Regeneration Potential *via* Somatic Embryogenesis in Cotton (*Gossypium hirsutum* L.)

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Abstract

Agrobacterium–mediated transformation and regeneration *via* somatic embryogenesis remains the method of choice for regenerating of transgenic cotton plants. Cotyledon and hypocotyl explants of three cotton genotypes *viz.*, MCU 12, Coker 201 and Coker 310 were cultured on different callus induction media. CIM2 medium containing 0.1 mg/l of 2, 4-D and 0.5 mg/l of kinetin recorded maximum callus induction frequency. Regeneration potential *via* somatic embryogenesis was found to be highly genotype dependent. Coker 310 and Coker 201 were able to produce somatic embryos, whereas MCU 12 did not evoke somatic embryogenesis. SEM8 medium containing an additional supplement of KNO_3 (1.9 g/l) recorded the highest frequency of somatic embryogenesis irrespective of explant types used in Coker genotypes. However, cotyledon was more responsive to somatic embryogenesis than hypocotyls. Between the Coker genotypes, Coker 310 recorded higher frequency of embryogenic calli induction (92.7 %), somatic embryo induction (76.7 %) and somatic embryo maturation (43.0 %). The best regeneration response was observed on PRM3 medium containing 0.1 mg/l of GA3 and 1.0 mg/l of IAA. Coker 310 recorded the highest regeneration frequency of 42.7% with cotyledon explants. The complete protocol for successful regeneration of Coker genotypes through somatic embryogenesis was optimized in this study.

Key worlds: Cotton, Plant regeneration, Somatic embryogenesis, Coker genotypes.

Introduction:

Cotton (*Gossypium* spp.) is a miracle of nature. It is a fiber, feed and food crop. Cotton is the most world's important source of natural fiber and the second largest oilseed crop in production. It is playing a vital role in the economic, political and social affairs of the world (Kumria *et al.*, 2003). It has been estimated to contribute US $ 15-20 billion to the world's agricultural economy with over 180 million people depending on it for their livelihood (Bendict and Altman, 2001). It is growing in more than 80

countries with an annual production of 20 million tones (Li *et al*., 2004). Many factors can be attributing for the low yield and among them biotic and abiotic are the most important. For the past few decades, plant breeders have developed numerous varieties/hybrids with excelling yield, fiber quality and pest and disease resistance by exploiting the naturally available variability, but it appears that a Figureau has almost been reached and routine breeding methods may not be useful to break the impasse due to lack of genetic variation and practical problems like ploidy differences facing the utilization of wild species. Thus, for any kind of genetic improvement led to develop transgenic plants with agronomy traits, an effective regeneration system of the crop is a pre-requisite. Plant regeneration can be achieved either *via* somatic embryogenesis or organogenesis. Regeneration through somatic embryogenesis is preferred over organogenesis because regenerants have a probable single cell origin and the somatic embryos have no vascular connection with the maternal tissue, indicating that they are more amenable to *in vitro* manipulation (Shoemaker *et al*., 1986).The first significant work in cotton somatic embryogenesis was reported by Price and Smith (1979), who successfully induced embryoids from *Gossypium klotzschianum*, although complete plants could not be regenerated. Davidonis and Hamilton (1983) first described plant regeneration from two-year-old callus cultures of *G. hirsutum* L. Then comprehensive studies led to plant regeneration *via* somatic embryogenesis from several cultivars using various explants and growth regulators combinations (Trolinder and Goodin, 1987; Trolinder and Goodin, 1988 a,b; Trolinder and Xhixian, 1989; Firoozabady and DeBoor, 1993). After these initial reports, several cotton workers succeeded in regenerating cotton plants through somatic embryogenesis. *In vitro* regeneration of cotton has been a difficult goal to achieve, because morphogenic response is genotype dependent and most of the elite cultivars are recalcitrant to genetic manipulation. The recalcitrance of cotton to the tissue culture has not only slowed the development of transgenic cotton but has also narrowed their genetic base. Therefore, identification of additional regenerable commercial cultivars of cotton would be highly beneficial to accelerating the development of transgenic cotton plants.

With the above background, the present study was undertaken to develop an efficient regeneration protocol *via* somatic embryogenesis in cotton which can be used to screen cultivated varieties for widening the scope of biotechnological manipulation in cotton improvement.

Materials and methods:

This investigation on somatic embryogenesis and plant regeneration of cotton was carried out in the Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, India according Randomized Completely Blocks Design with three replications. Acid–delinted seeds of three cotton genotypes MCU12, Coker310 and Coker201 were used. The seeds were surface sterilized with 70 % ethanol for 2 min and then washed three times with sterile distilled water. They were again surface sterilized with 0.1 % mercuric acid for 10 min followed by three washes with sterile distilled water. The sterilized seeds were germinated on half strength MS (Murashige and Skoog, 1962) and supplemented with 1% (w/v) sucrose and 0.8 % (w/v) agar. The pH of the medium was adjusted to 5.7 - 5.8 prior to autoclaving.

