10)	77.48 (0.89)	84.08 (1.01)	70.13 (0.79)	
	S.E.	C.D.(5%)	C.D.(1%)		
Genotypes(G)	0.12 (0.00)	0.32 (0.01)	0.43 (0.01)		
Explants(E)	0.12 (0.00)	0.32 (0.01)	0.43 (0.01)		
Media(M)	0.20 (0.00)	0.56 (0.01)	0.74 (0.01)		
GxE	0.16 (0.00)	ns	ns		
G x M	0.28 (0.01)	0.79 (0.01)	1.05 (0.02)		
G x E x M	0.40 (0.01)	1.12 (0.02)	1.49 (0.03)		

* Values represent means of four replicates
Figures in parenthesis are transformed values

Hence, it is an indication that further minor manipulations and alterations in hormonal/ vitamin combinations of sub-culture media will bring the desirable changes in biochemical as well as physiological path way and thereby mature and friable somatic embryo can be obtained. Application of this simple histological tool will guide the scientists involved in tissue culture and genetic transformation to know the progress of somatic embryogenesis and to proceed tissue culture experiments in the right direction without wasting their valuable time and energy.

REFERENCES

Beasley, C. A. and Ting, I. P. 1973. The effects of plant growth substances on *in vitro* fiber development from fertilized cotton ovules. Amer. J. Bot., 60: 130-139.

Davidonis, G. H. and Hamilton, R. H. 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Sci. Lett., 32: 89-93.

Finney, D. J. 1952. Statistical Methods in Biological Assay. Charles Griffin & Company Ltd., London.

Firoozabady, E. and DeBoer, D. L. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). *In Vitro* Cell. Dev. Biol.,29P: 166-173.

Gamborg, O., Miller, R. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Johanson, D. A. 1940. Plant microtechnique. McGraw Hill, New York.

Katageri, I. S. and Khadi, B. M. 1998. Somatic embryogenesis in cultivated cotton (*Gossypium* Spp.). J. Indian Soc. Cotton Improv., 184-191.

Koonce, L., Dever, J., Bums, T. and Trolinder, N.L. 1996. Progress towards genotype independent transformation. Proc. Beltwide Cotton Prod. Res. Conf., p. 1173.

Mitten, D. H. 1985. Somatic embryogenesis in *Gossypium hirsutum* L. Proc. Beltwide Cotton Prod. Res. Conf., National Cotton Council, Memphis, TN, 57-58.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 80: 662-668.

Rajasekaran, K. 1996. Regeneration of plants from cryopreserved embryogenic cell suspension and callus cultures of cotton (*Gossypium hirsutum* L.). Plant Cell Rep., 15: 859-864.

Rajasekaran, K., Chlan, C. and Cleveland, T. E. 2001. Tissue culture and genetic transformation of cotton. In: Genetic Improvement of Cotton: Emerging Technologies. J.J. Jenkins and S. Saha (eds.), Science Publishers, Enfield, NH. pp. 269-290.

Rangan, T.S. 1993. Regeneration of cotton, USA Patent 5, 244, 802.

Rangan, T. S. and Rajasekaran, K. 1996. Regeneration of cotton plant in suspension culture. USA Patent 5,583,036.

Rangan T. S., Zavala, T. and Ip, A. 1984. Somatic embryogenesis in tissue cultures of *Gossypium hirsutum* L. *In vitro*, 20:256.

Sakhanokho, H. F., Sharma, G. C., Zipf, A., Sana, S. and Rajasekaran, K. 1998. Tissue culture potential of diverse diploid and tetraploid cotton genotypes. Proc. Beltwide Cotton Prod. Res. Conf., Pp. 590-593.

Sakhanokho, H. F., Zipf, A., Sharma, G.

C., Karaca, M., Saha, S. and Rajasekaran, K. 2000. Induction of highly embryogenic calli and plant regeneration in diploid and tetraploid cottons. Proc. Beltwide Cotton Prod. Res. Conf., National Cotton Council, Memphis, TN, pp. 570-575.

Sakhanokho, H. F., Zipf, A., Rajasekaran, K., Saha, S. and Sharma, G. C. 2001. Induction of highly embryogenic calli and plant regeneration in Upland and Pima cottons. Crop Sci., 41: 1235-1240.

Sandstedt, R. 1975. Habituated callus cultures of two cotton species. Pro. Beltwide Cotton prod. Res. Conf., National Cotton Council, Memphis, TN, pp. 52-53.

Shoemaker R. C., Couche, L. J. and Galbraith, D. W. 1986. Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant cell Rep., 3: 178-181.

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

Suresh kumar, M. V., Katageri, I. S., Vamadeviah, H. M., Khadi, B. M. and Soddi, R. R. 2002. Callus induction and regeneration studies in *Gossypium hirsutum* L. cottons. J. Cotton Res. Dev., 16 (1): 8-12.

Suresh kumar, M. V., Katageri, I. S., Vamadeviah, H. M., Khadi, B. M. and Salimath, P. M. 2003. *In vitro* screening for regeneration in cotton (*Gossypium* Spp.). Indian J. Genet., 63 (1): 54-58.

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

Trolinder, N. L. and Goodin, J. R. 1988a. Somatic embryogenesis in cotton (*Gossypium*). I. Effects of source of explant and hormone regime. Plant Cell Tiss. Organ Cult., 12: 31-42.

Trolinder, N. L. and Goodin, J. R. 1988b. Somatic embryogenesis in cotton (*Gossypium*). II. Effects of source of explant and hormone regime. Plant Cell Tiss. Organ Cult., 12: 43-53.

Trolinder, N. L. and Xixian, C. 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep., 8: 133-136.

Umbeck, P., Johnson, G. and Barton, K. 1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/ technology, 5: 235-236.