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High Frequency of Callus Induction, its Proliferation and Somatic Embryogenesis in Cotton (*Gossypium hirsutum* L.)

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Abstract

Callus induction and somatic embryogenesis are fundamental to cotton tissue culture biotechnology. An efficient protocol for callus induction, somatic embryogenesis and their maturation have been developed to regenerate plantlets from cotton (*Gossypium hirsutum* L.) variety coker 312. Embryogenic callus was initiated from hypocotyl region that was used as an explant at seedling stage when it was about 7-8 days old. Callus induction was achieved through culturing hypocotyls (5-7 mm) on MS_{1a} medium supplemented with 2,4-D (0.1 mg/L) and KT (0.5 mg/L) for six weeks. A friable, colorless, bulky and well proliferating callus becomes greenish with the addition of NAA (2.0 mg/L), ZT (0.1 mg/L) and removal of 2,4-D (MS_{1b}) cultured for two weeks then again transferred to MS_{1a}. 2,4-dichlorophenoxyacetic acid (2,4-D) promoted the proliferation of embryogenic callus, but had a negative effect on the differentiation and germination of somatic embryos. ZT (0.1 mg/L) and activated charcoal (2g/L), both hormones play an important role in differentiation and germination of somatic embryos in hypocotyls derived embryogenic callus but in case of cotton, such a capability have been observed on MS medium with 1.92 g/L KNO_3, but it is considered to attain somewhat more improvement. High embryogenesis frequency was achieved through nutrient deficient stress treatment. The frequency of globular embryogenesis (two-three folds) was achieved when well proliferating callus was (from MS_{1a} media) cultured on MS (1/5 strength) medium for four weeks. Here the development of anthocyanins is the best indicator for somatic embryogenesis. However, when embryoid callus was cultured on MS (full strength) medium, the globular embryos were developed into normal plantlets immediately. In this procedure 27.49% cotyledenary embryos were developed. Of that 70% cotyledenary embryos were developed not only into normal plantlets but rooted simultaneously, when cultured on MS (with 0.05 mg/L giberrelic acid) medium. So complete plants could be regenerated through somatic embryogenesis from hypocotyl explants within 6 months.

Key words: *Gossypium hirsutum* L.; Plant regeneration; Coker 312; Callus induction; Somatic embryogenesis; *In vitro* regeneration

Introduction

Cotton is one of the most important cash crop, playing a vital role in economic, political and social affaires of the world. Chiefly a fiber crop, it has been estimated to contribute US $ 15-20 billion to the world s agriculture economy with over 1 million people depending on it for their livelihood (Benedict and Altman 2001). Since genetic improvement of cotton through conventional breeding is limited by several factors such as lack of useful variation and long time periods that are required. Although plant biotechnology is an attractive means for improving cotton, its use requires an effective regeneration system from somatic tissues of cotton plants. Compared with many other crops, it is more difficult to obtain somatic embryogenesis and plant regeneration from cotton.

Thus reducing the chimeric transformation events, however efficient in vitro techniques for regeneration of large numbers

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of plantlets from cotton are limited when compared to other major commercial crops. Price and Smith (1979) were the first to report somatic embryogenesis in cotton *Gossypium koltzchianum,* although complete plants could not be regenerated. Davidonis and Hamilton (1983) first described plant regeneration from two year old callus of *Gossypium hirsutum* L. cv Coker 310 via somatic embryogenesis. Since then, significant progress has been reported in cotton tissue culture (Zhang and Feng 1992; Zhang 1994b). *In vitro* cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies (Shoemaker et al. 1986; Chen et al. 1987; Trolinder and Goodin 1987; Zhang and Wang 1989; Voo et al. 1991; Zhang 1994a; Zhang et al. 1996; Zhang and Konzak 1999).

Regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root (Zhang 1994a; Zhang et al. 2000) and anther (Zhang et al. 1996) and from various cotton species (Zhang 1994b). Somatic embryogenesis and plant regeneration systems have been established from cotton tissue, protoplasts and ovules (Zhang and Li 1992; Feng and Zhang 1994; Zhang 1995). Regeneration procedures have been used to obtain genetically modified plants after *Agrobacterium*-mediated transformation of hypocotyls (Umbeck et al. 1987; Lyon et al. 1993; Chen et al. 1994) and cotyledons (Firoozabady et al. 1987) or by transformation of particle bombardment (Finer and McMullen 1990). Although efficient plantlets regeneration from embryogenic calli through somatic embryogenesis has been improved significantly in recent years but some difficulties still remain.