Explants from 5 to 7 day old seedlings were used for inducing callus. Both hypocotyl (4-6 mm) and cotyledonary leaf (16 mm^2) sections were Figured onto callus induction medium supplemented with different concentrations and combinations of plant growth regulators to identify a medium that would produce, proliferate and maintain the callus (Table 1).

Table 1. The different media compositions tried to test their effect on callus induction

medium	compositions	medium	compositions
CIM1	MS+0.1mg/l 2,4-D+0.1 mg/l Kinetin	CIM13	MS+0.1 mg/l Kinetin+0.5 mg /l NAA
CIM2	MS+0.1mg/l 2,4-D+0.5 mg/l Kinetin	CIM14	MS+0.5 mg/l Kinetin+0.5 mg /l NAA
CIM3	MS+0.1mg/l 2,4-D+ 1 mg/l Kinetin	CIM15	MS+1 mg/l Kinetin+0.5 mg /l NAA
CIM4	MS+0.5mg/l 2,4-D+0.1 mg/l Kinetin	CIM16	MS+0.5 mg/l Kinetin+1 mg /l NAA
CIM5	MS+0.5mg/l 2,4-D+0.5 mg/l Kinetin	CIM17	MS+1 mg/l Kinetin+1 mg /l NAA
CIM6	MS+0.5mg/l 2,4-D+ 1 mg/l Kinetin	CIM18	MS+0.5 mg/l Kinetin+2 mg /l NAA
CIM7	MS+0.1mg/l 2,4-D+0.1 mg/l 2-ip	CIM19	MS+1mg/l Kinetin +2 mg/l Kinetin
CIM8	MS+0.1mg/l 2,4-D+0.5 mg/l 2-ip	CIM20	MS+0.1mg/l Kinetin +0.2 mg/l Picloram
CIM9	MS+0.1mg/l 2,4-D+1 mg/l 2-ip	CIM21	MS+0.5mg/l Kinetin +0.2 mg/l Picloram
CIM10	MS+0.1 mg/l Kinetin+0.1 mg /l NAA	CIM22	MS+1mg/l Kinetin +0.2 mg/l Picloram
CIM11	MS+0.5 mg/l Kinetin+0.1 mg /l NAA	CIM23	MS+0.1mg/l 2,4-D+0.1 mg/l Kinetin+0.1 mg/l NAA
CIM12	MS+1 mg/l Kinetin+0.1 mg /l NAA		

All media were supplemented with MS salts, B5 vitamins and 30 g/l glucose and solidified with 0.8 % agar.

After two months, the calli were separated from the explants and were transferred onto a hormone free MS basal medium to allow them for maturation. The calli were maintained on the same medium for about two months before transferring onto the somatic embryo induction medium. After the maturation of calli on callus maturation medium, they were transferred onto different media (Table2).

Table 2. The different media compositions for the induction of somatic embryogenesis

medium	compositions	medium	compositions
SEM1	MS basal	SEM6	MS+1.65 g/l NH_4NO_3
SEM2	MS +0.1 mg/l Zeatin	SEM7	½ MS +1.65 g/l NH_4NO_3
SEM3	MS +0.1 mg/l Zeatin + 2.0 g/l Activated charcoal	SEM8	MS+1.9 g/l $KN0_3$
SEM4	MS + 0.5 mg/l Zeatin	SEM9	½ MS + 1.9 g/l $KN0_3$
SEM5	MS + 1 mg/l Zeatin	SEM10	MS +0.3 mg/l picloram +0.1 mg/l Kinetin+400mg/lhaemoglobin

All media were supplemented with MS salts and 30 g/l maltose and solidified with 0.4% phytagel.

The observations on embryogenic calli induction and somatic embryo induction were recorded after six and seven months of culture respectively, while the somatic embryo maturation was assessed after eight months of culture. The bipolar torpedo stage and cotyledonary stage somatic embryos (more than 5 mm) with well-developed cotyledons or with just an apical dome and with or without root system were transferred to regeneration medium for shoot elongation and root development (Table 3). When the plantlets attained adequate growth by producing 4-6 leaves with sufficient root system, they were removed from the jam bottles and washed in running tap water carefully to remove the media adhering to roots. They were subsequently transplanted in pots containing sterilized soil. The plants were covered with polyethylene bags and kept in the culture room. After 15 days, the polyethylene bags were removed and well-established plants were transferred to green house.

Table 3. Different media compositions tried to regenerate complete plantlets

Medium	Compositions	Medium	Compositions
PRM1	MS + 0.05 mg/l GA3	PRM3	MS + 0.1 mg/lGA3+1.0 mg/l IAA
PRM2	MS + 0.05 mg/l GA3+0.1 mg/l IAA	PRM4	MS + 0.1 mg/lGA3+1.0 mg/l Kinetin

All media were supplemented with MS salts, B5 vitamins and 30 g/l sucrose and solidified with 0.4% phytagel.

