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Full Length Research Paper

Callus proliferation and somatic embryogenesis in cotton (*Gossypium hirsutum* L.)

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Somatic embryogenesis and plant regeneration are fundamental to tissue culture biotechnology in cotton (*Gossypium hirsutum* L.) cv. Coker 312. Callus proliferation was considered best on MS_{1a} (2.0 mg/L NAA; 0.1 mg/L ZT; 0.1 mg/L KT) when 6 weeks old callus was cultured from MS_{1b} (0.1 mg/L 2, 4-D; 0.5 mg/L KT) medium, there is no need to select embryogenic calli for somatic embryogenesis, as all of them were converted to somatic embryos. NH_4NO_3 play an important role in differentiation of callus into somatic embryos but is lethal for embryos just after two weeks. However, KNO_3 is less efficient for somatic embryo induction but is best for embryo maturation. By this procedure 56.51% cotyledenary embryos were developed within 5 weeks. Of that, 82.05% cotyledenary embryos were developed not only into normal plantlets, but rooted simultaneously when cultured on MS (with 0.05 mg/L GA_3) medium. A complete plant of Cocker-312 could be regenerated through somatic embryogenesis within 4 to 5 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 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 cotton is highly susceptible to biotic and abiotic stresses, it requires intensive crop management. Although conventional breeding programs have made steady improvements in agronomic traits, not much genetic diversity exists for further improvement. However, gene transformation techniques have provided for the introduction of foreign genes into cotton through either *Agrobacterium* or biolistic transformation, which involves the development of an efficient regeneration system from the transformed tissues. Regeneration through somatic embryogenesis is preferred over organogenesis because of single-cell origin of the somatic embryos (Merkle et al., 1995), thus reducing the chimeric transformation events. However, efficient *in vitro* techniques for the regeneration of large numbers 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 klotzchianum*), although complete plants could not be regenerated. Davidonis and Hamilton, (1983) subsequently described plantlet regeneration via somatic embryogenesis from a 2-year-old callus culture of *G. hirsutum* var. Coker 310. This procedure involved a lengthy culture period and was difficult to repeat. Since then, several investigators have worked extensively on plant regeneration through somatic embryogenesis in different cotton cultivars

Table 1. Callus induction (%) and callus growth ratio (mg/day) after4 weeks of culture in cotton (Gossypium *hirsutum L.*) cv Coker-312.

Medium	Hormones (mg/L)				No. of Explants	Callus induction (%)	Callus growth ratio (mg/day)
	2,4-D	NAA	KT	ZT			
MS_{1a}	0.0	2.0	0.1	0.1	30	29	48.32±0.9
Calli taken from MS_{1b} (2months old)							63.79±1.5
MS_{1b}	0.1	0.0	0.5	0.0	35	35	58.76±1.7

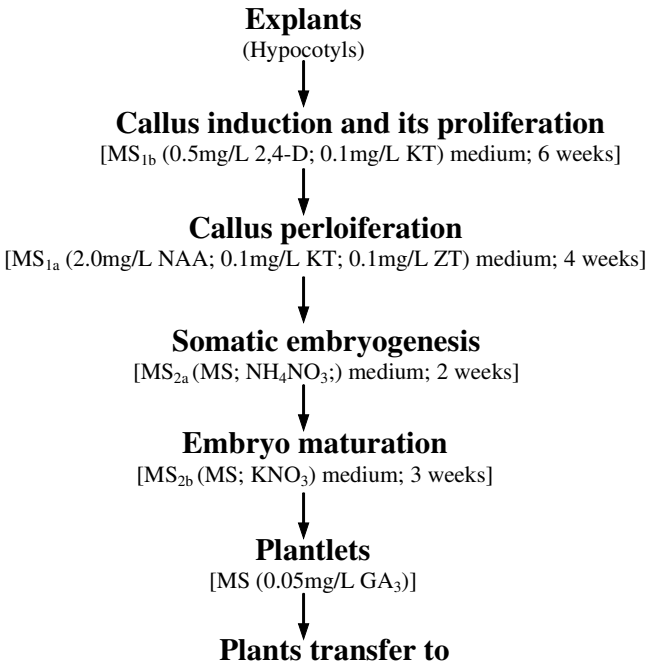

Figure 1. Protocol for somatic embryogenesis and plant regeneration in cv. Cocker-312.