Only a limited number of varieties can be induced to produce somatic embryos and regenerative plants, and the most responsive lines are Coker varieties, which are no longer under cultivation (Feng et al. 1998). Genotype dependent response restricts the application of cotton biotechnology to cotton breeding and production. Therefore, before plant tissue culture techniques are widely applied to cotton improvement programs, plant regeneration must be possible for a broad range of genotypes. Although in the past, several attempts have been made for in vitro regeneration from other cultivars, little success has been made.

Nitrogen has a key role in plant growth and development. As the nitrogen have direct effects on rate of cell growth, differentiation and cell totipotency (Kirbey et al. 1987). Nitrates a good source of nitrogen supply to plants then others like ammonium nitrates or glutamine etc. (Parvin 2003) because it is readily taken up and metabolized by the cells and affects a number of developmental processes leading to root branching, seed breaking, bud dormancy and apical domonance. With the reduction of nitrogen supply, often initiates sexual development (Trewavas 1983).

In this manuscript, we studied, callus induction, callus proliferation, embryogenesis and development of plantlets of cocker-312. Which can be helpful in the study of cotton tissue culture and will be of great value in research field.

Materials and Methods

Plump, mature seeds of cotton (*Gossypium hirsutum* L.) variety Coker 312 were chosen, delinted with commercial HCl then surface sterilized by 30% commercial bleach [5.25% (v/v) NaOCl] for 30 minutes and then washed three times in sterile distilled water, then surface sterilized seeds were soaked overnight for softening of seed coat. The seeds were sown on MS [MS (Murashige and Skoog 1962) salts with B5 (Gamberg et al. 1968) vitamins] medium supplemented with 3% sucrose 2.60 g/L phytagel. For germination, culture was placed under dark conditions at 28±2℃ for 72 hours then shifted to photoperiodic conditions (approximately 2,000 lx). After radical emergance from seeds. All plant growth regulators (Zeatin, 2,4 Dicholorophenoxyacetic acid and Napthaleneacetic acid were added and pH was adjusted prior to autoclaving the medium. Hypocotyl (3-5 mm) sections were excised from 6-8 days old sterile seedlings that were used for callus induction culturing on MS (MS salts with B_5 vitamins) medium supplemented separately and combination of auxin and cytokinin (0.1 mg/L 2, 4-D; 0.5 mg/L KT; 0.1 mg/L Zeatin; 2.0 mg/L NAA) and 3% maltose for 8 weeks.

The embryogenic callus proliferation was observed through application of a single orwthin a combination of harmones in MS medium for 4 weeks.

Embryogenic callus cell lines with high frequency of proliferation were chosen and transferred onto embryo induction medium, so somatic embryogenesis was induced hrough the application of hormones (ZT and activated charcoal) and also with the changes in microenvironment by various means like as metabolic stress. First they were cultured on whatman filter paper (Cat no. 1004070) placed on MS medium [(full-, ½-,1/5-strength) (Kumria et al. 2003)], secondly by using the double concentration of KNO_3 (Trolinder and Goodin 1987). The calli was cultured as 7 replicates (100±10) per plate.

Three methods were employed independently and were compared with control culture MSo (Full strength). The germination and maturation frequencies were recorded for each of the media. During the six weeks of culture under stress conditions, of that tremendous growth was observed with in 20 days, after each two weeks media was refreshed. The cotyledonary embryos initially cultured onto MS (full-, ½-, and 1/5-strength) medium supplemented with 0.05 mg/L GA_3, for 10 days and later transferred to basal MS medium in culture tubes (15x50 mm). All of six cultures were maintained at 28

± 2°C under a light intensity of approximately 2000 lx provided by growth rooms with 16 h photoperiod.

All media were supplemented with 30 g/L maltose, and were solidified with 2.6 g/L phytagel. The pH of medium was adjusted to 5.7-5.8 before autoclaving at 121°C for 15 min.

Results and Discussion

There is a need of the time to develop a protocole to attain an efficient callus induction, proliferation and regeneration system for cotton, as the increasing percentage of transgenic cotton being grown worldwide. In this aspect, we have used a number of different hormonal combinations of auxin (NAA; ZT, and/or 2,4-D) and cytokinin (kinetin) that were used at varying concentrations in basal MS medium (Murashige and skoog 1992; Trolinder and Goodin 1988a, 1988b). of course two of them were most important for callus induction and its peroliferation that are discussed in this manuscrip. In this experiment 7 replicates for both hormonal combinations were scored for callus induction efficiency (%) that based on two components a) both hormonal concentrations and b) the means of the number of explants producing good quality callus.