Data were analyzed by analysis of variance and significant difference between treatment means were determined by Duncan's Multiple Range Test (DMRT) using statistical software Statgraphics Centurian version XV (www. statgraphics.com).

Results and discussion:

Highly significant difference was observed for callus induction due to media composition, genotype and explant types used. The interaction effect between media composition and genotypes was highly significant. Similarly, media composition and explant types showed high significant interaction effect. However, the interaction effect between the genotypes and explants was not significant for callus induction (Table 4).

Table 4. Analysis of variance for callus induction in cotton genotypes with different explants and media compositions

Source of Variance	Degrees of freedom	Mean square	F- ratio	P-value
Main effects				
A : Medium	22	845.23	53.12**	0.00
B : Genotype	2	1120.36	70.42**	0.00
C : Explant	1	14274.8	897.19**	0.00
Interaction effects				
AB : Medium x Genotypes	44	61.15	3.84**	0.00
AC : Medium x Explants	22	74.77	4.70**	0.00
BC : Genotypes x Explants	2	10.99	0.69NS	0.502
ABC: Medium x Genotypes x Explants	44	28.73	1.81*	0.002
Error	276	15.91		

***, ** Significant and High Significant at 5 and 1 % levels respectively; NS – Non significant at 5% level.**

Callus induction:

The frequency of callus and plant regeneration influenced by several factors, including composition of the culture medium, explant sources and genotypes (Shengwei and Jingson, 2000). In the present study, callus induction was observed on all media compositions with both explants cultured (Figure 1: A and B). The best callusing response was obtained on CIM2 medium containing 0.1 mg/l of 2, 4-D and 0.5 mg/l kinetin followed by CIM3 (0.1 mg/l of 2,4-D and 1.0 mg/l of kinetin) medium in all three genotypes (Figure 1: C and D). On CIM2, higher callus induction frequency was observed in hypocotyl explants (98.3 %) and it was 93.3 % for cotyledon explants (Table 5).

Table 5. Effects of plant growth regulators and explant types on callus induction of genotypes

Medium	Frequency of callus induction (%)					
	MCU 12		Coker 201		Coker 310	
	Cotyledon leaf	Hypocotyl	Cotyledon leaf	Hypocotyl	Cotyledon leaf	Hypocotyl
CIM1	49.7±0.2^{i}	70.3±0.9^{gh}	55.7±0.3^{j}	76.3±0.4^{j}	63.3±0.2^{jk}	83.7±0.3^{ef}
CIM2	80.7±0.8^{ab}	97.7±0.8^{a}	93.3±0.6^{a}	98.3±0.3^{a}	93.3±0.2^{a}	97.7±0.3^{a}
CIM3	87.3±0.6^{a}	93.3±0.6^{ab}	91.3±0.8^{a}	95.3±0.7^{ab}	92.3±0.7^{ab}	97.0±0.5^{a}
CIM4	59.3±0.9^{gh}	70.0±0.7^{gh}	68.3±0.4^{f-h}	82.7±0.8^{e-i}	74.7±0.8^{e-h}	87.7±0.2^{b-d}
CIM5	59.0±0.5^{h}	78.0±0.5^{e-g}	70.3±0.8^{d-h}	84.0±0.5^{d-g}	73.7±0.8^{f-h}	87.3±0.7^{b-d}
CIM6	59.3±0.2^{gh}	65.0±0.3^{h}	66.3±0.3^{g-i}	76.0±0.7^{j}	74.0±0.9^{f-h}	85.7±0.3^{b-d}
CIM7	75.3±0.8^{bc}	88.7±0.3^{b-d}	80.3±0.9^{bc}	92.3±0.6^{bc}	80.0±0.7^{c-f}	86.3±0.9^{b-d}
CIM8	70.0±0.5^{c-f}	86.7±0.7^{b-d}	74.7±0.4^{c-f}	89.3±0.6^{cd}	77.3±0.8^{c-h}	90.0±0.8^{b}
CIM9	61.3±0.8^{f-h}	83.3±0.4^{c-e}	72.0±0.7^{d-g}	87.3±0.6^{c-f}	72.0±0.5^{gh}	85.7±0.7b-d
CIM10	84.7±0.8^{a}	88.7±0.8^{b-d}	84.7±0.6^{b}	88.0±0.9^{c-e}	84.3±0.4^{bc}	89.0±0.6^{bc}
CIM11	81.3±0.3^{ab}	91.0±0.7^{a-c}	79.3±0.6^{bc}	87.7±0.7^{c-f}	83.7±0.8^{cd}	87.3±0.7^{b-d}
CIM12	72.0±0.5^{c-e}	87.0±0.5^{b-d}	77.3±0.1^{cd}	83.3±0.7^{d-h}	81.3±0.8^{c-e}	84.3±0.3^{c-e}
CIM13	74.3±0.3^{bc}	84.0±0.2^{c-e}	67.3±0.9^{f-h}	85.7±0.3^{d-f}	78.3±0.3^{c-h}	83.7±0.8^{ef}
CIM14	71.7±0.8^{c-e}	81.3±0.4^{d-f}	69.3±0.7^{e-h}	88.7±0.8^{c-e}	72.0±0.9^{g-h}	86.3±0.7^{b-d}
CIM15	68.3±0.7^{c-f}	80.3±0.4^{d-f}	68.0±0.5^{f-h}	86.0±0.9^{d-f}	70.7±0.7^{h-j}	87.7±0.1^{b-d}
CIM16	69.7±0.4^{c-f}	76.7±0.8^{e-g}	67.7±0.6^{f-h}	79.0±0.5^{g-j}	70.0±0.5^{ij}	86.7±0.3^{b-d}
CIM17	68.0±0.5^{c-g}	75.3±0.6^{fg}	65.3±0.4^{g-i}	76.3±0.8^{j}	74.0±0.8^{f-h}	84.3±0.2^{c-e}
CIM18	64.7±0.8^{d-h}	76.7±0.4^{c-g}	65.7±0.4^{g-i}	78.0±0.9^{h-j}	59.7±0.3^{k}	83.3±0.8^{ef}
CIM19	63.67±3^{e-h}	75.0±0.5^{fg}	60.0±0.8^{ij}	78.7±0.7^{g-j}	60.3±0.9^{k}	79.7±0.8^{f}
CIM20	73.7±0.9^{b-d}	84.0±0.6^{c-e}	76.0±0.5^{c-e}	81.7±0.6^{f-j}	74.7±0.5^{e-h}	87.0±0.6^{b-d}
CIM21	69.7±0.8^{c-f}	84.0±0.5^{c-e}	71.0±0.5^{d-h}	81.7±0.6^{f-j}	81.3±0.4^{c-e}	87.3±0.5^{b-d}
CIM22	68.3±0.3^{c-f}	84.3±0.3^{c-e}	64.3±0.7^{hi}	77.0±0.5^{ij}	84.3±0.9^{bc}	87.7 ± 0.4^{b-d}
CIM23	82.3±0.2^{ab}	88.3±0.2^{b-d}	77.3±0.8^{cd}	92.0±0.9^{bc}	79.7±0.8^{c-g}	95.0±0.5^{a}