(Leelavathi et al., 2004; Chaudhary et al., 2003; Trolinder and Goodin, 1987; Cousins et al., 1991; Kumeria et al., 2003; Rajasekaran et al., 1996. Although regeneration efficiency via somatic embryogenesis has been improved, problems remained as high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and lack of shoot elongation are often associated with cotton regeneration. We describe here a protocol for efficient callus proliferation with high frequency of development of somatic embryos that developed into normal plantlets within 4–5 months in cotton (*Gossypium hirsutum* L.) cv. Coker-312.

MATERIALS AND METHODS

Mature seeds of cotton (*Gossypium hirsutum* L.) cv. Coker-312 were surface sterilized by 30% commercial bleach [5.25% (v/v) NaOCl] by stirring for 30 min and then washed three times with sterile distilled water. The surface sterilized seeds were germinated on MS [MS (Murashige and Skoog, 1962) salts with B5 (Gamberg et al., 1968) vitamins] medium. For germination, culture was placed under dark conditions at 28±2ºC for 72 h, after radical emergence, culture was transferred to growth room.

Hypocotyl (3-5 mm) sections were excised from 6-8 days old sterile seedlings that were used as explants for callus induction by culturing on MS (MS salts with B_5 vitamins) medium supplemented with a combination of different auxin and cytokinin hormones for 6 weeks (Table 1).

The relative growth ratio in callus was calculated by culturing embryogenic calli as 7 replicates with initial weight 100±10 mg per plate per medium for 4 weeks.

Embryogenic callus with high proliferation rate was chosen and transferred onto embryo induction medium for somatic embryogenesis. Three different embryo induction media were used for somatic embryogenesis (Table 2). The cotyledonary embryos were cultured onto MS medium for root/shoot induction supplemented with 0.05 mg/L GA_3.

All cultures were supplemented with 1.60 mg/L $MgCl_2$, 30 g/L glucose, and were solidified with 3.60 g/L phytagel. The pH of each medium was adjusted to 5.7-5.8 before autoclaving at 121℃ for 15 min. Each culture was maintained at 28 ± 2℃ under a light intensity of approximately 2000 lx provided by growth rooms with 18/6 h photoperiod.

RESULTS AND DISCUSSION

There is a need at this time to develop a protocol to attain an efficient callus induction, proliferation and plant regeneration system for cotton. Different hormonal combinations [auxin (NAA; 2,4-D) and cytokinin (Kinetin; ZT)] were used in basal MS medium (Murashige and skoog, 1962), two of them considered best are discussed here. The 7 replicates per culture were maintained for calculating callus induction, its proliferation, callus growth ratio and embryo maturation (%). Highly friable greenish-white callus obtained from hypocotyl sections when cultured on MS_{1b} medium (Table 1; Figure 2a). After 6 weeks such calli was subcultured for 4 weeks on MS_{1a} (Table 1; Figure 2b) resulted in the development of various sectors of embryogenic calli as well as greenish-yellow calli with varying degrees of compactness. This variation with respect to color and texture of embryogenic calli has been observed by several authors (Finer, 1988; Firoozabady et al., 1987; Gawel and Robacker, 1990; Shoemaker et al., 1986; Trolinder and Goodin (1987). A rapid increase in callus growth ratio (63.79 ± 1.5) was

Table 2. The somatic embryogenesis and their maturation in Cocker-312.

Medium	Treatments	Embryo maturation (%)	Plantlets development(%)
MS_{2a}	MSo+NH_4NO3	20.94	30.32
MS_{2b}	MSo+KNO3	27.49	46.24
Callus taken from MS_{2a} after 2 weeks		5 6.51	82.05

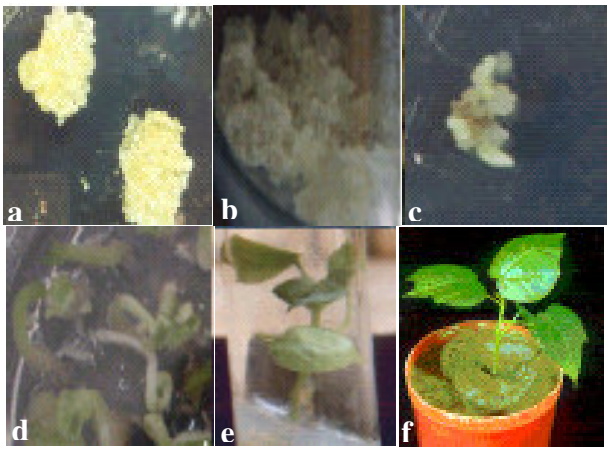

Figure 2. Callus proliferation and plant regeneration via somatic embryogenesis in cotton (*Gossypium hirsutum* L.) cv. Cocker-312, a and b: Callus proliferation on MS_{1b} (0.5mg/L 2,4-D; 0.1mg/L KT) and MS_{1a} (2.0mg/L NAA; 0.1mg/L KT; 0.1mg/L ZT) respectively; c: Embryogenic culture, with immature embryos; d: Dicotyledenary/ mature embryos on MS_{2b} (MS+KNO_3) medium; e: Rooted plantlets on MS medium supplemented with 0.05mg/L GA_3; f: A regenerated plant transferred to soil.