Highly friable yellowish-white callus was obtained from hypocotyls explants (Figure 1a) from MS medium supplemented with 3% maltose, 0.5 mg/L kinetin + 0.1 mg/L 2, 4-D but callus developed on MS_{1b} supplemented with 3% maltose, 0.5 mg/L kinetin + 0.1 mg/L ZT and 2.0 mg/L NAA was compact, greenish and with slow growing calli. (Figure 1b; Table 1). This variation with respect to color and texture of embryogenic calli has been observed by several authors (Finer 1988; Trolinder and Goodin 1988a, 1988b). During study, the normal treatments indicated that the 2,4-D is essential for callus induction in cocker-312 hypocotyls, six weeks old callus from MS_{1a} was subcultured on MS_{1b} for two weeks, which begins to be greenish but proliferate at very high rate, as it becomes doubled in weight (Figure 1c), so NAA and ZT is highly beneficial in callus induction in cotton.

The use of glucose causes severe browning of explants

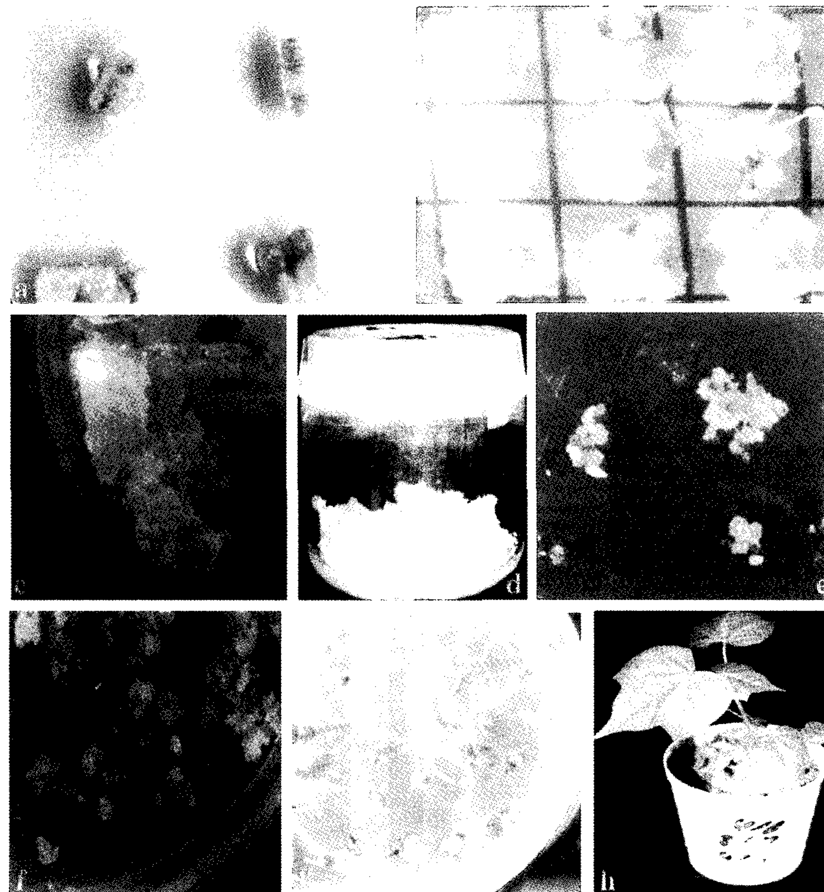

Figure 1. Different steps of tissue culture from callus induction to the developments of plantlets in cotton; a and b: Callus induction and proliferation in hypocotyls on MS_{1a} medium; c: Callus proliferation on MS_{2a} for two weeks; d: Callus proliferation for three weeks again on MS_{1a}; e and f: Embryo induction, maturation and development into plantlets; g: Plantlets on rooting media; h: Plant in soil.

due to phenolic oxidation in cotton tissue culture has been emphasized in the past (Beasley and Ting 1973; Smith et al. 1977). Unlike earlies investigators who regenerate plantlets on medium containing glucose (Firoozabady et al. 1987; Rajasekaran 1996). We have been used maltose as a carbon source in this study that is utilized more efficiently then glucose as in rice (Ghosh-Bswas and Zapata 1993). Brownish calli was not observed at any stage of callus growth in the presence of maltose as a carbon source in the MS medium.