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

Most of the published works have also reported MS based medium containing 2,4-D (auxin) and kinetin (cytokinin) for callus induction (Kumria *et al*., 2003; Choudhary *et al*., 2003; Haq and Zafar, 2004; Haq, 2005; Tohidfar *et al*., 2005; Zhao *et al*., 2006; Qayyum *et al*., 2006; Koukou *el al*., 2007; Zouzou *et al*.,2008; Rajeswari.,2010 Ge *et al*., 2018 and N'guissan *et al.*, 2020). The auxin 2, 4-D is more effective than NAA in producing a high-quality callus. Moreover, low levels of 2, 4-D induced callus quickly and readily, whereas NAA required a longer time to produce significant callus. Callus selection is an important step in cotton tissue culture considering diverse nature of callus morphologies and characteristics, such as colour, texture, friability and size play a major role in the successful regeneration of cotton *via* somatic embryogenesis. In the present investigation, the callus characteristics differed significantly depending on the media compositions and explants used. Treatment containing 2, 4-D and kinetin produced smooth, friable medium sized callus rapidly and the calli were of yellowish green or light brown to creamy in colour. This type of callus was also reported by Gawel *et al*., (1986), Shoemaker *et al*., (1986), Firooxabady *et al*., (1987) and Kumria *et al*., (2003). On the other hand, hard, compact, non-friable, large and dark green callus produced in treatment containing NAA as auxin and kinetin or 2ip as cytokinin sources. Similar observations were also reported by Nobre *et al*., (2001). Treatment containing 2,4-D and 2ip produced hard, compact and yellowish green callus with varying

degrees of friability, whereas the treatment containing picloram and kinetin produced white or brown callus which were smooth, friable and medium sized. After induction of callus, its maturation appeared to be a prerequisite for regeneration. This meant that any young callus line needed to become mature enough. After two months of culture on callus induction media, the calli were subcultured on MS basal medium lacking plant growth regulators for about two months. During this stage, the calli became smooth and turned black in colour (Figure 1: E and F). These results were in accordance with Finer (1988) and Kumar and Tuli (2004).

