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Optimal factors for callus proliferation of cotton (*Gossypium hirsutum L.*)

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ABSTRACT : *In vitro* regeneration of cotton (*Gossypium* spp) has been a subject of intense research for the last two decades because of the commercial value of the crop. A study was conducted to assess the callusing potential of two local cotton varieties *viz.*, MCU 5 and SVPR 2 and two exotic genotypes Coker 310 and 312. High significant difference was observed between media composition, genotype and explant types used for callus induction. Among the 24 media composition screened for callusing, the medium CIM3 (MS + 0.1 mg/l 2,4-D + 0.5 mg/l kinetin) exhibited successful and faster induction of calli. Callus initiation was found to be quicker from hypocotyls than cotyledons. Calli developed from hypocotyl explants were large, rough and friable while cotyledon derived calli were friable and medium sized. Higher callus induction frequencies were observed significantly in hypocotyl (97.3%) than cotyledon (95.8%) explants which were collected from 7 day old seedlings irrespective of genotype. Explants collected from younger seedlings (4 day old) and older (14 day old) showed poor and low callus induction frequencies.

Key words : Cotyledon, *Gossypium hirsutum,* hypocotyls, media composition

Genetic improvement of cotton through conventional breeding is limited by several factors including incompatibility barriers and time period for improved variety development. Although plant biotechnology seems to be an attractive way to improve cotton plant, its use requires an effective *in vitro* culture system using somatic tissues of plant. *In vitro* culture allows circumventing these difficulties; *e.g.* callus obtained from explant is an ideal material for genetic transformations. Cotton somatic embryogenesis was first observed in *Gossypium klotzschianum*, but complete plantlets could not be regenerated from somatic embryos. Plant regeneration in cotton through somatic embryogenesis was first reported in two year old calli derived from cotyledons. Since then, numerous reports on somatic embryogenesis and regeneration have been published. With regard to Indian cotton varieties, success was reported so far only in two varieties *viz.*, MCU 5 and SVPR 2 (Ganesan and Jayabalan, 2004). Factors involved in the initiation and maintenance of callus, *Gossypium* species have been investigated by number of laboratories (Rao *et al.*, 2006; Sun *et al.*, 2006). The main factors determining the tissue culture response in cotton and other recalcitrant crops include genotype donor plant type of growth regulators (Sun *et al.*, 2006). An in depth study of such factors would enable the development of genotype specific culture methods to enhance the tissue culture response of the recalcitrant crops. The purpose of this study is to screen different cotton genotypes, media composition, explant age and type of explant (cotyledon, hypocotyls) for cotton callus induction.

MATERIALS AND METHODS

Genotype : Acid delinted cotton seeds of elite cotton varieties, MCU 5, SVPR 2 and two Coker genotypes *viz.*, Coker 310 and 312 in which genetic transformation work was reported earlier were obtained from the germplasm collections of the Department of Cotton, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore.

Surface sterilization: Delinted seeds were surface sterilized with 70 per cent ethanol for 2 min and then washed three times with sterile distilled water. They were again surface sterilized with 0.1 per cent mercuric chloride for 10 min followed by three washes with sterile distilled water.

Seed germination: The surface sterilized seeds were germinated on half strength MS medium supplemented with one per cent (w/v) sucrose and 10 g/l agar. The pH of the medium

was adjusted to 5.7-5.8 (by using 0.1N KOH or 0.1N HCl) prior to autoclaving.

Explant type: Hypocotyl and cotyledonary explants were obtained from (5 to 7 day old) seedlings used for callus induction. Both hypocotyl (4-6 mm) and cotyledonary leaf (16 mm^2) sections were plated onto callus induction medium (Fig.1.). The explants of each genotype were plated on the following MS salts based compositions in three replications (Table 1) and subculture on same medium was done once in two weeks. The observations were recorded in each replication for all the genotypes and percentage of callusing was worked out.