According to the previous reports, 2,4-D was an essential hormone for embryohenesis in cotton and other plants (McKersie and Brown, 1996; GonzalezBenito et al. 1997; Guis et al. 1998; Choi et al. 1999; Zhang, 2000). However, in this study, we used to induce embryogenic callus with auxin (especially 2,4-D) and cytokinin combinations to compare this method with others. According to our and other's results (Davidonis and Hamilton 1983; Trolinder and Goodin 1986; Chen et al. 1987; Zhang and Li 1992; Kumar and Pental 1998), indicated that the addition of 2,4-D could promote the induction and growth of cotton callus, as it has a negative effect on differentiation and germination of somatic embryos so embryogenic callus was not observed within 11 weeks of culture (Table 1).

The production and proliferation of callus considered of good quality that based on four criteria: color, texture, friable, size and shape of undifferentiated cells. After 6 weeks of culture on MS_{1a} medium, it was subcultured on MS_{1b} medium for two weeks. Within two weeks callus mass was increased about to be double, then again shifted to its origional callus induction media (MS_{1a}) for three weeks. Embryogenic callus from that of both mediums (MS_{1a} and MS_{1b}) were subjected for embryo induction medium.

Callus was cultured for embryogenesis on MS_{2a} (0.1 mg/L ZT and 2 g/L activated charcoal) After two weeks of subculture; embryogenic callus produced visible somatic embryos. After 28 days of culture, somatic embryos in various developmental stages

could be obtained (Kolganova et al. 1992; Zheng and Konzak 1999). Embryogenic callus with somatic embryos at various developmental stages were chosen and again subcultured on the same medium. Therefore, the high frequency embryogenic lines required more subcultures, more than 100 somatic embryos could be produced in one culture plate (Figure 1C). Such a capability has been observed on MS medium with 1.92 g/L KNO_3. Two kinds of calli wereobserved after 8 weeks of culture: embryogenic and non-embryogenic callus. Than embryogenic callus developed into somatic embryos at various stages after 28 days of subculture

Friable peripheral loose mass of calli (MS_{1a}) transferred to embryo induction media that were used for the improvement of the frequency of embryogenesis. Of that globular embryo formation was high but was 40% of the culture here cotyledenary staged embryos showed various degrees of abnormalities, lake of well defined shoot tip, fused or/and multipule cotyledons. Several authers have reported, a low frequency of embryo induction in cotton (Gawel and Robacker 1990: Kumar and Pental 1998).

There is a critical limiting step in genetic transformation for

Table 1. Callus induction and proliferation of a cotton (*Gossypium hirsutum* L.) variety Coker 312 from hypocotyl explant at various hormonal concentrations.

Medium	Hormones (mg/L)				Callus induction and its proliferation, 8 weeks culture					
	2,4-D	NAA	KT	ZT	No. of Explants	No. of calli induced	Callus induction (%)	Callus (mg)	Callus Wt (g)	Callus growth ratio
MS_{1a}	0.0	2.0	0.1	0.1	30	28	93.33	100.40	3.80	37.84
MS_{1b}	0.1	0.0	0.5	0.0	35	35	100.0	98.70	5.80	58.76

Calli was cultured as 7 replicates (100±10) per plate
Growth ratio was calculated by following formula: (Growth ratio)=(wet weight of callus after six weeks of culture)/(original weight of callus). For each treatment, 7 samples were recorded and averaged.
Values represent means of seven replicates

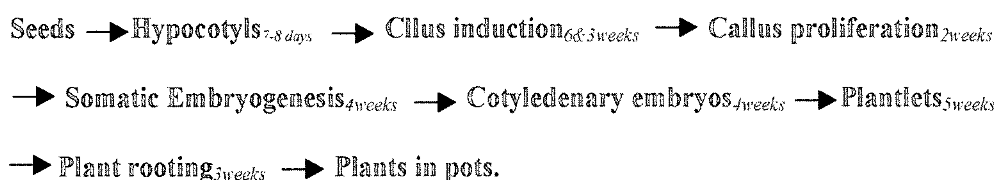

Figure 2. A layout for somatic embyogenesis and plant regeneration of cotton (Gossypium hirsutum L.) variety Coker 312.

Table 2. The effect of various media on Cocker-312 somatic embryogenesis and its maturation (after 36 day of culture).

Medium	Treatments	Number of embryos and embryogenic callus 5 Weeks of culture		Embryo maturation (%)
		Globular	Cotyledenary	
MS_{2b}	*MSo+KNO₃*	70.2	19.30	27.49
MS_{2c}	*1/2MSo+KNO₃*	95.5	6.22	6.51
MS_{2d}	*1/2MSo+KNO₃*	101.5	6.01	5.92
MS_{2e}	*1/5MSo+KNO₃*	107.8	5.22	4.84
MS_{2f}	*1/5MSo+KNO₃*	120.2	5.01	4.16

the production of a large number of transgenic plants, because of lake of the development and maturation of somatic embryos. For that to develop somatic embryos into plantlets, globular somatic embryos were subjected to metabolic stresses by means of reducing major and minor salts in culture media. The friable embryogenic calli were plated directly on the media or on sterile filter paper placed on media of varying concentrations of MS salts (Saito et al. 1991).