Somatic embryogenesis:

The matured calli obtained from different callus induction media were cultured on ten different media compositions to induce embryogenic callus in MCU 12 genotype. However, browning and later drying of the calli were regularly observed on embryogenic induction media. Recalcitrance to regeneration through somatic embryogenesis in Indian cultivars like MCU 7, MCU 11, MCU 5, SVPR2, MCU13, G. Cot.10, BC-68-2 was reported by Kumar and Pental (1998), Kumar *et al.,* (2003), Raeswrai *et al.,* (2010) and Kumar *et al.,* (2013). The effect of different somatic embryo induction media on somatic embryogenesis of Coker genotypes was studied in this investigation (Tables 6 and 7). The results showed that the highest frequency of embryogenic calli induction (92.7%), somatic embryo induction (76.0%) and somatic embryo maturation (43.0%) was obtained on SEM8 medium containing MS salts with extra quantity of KNO_3 (1.9 g/l) in cotyledon of Coker 310 genotype followed by SEM9 medium containing half strength MS salts with extra quantity of KNO_3 (1.9 g/l). These results are in agreement with the findings of Davidonis and Hamilton (1983), Trolinder and Goodin (1988b), Haq and Zafar (2004) and Nandini and Soumitra (2016). Nitrogen has a key role in plant growth and development because it has direct effect on rate of cell growth, differentiation and totipotency.

Table 6. Effect of media compositions on induction and maturation of somatic embryos of Coker 201

Medium	Frequency of embryogenic calli induction (%)		Frequency of somatic embryo induction (%)		Frequency of somatic embryo maturation (%)	
	Cotyledonary leaf	Hypocotyls	Cotyledonary leaf	Hypocotyls	Cotyledonary leaf	Hypocotyls
SEM1	0	0	0	0	0	0
SEM2	0	0	0	0	0	0
SEM3	0	0	0	0	0	0
SEM4	0	0	0	0	0	0
SEM5	0	0	0	0	0	0
SEM6	25.3±0.8c	17.7±0.8c	0	0	0	0
SEM7	16.0±0.8d	11.7±0.3c	0	0	0	0
SEM8	81.0±0.1a	76.3±0.4a	37.7±0.8a	28.3±0.4a	19.0±0.7a	14.0±0.9a
SEM9	68.3±0.7b	61.3±0.3b	26.7±0.9b	18.7±0.4b	14.0±0.2b	8.7±0.1b
SEM10	0	0	0	0	0	0

Values represent the mean ± standard error of three replications. In a column, means followed by same letters are not significant at 5% level by DMRT. Number of explants in each case – 100.

Table 7. Effect of media compositions on induction and maturation of somatic embryos of Coker 310

Medium	Frequency of embryogenic calli induction (%)		Frequency of somatic embryo induction (%)		Frequency of somatic embryo maturation (%)	
	Cotyledonary leaf	Hypocotyls	Cotyledonary leaf	Hypocotyls	Cotyledonary leaf	Hypocotyls
SEM1	0	0	0	0	0	0
SEM2	0	0	0	0	0	0
SEM3	0	0	0	0	0	0
SEM4	0	0	0	0	0	0
SEM5	0	0	0	0	0	0
SEM6	17.0±0.6d	14.3±0.7c	0	0	0	0
SEM7	26.3±0.7c	20.3±0.2c	0	0	0	0
SEM8	92.7±0.8a	88.0±0.4a	76.7±0.3a	65.0±0.5a	43.0±0.1a	26.0±0.5a
SEM9	80.0±0.3b	71.0±0.7b	66.3±0.2b	53.3±0.1b	36.3±0.7b	18.3±0.4b
SEM10	0	0	0	0	0	0

Values represent the mean ± standard error of three replications. In a column, means followed by same letters are not significant at 5% level by DMRT. Number of explants in each case – 100.

The addition of 1.9 g/l of KNO_3 to MS medium appears to have some beneficial effect on embryo development in cotton. Besides increasing the frequency of conversion to embryogenic calli (Figure 1: J and K), doubling the quantity of KNO_3 produces somatic embryos in short time. However, full strength or half strength MS basal medium with 1.65 g/l of NH_4NO_3 did not evoke somatic embryogenesis. Moreover, with the addition of NH_4NO_3, the rate of the cell growth in embryogenic calli was considerably affected, it was observed that cells were almost dying. Out of the two types of explants used in the present study, cotyledon explants were found to be more responsible for the production and maturation of somatic embryos than the hypocotyl explants irrespective of media compositions and genotypes studied. The callus induction was more in hypocotyl explants than cotyledon explants indicating that the callus induction frequency had no direct relation to somatic embryogenesis frequency. Between the genotypes, Coker 310 showed higher embryogenic potential than Coker 201.Variation among genotypes for response to embryogenesis was reported by Trolinder and Xhixian (1989) and Zhang *et al*., (2000)). In the present study, characteristics of embryogenic calli of Coker genotypes were yellowish green, light yellow or creamy, highly friable, vigorous, granular and loose. While non-embryogenic calli could be distinguished by dark green colour, non-friable, hard and slow growing nature. These characteristics were also reported by Rajasekaran *et al*., (1996) and Sakhanokho *et al*., (1998). The non-embryogenic calli were also found to occur along with embryogenic calli (Figure 1: H). It was noticed that the existence of embryogenic calli significantly affected the embryogenic nature by rendering them into non embryogenic calli. Therefore, during the sub-culturing, the non-embryogenic calli were removed from the cultures then and there only embryogenic calli were allowed to proliferate on SEM8 medium. Sakhanokho *et al*., (2001) observed the co-existence of embryogenic calli and non-embryogenic calli in the cotton cultures. The accumulation of small amount of anthocyanin was observed in callus and embryo cultures (Figure 1: I). It may be a good indicator of regeneration because the embryogenic calli under stresses of KNO_3 converted into somatic embryos