Effect of plant growth regulators on callus induction: Both hypocotyl and cotyledon explants were cultured on MS basal medium supplemented with different concentrations and combinations of plant growth regulators to identify a medium that would produce, proliferate and maintain the callus cultures. The different media compositions and explants tried to test their effect on callus induction frequency were listed in Table 1. After autoclaving the media,

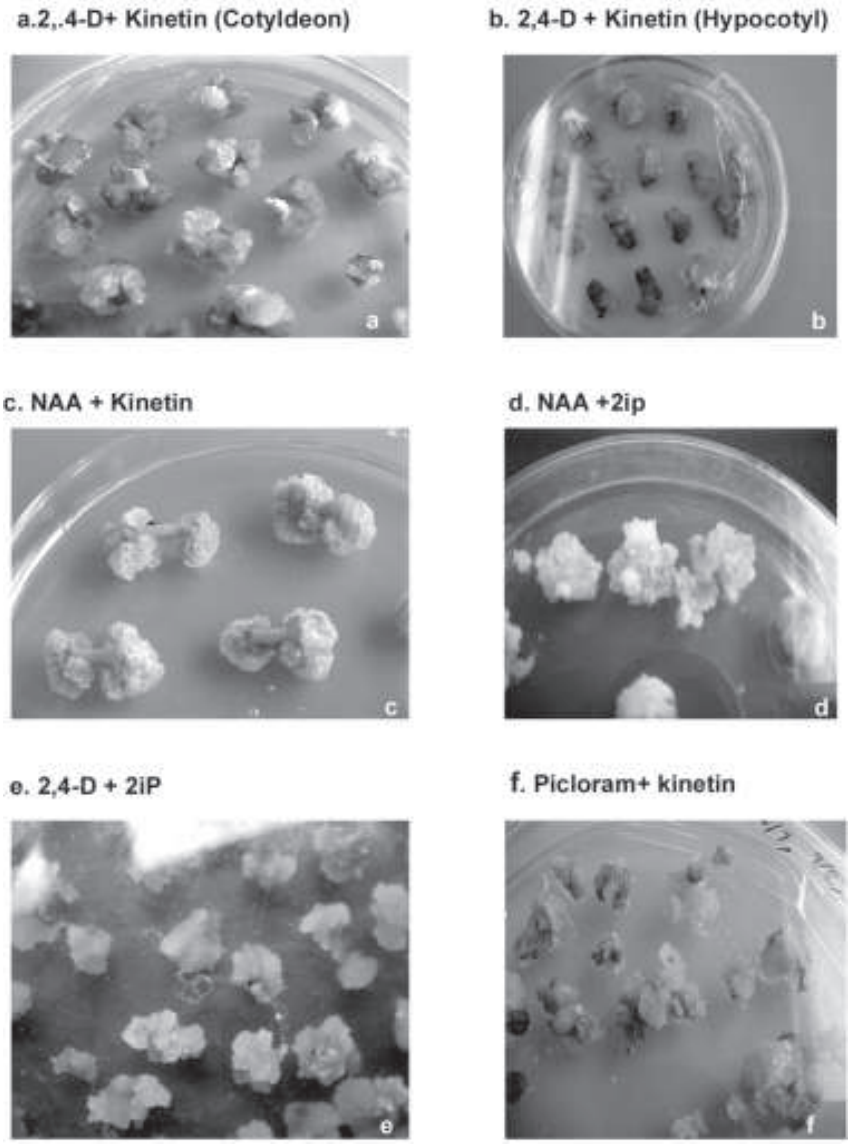

Fig. 1. Effect of different plant growth regulators on callus induction

the filter sterilized plant growth regulators were added onto the media at 42°C. The pH of the media was adjusted to 5.8 prior to autoclaving the media for 15 min at 121°C and 15 psi pressure. Callus initiation was evaluated one month after culture on the callus induction medium. The frequency of callus induction was expressed in percentage on the number of explants cultured and was calculated as given below :

$$\text{Frequency of callus induction (\%)} = \frac{\text{Number of explants produced calli}}{\text{Total number of explants cultured}} \times 100$$

Effect of explant age: To test the effect of age of the explants on callusing response, hypocotyls and cotyledon explants were excised from 5, 7, 10 and 12 day old seedlings. The callus induction frequency of hypocotyls and cotyledons was assessed on CIM3 medium.