A large number of embryos were developed on MS (1/5-strength with KNO_3) media. Non-embryogenic calli are also present among globular embryos that could be due to the lowering of maturation frequencies, as majority of globular embryos formed did not develop further to form cotyledenary embryos that suggest us the high nutrient level might be required for the maturation of embryos. Davidonis and Hamilton (1993) reported, decrease in nitrogen and sugar triggers embryo maturation. The dilution of the media concentration sustained the cell division and growth of the calli but lead to direct differentiation. Further, the full strength MS medium, high embryo maturation was noted, because of higher nutrient requirement for the maturation of somatic embryos.

So the use of macro and micronutrient stress i.e. MS basic salts strength that was beneficial for embryo development which is a better response for embryo induction then others as mentioned earlier but the rate of and embryo maturation and development into plantlets was more efficient on full strength MS media (Table 2).

On embryo induction media, with the accumulation of small amounts of anthocyanins (red pigmentation) in callus and embryo cultures was a good indicator for embryogensis in callus because of, after that cocker plantlets regenerated (Mishra et al. 2003) but Anthocyanin production may be influenced by different factors such as UV, light, nitrogen source, type of sugar, osmotic stress, temperature, elicitor conditioning and phytoharmone conditions (Zhang et al. 1998). According to Kim and Kim (2002) when either NH_3 or NO_3 was lacking, cell growth decreased that leads to anthocyanin development. When NO_3 was lacking, cell growth increased slightly and anthocyanin contents become relatively low. It was thought that NO_3 affected cell growth while NH_3 affected antho-cyanin production. Anthocyanin accumulation began when there was no multiplication of cells, and when cell multiplication began, anthocyanin accumulation was diminished.

Extracellular anthocyanin in the medium during the course of culture as was pointed out in suspension cultures of *Vitis vinifera*, antho-cyanin catabolism is closely related to cell membrane integrity. With cellular lysis, the anthocyanins stored in anthocyanoplasts or vacuoles are released into the culture medium and are quickly metabolized (Guardiola et al. 1995). So it is confirmed that during embryoid development the cell growth inhibited that may be caused to the synthesis of anthocyanin. As the activity of cells for synthesis of secondary products lost in most cases when the cells are differentiated and grow rapidly in cultures (Ozeki and Komamine 1981).

Hypocotyl explants excised from seedlings and cultured on callus induction, proliferation and then the embryo induction media. After every 2-3 weeks callus was subcultured on fresh media. A considerable improvement in the quality of callus and somatic embryogensis was achieved through micro-environmental changes. So the somatic/cotyledenary embryos were germinated on varying strengths of MS media in the presence of GA3 (0.05 mg/L) (Figure 1g). More then 70% of the cotyledenary embryos germinated on full strength MS medium. Whereas 40-50% were developed into plantlets on ½ and 1/5-strength MS medium that s perhaps because of prerequisite for normal plant development. Germinated embryos [(2-3 cm) Figure] were transferred onto MS basal media in pint jars to establish rooted plantlets prior s to transfer to soil. There is not required any additional treatment root formation that occur simultaneously with shoot elongation as reported earlier (Gould et al. 1991; Kumar and Pental 1998).

The recalcitrance of commercial cotton varieties to tissue culture has been a major stumbling block for transgeneic cotton development. In addition the fact that the current regeneration of transgenic cotton is based on only the cocker lines could lead to a genetic bottleneck problem. Development of an efficient

tissue culture and plant regeneration protocol for cotton varieties is the first step towards the application of transgenic technology to improve cotton breeding. Somatic embryogenesis in cotton is hampered by an extended culture period, low frequency of normal embryos and low number of embryos. Meanwhile, the established cotton tissue culture protocol for cotton variety Coker 312, hundreds of regenerated plants were obtained from Coker 312 via through somatic embryogenesis. Here nutrient manipulation have a key role to trigger the process of differentiation in the embryogenic callus that lead to reduce the embryo induction period. A reduced cultured period allows for the convension of a large competent cell population into embryos and as well as maturation into normal plants. The efficient embryo maturation observed in present study that can be applied to regenerate embryogenic calli into a large number of plants in genetic transformation of cotton.

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