with the development of anthocyanin (Mishra *et al*., 2003). Therefore, biochemical screening of local cotton genotypes for anthocyanin content may give some indication on their regeneration ability.

Plantlet regeneration:

Among the different media tried, the best regeneration response was observed on PRM3 (0.1 mg/l of GA_3 and 1.0 mg/l of IAA). Upon transfer, the mature somatic embryos at torpedo and cotyledonary stage were found to initiate root growth within one week and shoot growth within 10 to 12 days. The roots produced were either of a taproot or adventitious roots. The elongation of shoot and root development took place simultaneously resulting in the normal plantlet regeneration (Figure 1: L). Similar results were also observed by Zhang *et al*., (2000) and Kumria *et al*., (2003). The regeneration frequencies of 42.7% and 27.1 % were achieved from cotyledon explants of Coker 310 and Coker 201 respectively. With hypocotyl explants, the regeneration frequencies were 20.3 % and 18.0 % respectively for Coker 310 and Coker 201(Table 8). The regenerated plantlets produced 4-6 leaves and sufficient root system were maintained in the culture room for about 15 days to allow for gradual acclimatization (Figure 1: M and N) before their transfer to the green house for seed production (Figure 1: O).

The regenerated plants showed normal growth, morphology, flowering and boll setting as compared to seed derived cotton plants. With this protocol, the period from callus induction to establishment of plants will be about 10 months. From this study, it can be concluding that the tissue culture protocol of Coker genotypes for regeneration through somatic embryogenesis was standardized. Among the different media compositions tried for callus induction, CIM2 medium was superior in callusing response irrespective of the genotypes studied. The highest callus induction frequency was recorded for hypocotyls in Coker 201. SEM8 medium containing 1.9 g/l KNO_3 was the best for embryogenic calli induction, somatic embryo induction and maturation. The genotype MCU 12 did not produce embryogenic calli when cultured on different embryogenic induction media Coker 310 recorded higher frequency of embryogenic calli induction, somatic embryo induction and somatic embryo maturation. The complete protocol for successful *in vitro* regeneration of Coker genotypes through somatic embryogenesis was optimized and can provides technical support that will facilitate *Agrobacterium*-mediated transformation of Coker genotypes and screening local genotypes for regeneration ability.

Table 8. Regeneration frequency in Coker genotypes

Genotype (s)	Number of explants cultured		Number of plants regenerated		Regeneration frequency (%)		Number of plants survived
	Cotyledon leaf	Hypocotyl	Cotyledon leaf	Hypocotyl	Cotyledon leaf	Hypocotyl	
Coker 201	85	50	23	9	27.1	18.0	24
Coker 310	96	64	41	13	42.7	20.3	38

References

Benedict, J.H.; and D.W. Altman (2001). Commercialization of transgenic cotton expressing insecticidal crystal protein. In: Jenkins, J.N. and Saha, S. Genetic improvement of cotton. USDA-ARS. Oxford & IBH, New Delhi, pp.136-201.

Chaudhary, B.; S. Kumar; K.V.S.K. Prasad; G.S. Oinam; P.K. Burma; and D. Pental (2003). Slow desiccation leads to high – frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310 FR). Plant Cell Rep., 21:955-960.

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

Finer, J.J.; and R.H. Smith (1984). Initiation of callus and somatic embryos from explants of mature cotton (Gossypium Klotzs Chianum Anderss). Plant Cell Rep. 3: 41-43.

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., 29: 166-173.

Firoozabady, E.; D.L. DeBoer; D.J. Merlo; 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 Mol. Biol., 10: 105-116.

Gawel, N.J.; A.P. Rao; and C.D. Robacker (1986). Somatic embryogenesis from leaf and petiole callus cultures of *Gossypium hirsutum*. Plant Cell Rep., 5: 457-459.

Ge, S.; Q. Shen; J. He; Z.F. LU; H. Zheng; F. and Li (2018). A regeneration system for cotton variety Xinluzao 45 *via* somatic embryogenesis. Cotton Science. 30(6):492-497.