All tissue culture media were carried out in a completely randomized block design with three replications. Statistical analysis of the experiments were performed using AGRES-AGDATA software

RESULTS AND DISCUSSION

The frequency of callus induction and plant regeneration influenced by several factors, including composition of the culture medium, explant sources and genotypes. The analysis of variance (Table 2) revealed significant differences in callus induction and callus characteristics due to media compositions, genotypes, and explant types. The interaction effects were also significant.

Callus induction was observed on all media compositions in both explants. Among the various media compositions tested, CIM3 (MS+0.1 mg/l, 2,4-D +0.5 mg/l kinetin) recorded maximum callus induction frequency with hypocotyl and cotyledon explants irrespective of genotypes studied (Table 3). Among the four genotypes, highest callus induction frequency of 97.7 per cent was observed in Coker 310. Callus maturation was achieved on MS basal medium in duration of 2 months. Most of the published works had also reported MS based medium containing 2,4-D and kinetin as the best for callus induction (Kumria *et al.*, 2003; Choudhary *et al.*,

Table 1. Different media compositions used for callus induction from seedling explants

Medium	Media composition*				
	2,4-D mg/l	Kinetin mg/l	NAA mg/l	2-ip mg/l	Picloram mg/l
CIM1	0.1	0.1	-	-	-
CIM2	0.1	0.3	-	-	-
CIM3	0.1	0.5	-	-	-
CIM4	0.5	0.1	-	-	-
CIM5	0.5	0.5	-	-	-
CIM6	0.5	1	-	-	-
CIM7	-	0.1	2	-	-
CIM8	-	0.5	2	-	-
CIM9	-	1	2	-	-
CIM10	-	0.1	1	-	-
CIM11	-	0.5	1	-	-
CIM12	-	1	1	-	-
CIM13	-	0.5	0.1	-	-
CIM14	-	0.5	0.5	-	-
CIM15	-	-	1	0.5	-
CIM16	-	-	0.5	0.1	-
CIM17	0.1	-	-	0.1	-
CIM18	0.1	-	-	0.5	-
CIM19	0.1	-	-	1	-
CIM20	0.5	-	-	0.1	-
CIM21	0.5	-	-	0.5	-
CIM22	0.5	-	-	1	-
CIM23	-	0.3	-	-	0.5
CIM24	-	0.3	-	-	1

* All media were supplemented with MS salts and 30 g/l maltose and solidified with8 g/l agar (CIM: Callus Induction Medium)

2003; Haq and Zafar, 2004; Tohidfar *et al.*, 2005 and Zhao *et al.*, 2006).

In the present study it was observed that 2,4-D was more effective than NAA in producing embryogenic calli. Moreover, low levels of 2,4-D induced callus quickly and readily, whereas NAA required a longer time to produce significant amount of callus. Such high callusing in hypocotyls was also reported by Sakhanokho *et al.*, 2004. Rapid callus development in hypocotyl tissue may shorten the culture duration, thus reducing the occurrence of somaclonal variation, a major problem in cotton tissue culture (Sakhanokho *et al.*, 2004). Among the cotton genotypes studied, Coker 310 exhibited highest callus induction frequency than MCU 5, SVPR 2 and Coker 312. Callus induction frequency was more in hypocotyl explants than cotyledon explants isolated from 7 day old seedlings. Significantly higher callus induction frequencies

Table 2. Analysis of variance for callus induction in cotton genotypes with different explants and media compositions

Source	Degrees of freedom	Mean square	F ratio	P value
Main effects				
A : Medium	23	708.5	185.7**	0.00
B : Genotype	3	2044.2	536.0**	0.00
C : Explant	1	3835.5	1005.7**	0.00
Interaction effects				
AB : Medium x Genotypes	69	25.9	6.81**	0.00
AC : Medium x Explants	23	17.8	4.68**	0.00
BC : Genotypes x Explants	3	12.2	3.20*	0.023
ABC: Medium x Genotypes x Explants	69.0	11.30	2.96**	0.00
Error	384	3.81		