observed in the calli cultured on MS_{1a} from MS_{1b}. With the passage of time such calli changes its color, whitish to green, when this culture was maintained up to 6 weeks. The developments of roots in the calli were also observed, but this is not beneficial for the cotton tissue culture aspect. After 4 weeks, the calli was subcultured again for 2 weeks on MS_{1b} medium. In calli directly subjected to embryogenesis, rooting was observed. There is no need to identify and select embryogenic callus from the culture on MS_{1b} medium, because all of them are able to develop embryos on embryo induction medium.

There is a critical limiting step in genetic transformation for the development of a large number of transgenic plants, because of the lack of development and maturation of somatic embryos. In order to develop somatic embryos, the embryogenic calli was subjected to harmone free MS_{2a} medium (Table 2) containing higher concentration of ammonium nitrate (1.90 mg/L NH_4NO_3). Whitish globules on the embryogenic calli were observed under light microscope, while the adventitious roots were developed from the calli that was induced and proliferated on $MS_{1a,}$ but not in calli from MS_{1b} media. So the callus from MS_{1b} medium was considered best for somatic embryogenesis.

After 2 weeks of culture, the embryogenic calli with globular embryos were subcultured on MS_{2b} from MS_{2a} medium, and each of the two cultures was constantly refreshed. The calli on MS_{2a} medium become reddish due to the sythesis of anthocyanin (red pigmentation), and had a limited number of embryos. Most of the embryos were abnormal in morphology and that dies later on. While in calli induced and proliferated on MS_{2b} medium, there was no embryogenesis in the calli. However, in the calli that was cultured from MS_{2a} onto $MS_{2b,}$ many healthy embryos developed (Table 2; Figure 2c), and these become dicotyledenary prior to the appearance of mature embryos on the MS_{2b} medium. No anthocyanin synthesis was observed here. Globular embryo formation was low in the MS_{2b} medium, but 40% of the culture showed various degrees of abnormalities such as lake of well defined shoot tip, fused or/and multipule cotyledons (Gawel and Robacker, 1990: Kumar and Pental, 1998).

The mature and well developed dicotyledenary embryos from each medium were cultured on MS medium for the development of rooting and shooting. More then 82.05% of the cotyledenary somatic embryos germinated on MS media in the presence of GA_3 (0.05 mg/L) all of them were normal in morphology (Kumeria et al., 2003). So the protocol scheme as presented in (figure. 1) is the fastest and most reliable way for the establishment of plant regeneration system via somatic embryogenesis in Cocker-312 cultivar.

On embryo induction medium, the accumulation of small amounts of anthocyanins (red pigmentation) among the developed embryo is considered a good indication for Cocker regeneration (Mishra et al., 2003). According to Zhang et al. (1998), anthocyanin production may be influenced by different factors such as UV, light, nitrogen source, type of sugar, osmotic stress, temperature, elicitor and/or phytoharmone conditions. Kim and Kim (2002) observed that when either NH_3 or NO_3 was lacking, cell growth decreased leading to anthocyanin development. When NO_3 contents are high then NH_3 becomes lacking causing cell growth to increase slightly and anthocyanin contents become relatively low. It was thought that NO_3 increased cell growth while NH_3 influenced anthocyanin production. Anthocyanin accumulation began when there was no multiplication of cells, and when cell multiplications occur, anthocyanin accumulation diminishes (Kirby et al., 1987).

Therefore, cell growth inhibition may be the cause of anthocyanin synthesis. The synthesis of secondary products reduces in most cases when the cells are under differention process with rapid cell division (Ozeki and Komamine, 1981).

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 and low frequency of normal embryos. In our tissue culture protocols for Coker 312, hundreds of normal plants were regenerated through somatic embryogenesis. Here ammonium nitrate plays a key role in the triggering of the process for differentiation in the embryogenic callus that lead to reduction in the embryo induction period. A reduced cultured period allows for the conversion of a large competent cell population into embryos and as well as development into normal plants.

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