Haq, I.; and Y. Zafar (2004). Effect of nitrates on embryo induction efficiency in cotton (*Gossypium hirsutum* L. African Journal of Biotechnology. 3: 319-323.

Haq, J. (2005). Callus proliferation and somatic embryogenesis in cotton (*Gossypium hirsutum* L.). African Journal of Biotechnology. 4(2): 206-209.

Kouakuo, T.H.; T.P. Waffo; Y.J. Kouadio; J. Valls; R. Tristan; A. Decendit; and J.M. Merillon (2007). Phenolic compounds and somatic embryogenesis in cotton (*Gossypium hirsutum* L.). Plant Cell Tissue Org Cult., 90:25-29

Kumar, M.; and R. Tuli (2004). Plant regeneration in cotton: A short-term inositol starvation promotes developmental synchrony in somatic embryogenesis. *In vitro* Cell Dev. Biol., 40: 294-298.

Kumar, S.; and D. Pental (1998). Regeneration of Indian cotton variety MCU-5 through somatic embryogenesis. Curr. Sci., 74: 538-540.

Kumar, M.; H. Singh; A.K. Shukla; P.C. Verma; and P.K. Singh (2013). Induction and establishment of somatic embryogenesis in elite Indian cotton cultivar (*Gossypium hirsutum* L.cv Khandwa2). Plant Signaling and Behavior. 8(10):26762-26767.

Kumria, R.; S. Leelavathi; R.K. Bhatnagar; and V.S. Reddy (2003). Regeneration and genetic transformation of cotton: present status and future perspectives. Plant Tissue Cult., 13(2): 211-225.

Li, X.; X.D. Wang; H. Zhao; and Y. Dutt (2004). Improvement of cotton fiber quality by transforming the *acsA* and *acs*B gene into *Gossypium hirsutum* L. by means of vacuum infiltration. Plant Cell Rep., 22: 691-697.

Mishra, R.; H. Wang; N.R. Yadav; and T. Wilkins (2003). Development of a highly regenerable elite Acala cotton (*Gossypium hirsutum* cv. Maxxa) – A step towards genotype independent regeneration. Plant Cell Tiss. Org. Cult., 73: 21-25.

Murashige, T.; and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 474-497.

N'guissan, S.R.; K. Ayolie; Y.K.F. Kanan; S.E.S. Yapo; Y.J. Kouadio; and T.H. Kouakou (2020). Optimization of somatic embryogenesis culture medium induction in cotton (*Gossypium hirsutum* cv. Y331B-R5) growth in Cote D'ivore. IJRDO Journal of Agricultural and Research. 6(7):1-12

Nandini, B.; and K.S. Soumitra (2016). Development of a suitable plant regeneration protocol of cotton cutivar variety in comparison to non-cultivar Coker. IOSR Journal of Biotechnology and Biochemistry. 2(5):14-20

Nobre, J.; D.J. Keith; and J.M. Dunwell (2001). Morphogenesis and regeneration from stomatal guard cell complexes of cotton (*Gossypium hirsutum* L.). Plant Cell Rep., 20: 8-15.

Price, H.J. and R.H. Smith (1979). Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anderss. Planta. 145: 305-307.

Qayyum, R.A.; S.S. Hussain; M.S. Shahazad; S.Y.A. Bokhari; M.H. Raza; A. Rakha; A. Majeed; A.A. Shahid; Z. Saleem; T. Husnain; and S. Riazuddin (2006). Somatic embryogenesis in wild relatives of cotton (*Gossypium* spp.). Journal of Zhejiang University Science B. 7(4): 291-298.

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.

Rajeswari, S.; S. Muthuramu; R. Chandirakala; V. Thiruvengadam; and T.S. Raveendran (2010). Callus induction, somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Electronic Journal of Plant Breeding. 1(4):1186-1190

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

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

Shengwei A.; and F.; Jingsan (2000). Rapid plant regeneration from cotton (*Gossypium hirsutum* L.). Chinese Sci. Bull., 45: 1771-1773.

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

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

Tohidfar, M.; M. Mohammadi; and B. Ghareyazie (2005). *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a heterogous bean chitinae gene. Plant Cell Tiss. Organ Cult., 83: 83-96.

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

Trolinder, N.L.; and J.R. Goodin (1988a). Somatic embryogenesis in cotton (*Gossypium*). I. Effects of source of explant and hormone regime. Plant Cell Tiss. Organ Cult., 12: 178-181.

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

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

Zhang, B.H.; F. Liu; and C.B. Yao (2000). Plant regeneration via somatic embryogenesis in cotton. Plant Cell Tiss. Organ Cult., 60: 89-94.

Zhao, F.Y.; Y.F. Li; and P. Xu (2006). *Agrobacterium* – mediated transformation of cotton (*Gossypium hirsutum* L. cv. Zhongmian 35) using glyphosate as a selectable marker. Biotechnol. Lett. 28: 1199-1207.