* Significant at 5 per cent level, ** Significant at 1 per cent level

were observed for hypocotyl (97.3%) than cotyledon (95.8%) explants collected from 7 day old seedlings irrespective of genotypes studied (Fig 2 and 3). Explants collected from younger and older seedlings of 4 and 12 days old exhibited only low callus induction frequencies. It was also observed that 7 day old seedlings provided explants (cotyledon and hypocotyls) which were superior in callusing response. Such variable responses in callus induction for different age have been reported in cotton and also in other species (Haq and Zafar, 2004 and Sakhanokho *et al.*, 2004). Such variations can be attributed to the physiological condition of the explant, which is determined by genetic factors. Younger explants exhibit greater morphogenic potential than older explants in view of their higher metabolically active cells. From the study it is clear that the effectiveness of various callus initiation media for each of the cotton genotypes tested suggested that the optimal media combinations and genotypes were dependent on each other. Hormonal and nutritional conditions could also contribute to the differential callusing response.

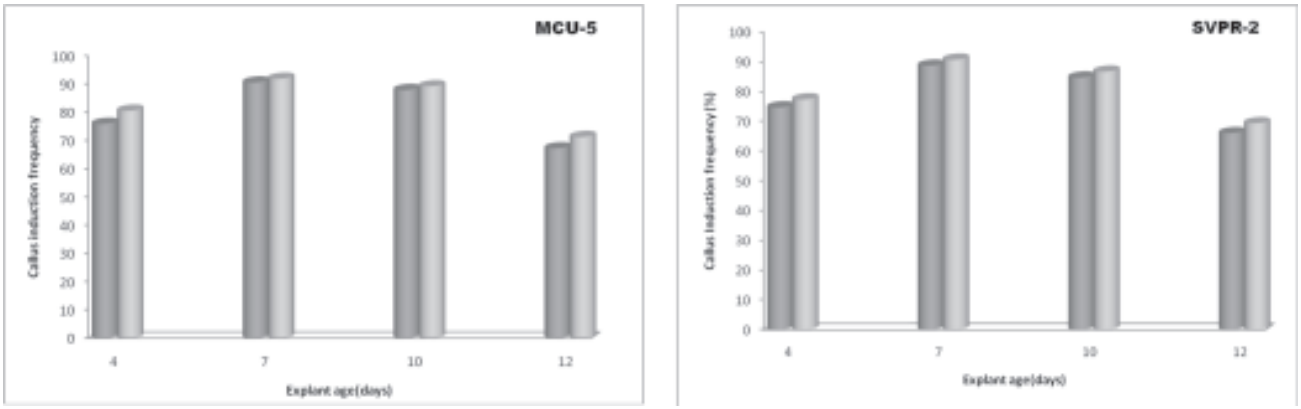

Fig. 2. Effect of age of explants on callus induction frequency on Indian genotype

Table 3. Effects of plant growth regulators on callus induction from cotyledon and hypocotyls explants of cotton varieties

Medium	Frequency of callus induction (%)							
	Coker 310		Coker 312		MCU 5		SVPR 2	
	Cotyledonary leaf	Hypocotyl	Cotyledonary leaf	Hypocotyl	Cotyledonary leaf	Hypocotyl	Cotyledonary leaf	Hypocotyl
CIM1	73.3±0.7^{i}	79.3±0.2^{i}	69.5±0.7^{kl}	75.3±0.2^{kl}	55.2±0.3^{m}	66.6±0.6^{h}	50.4±0.9^{k}	67.3±0.5^{k}
CIM2	75.5±0.2^{hi}	82.0±0.5i-^{k}	74.0±0.7^{i-l}	77.9±0.7^{jk}	65.9±0.3^{i-l}	72.6±0.4^{g}	64.4±0.5^{h-j}	69.2±0.6^{i-k}
CIM3	**95.8±0.3^{a}**	**97.7±0.2^{a}**	**93.3±0.3^{a}**	**96.1±0.2^{a}**	**91.6±0.3^{a}**	**95.5±0.2^{a}**	**90.0±0.5^{a}**	**93.8±0.5^{a}**
CIM4	74.6±0.6^{i}	84.6±0.2 $^{g-j}$	69.3±0.2^{kl}	78.9±0.4^{i-k}	64.0±0.2^{kl}	72.6±0.6^{g}	62.0±0.7^{ij}	68.6±0.5^{jk}
CIM5	73.6±0.7^{i}	82.4±0.2^{i-k}	68.8±0.8^{l}	80.6±0.2^{h-j}	65.1±0.3^{j-l}	76.3±0.6^{fg}	62.9±0.3^{h-j}	73.9±0.4^{hi}
CIM6	74.0±0.7^{i}	80.5±0.5^{jk}	70.3±0.7^{j-l}	72.5±0.3^{l}	62.2±0.5^{l}	66.6±0.4^{h}	60.0±0.5^{j}	64.8±0.8^{k}
CIM7	83.3±0.7^{fg}	91.6±0.3^{c-e}	81.1±0.5^{fg}	90.0±0.5^{de}	67.4±0.3^{h-k}	78.1±0.7^{f}	65.1±0.3^{hi}	73.9±0.4^{hi}
CIM8	83.7±0.3^{fg}	90.6±0.2^{de}	82.2±0.8^{fg}	89.3±0.2^{de}	65.9±0.3^{i-k}	75.7±0.4^{fg}	63.7±0.5^{h-j}	73.9±0.9^{hi}
CIM9	71.8±0.3^{i}	83.0±0.9^{h-k}	70.3±0.9^{j-l}	82.0±0.9^{g-j}	65.1±0.3^{j-l}	73.9±0.6^{fg}	62.9±0.7^{h-j}	72.2±1.6^{hi}
CIM10	75.5±0.5^{i}	86.6±0.2^{f-i}	71.1±0.5^{j-l}	85.3±0.2^{fg}	72.5±0.3^{fg}	78.0±0.7^{f}	70.3±0.7^{f}	76.0±0.8^{h}
CIM11	74.4±0.4^{i}	82.6±0.5^{i-k}	72.2±0.4^{j-l}	82.0±0.4^{g-j}	71.1±0.5^{f-h}	77.3±0.6^{f}	68.8±0.8^{fg}	75.3±0.8^{h}
CIM12	75.0±0.5^{i}	78.4±0.2^{k}	71.2±0.5^{j-l}	85.3±0.2^{fg}	69.6±0.3^{f-h}	76.0±0.4^{fg}	67.4±0.7^{fg}	74.0±0.4^{hi}
CIM13	75.7±0.5^{hi}	85.3±0.2^{g-j}	74.2±0.3^{i-k}	81.3±0.8^{g-j}	73.3±0.9^{ef}	83.3±0.6^{e}	71.21±0.5^{ef}	80.6±0.2^{e-g}
CIM14	76.2±0.3^{hi}	87.3±0.5^{f-h}	74.8±0.5^{h-j}	83.3±0.6^{f-h}	78.0±0.5^{cd}	85.3±0.5^{de}	75.7±0.3^{cd}	82.0±0.4^{de}
CIM15	87.9 ±0.5^{de}	93.90.4^{b-d}	89.3±0.4^{cd}	91.5±0.3^{cd}	82.2±0.5^{b}	84.6±0.2^{de}	80.1±0.3^{b}	82.6±0.2^{cd}
CIM16	90.7±0.2^{d}	94.8±0.2^{bc}	72.7±0.8^{j-l}	82.3±0.6^{g-i}	83.7±0.3^{b}	87.3±0.6^{cd}	81.5±0.2^{b}	85.3±0.2^{b}
CIM17	91.4±0.4^{b-d}	92.80.6^{b-d}	88.6±0.7^{c-e}	91.1±0.4^{cd}	77.2±0.5^{de}	91.3±0.6^{b}	75.0±0.5^{de}	88.6±0.2^{bc}
CIM18	89.6±0.3^{de}	91.5±0.4^{c-e}	86.6±0.5^{de}	90.6±0.5^{cd}	72.7±0.5^{g}	89.3±0.5^{bc}	70.4±0.5^{f}	87.3±0.2^{c-e}
CIM19	86.6±0.6^{ef}	88.7±0.3^{e-g}	85.3±0.2^{ef}	86.8±0.4^{ef}	64.3±0.3^{kl}	86.6±0.2^{c}	62.1±0.3^{ij}	84.6±0.2^{bc}
CIM20	93.6±0.4^{a-c}	95.8±0.3^{ab}	90.0±0.2^{b-d}	94.9±0.2^{ab}	82.9±0.8^{b}	89.3±0.2^{bc}	80.8±0.4^{b}	87.3±0.2^{c-e}
CIM21	94.3±0.5^{ab}	95.3±0.3^{ab}	92.9±0.3^{ab}	94.4±0.3^{ab}	81.5±0.7^{bc}	86.6±0.2^{c-e}	79.4±0.3^{b}	84.6±0.2^{d-f}
CIM22	92.9±0.5^{ab}	94.4±0.5^{bc}	90.7±0.5^{a-c}	93.5±0.3^{bc}	80.8±0.9^{bc}	83.3±0.6^{e}	78.7±0.4^{b-d}	81.3±0.2^{fg}
CIM23	82.2±0.5^{g}	86.6±0.6^{f-i}	79.2±0.5^{gh}	84.6±0.2^{gh}	71.9±0.4^{f}	75.3±0.2^{fg}	68.8±0.8^{fg}	77.3±0.2^{gh}
CIM24	80.0±0.5^{gh}	80.8±0.9^{jk}	77.7±0.9^{g-i}	78.6±0.9^{i-k}	68.9±0.2^{g-j}	76.6±0.6^{fg}	67.4±0.5^{f-h}	76.0±0.7^{fg}