Zouzou, M.; T.H. Kouakou; M. Kone; N.G. Amani; and Y.J. Kouadio (2008). Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). Australian J. Crop Sci., 2 (1):1-9.

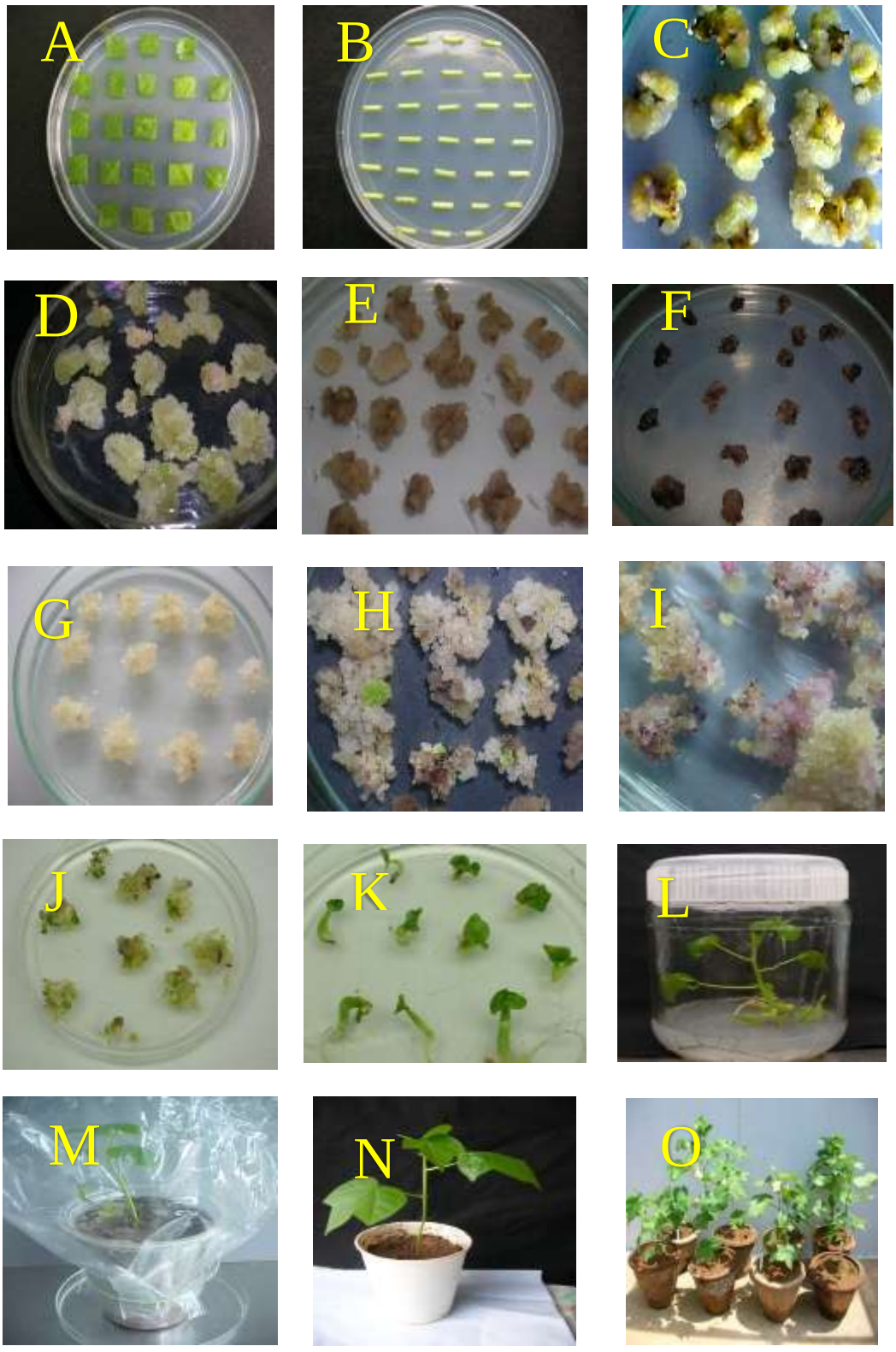

Figure 1. Somatic embryogenesis in Coker genotypes.

A: Cotyledon explants (16mm^2), B: Hypocotyl explants (4-6mm), C: Induction of callus from cotyledon, D: Induction of callus from hypocotyl, E and F Maturation of calli , G: Embryogenic calli, H: Co-existence of embryogenic calli and non-embryogenic calli in culture, I: Embryogenic calli showing red pigmentation (anthocyanin), J: somatic embryos, K: Development and maturation of somatic embryos, L: Regenerated plantlet, M and N: Hardening of regenerated plantlets in culture room, O: Hardening and establishment of regenerated plants in the green house.