Values represent the mean ± standard error of three replications.
In a column, means followed by same letters are not significant at 5% level by LSD

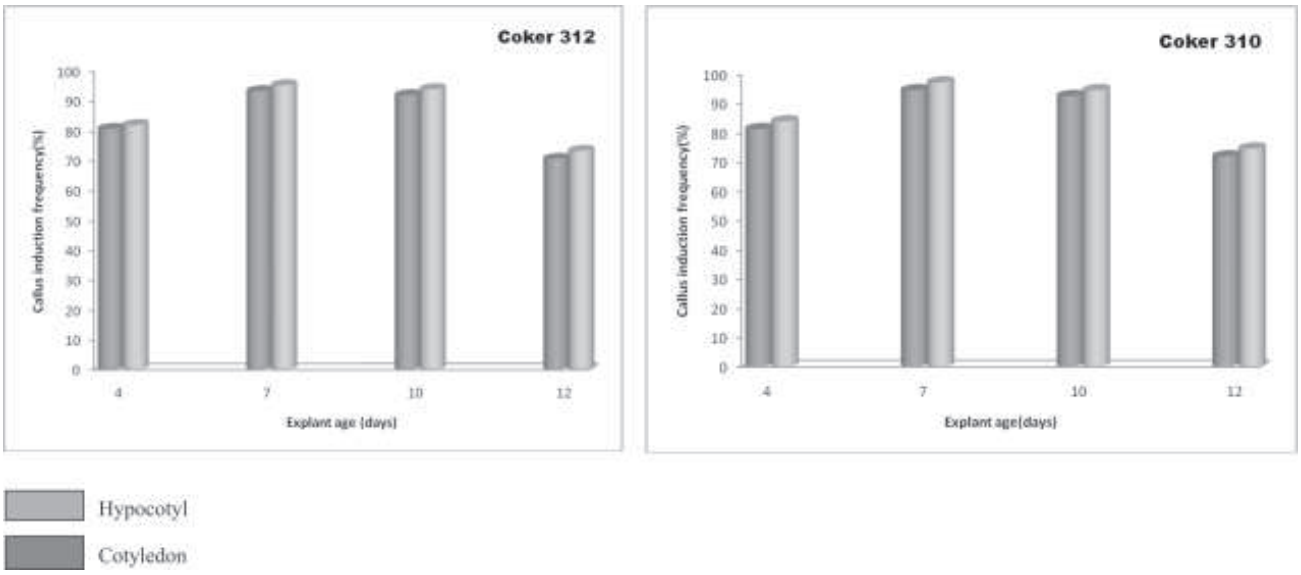

Fig. 3. Effect of age of explants on callus induction frequency on Coker genotype

Callus selection is an important step in cotton tissue culture which played a major role in the successful regeneration of cotton *via* somatic embryogenesis.

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