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Full Length Research Paper

Somatic embryogenesis and plant regeneration of recalcitrant cottons (*Gossypium hirsutum*)

G. Y. Han, X. F. Wang, G. Y. Zhang and Z. Y. Ma*

Key Laboratory of Crop Germplasm Resources of Hebei, Agricultural University of Hebei, Baoding 071001, People's Republic of China.

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Many restrictive factors still remain in cotton tissue culture such as long duration, unpredictability and a high degree of genotype dependence. The main objective of this study was to develop a protocol allowing consistent somatic embryogenesis and plant regeneration from five recalcitrant cotton cultivars. Our results showed that the best medium for calli induction is MSB (MS medium + vitamine B5) supplemented with indolebutyric acid (IBA, 0.1 mg.l^{-1}), kinetin (KIN, 0.1 mg.l^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg.l^{-1}). Embryogenic calli of all the five genotypes used were successfully from MSB medium supplemented with IBA (0.3 mg.l^{-1}) and KIN (0.05 mg.l^{-1}). Somatic embryos and transformation of somatic embryos into plants were successfully induced on MSB medium supplemented with ½×NH_4NO_3 (825 mg.l^{-1}), 2×KNO_3 (3800 mg.l^{-1}), glutamine (2.0 g/l) and asparagines (0.5 g/l). The protocol developed in this study for cotton plant regeneration could be shortened to 4 - 5 months. Furthermore, the firstly-obtained regenerated plants of above five cultivars will broaden the range of genotypes for *in vitro* manipulation for cotton improvement.

Key words: Cotton, callus induction, somatic embryogenesis, embryogenic callus, plant regeneration.

INTRODUCTION

Somatic embryogenesis and subsequent plant regeneration have been reported in most major crop species, including cotton with breeding purpose of both mutant screening and gene transfer (Wu et al., 2004). However, cotton has proved to be one of the most difficult crops to regenerate *in vitro*. The first significant result was reported by Price and Smith (1979), who successfully induced embryoids from *Gossypium klotzchianum*, although complete plants could not be regenerated. Plant regeneration from cotton cells was achieved from two-year-old callus of *G. hirsutum* cv. Coker310 via somatic embryogenesis (Davidons and Hamilton, 1983). Since then, *in vitro* somatic embryogenesis in cotton has been reported in many laboratories using different methods (Shoemaker et al., 1986; Finner, 1988; Gawel and Robacker, 1990; Chaudhary et al., 2003; Leelavathi et al., 2004; Sakhanokho et al., 2004b; Xie et al., 2007). It remains the case, however, that only a limited number of cultivars can be induced to produce somatic embryos and regenerated plants. The most responsive genotypes are model varieties, especially cultivars '*Coker*' lines that are no longer cultivated by farmers. Therefore, the number of commercial cultivars and elite germplasm lines with superior fiber and agronomic characters that can undergo plant regeneration remains low (Sakhanokho et al., 2004b).

Genotype dependence is one of the important factors that restrict somatic embryogenesis and plant regeneration. From 1971 to 1997, out of 100 cotton cultivars studied in tissue culture, 54% exhibited different somatic embryogenesis capability, while 46% could not proliferate to form somatic embryos (Feng et al., 1997). Since that time, about 150 additional cultivars were used in tissue culture within a decade, but only 20% of elite cotton cultivars could be regenerated successfully (Zhang et al., 2001; Wu et al., 2004; Sakhanokho et al., 2004b; Wang et al., 2006). Furthermore, apart from the model lines, cotton regeneration also takes a long time. Six to eight months has been reported as the shortest time for regeneration (Mishra et al., 2003; Wang et al., 2006).

Our laboratory has special interest in cultivars with

*Corresponding author. E-mail: mzhy@hebau.edu.cn. Tel: +86-312-7528401. Fax: +86-312-7528400.

Fusarium and/or *Verticillium* wilts resistance, but to date these also have proven to be very recalcitrant to regeneration via somatic embryogenesis (Wang et al., 2006). The present study was therefore conducted to develop a comprehensive tissue culture protocol for plant regeneration through somatic embryogenesis for cotton cultivars with *Fusarium* and/or *Verticillium* wilts resistance.

MATERIALS AND METHODS

Plant materials

Five upland cotton (*G. hirsutum*) cultivars (Shann724, Zhong6331, CCRI18, Liaomian12 and Jinmian14), provided by Cotton Genetics and Breeding Laboratory of Agricultural University of Hebei, China, were used in this study.

Seed germination and culture of sterile seedlings

Mature seeds of cotton cultivars were delinted with sulfuric acid (98%), then sterilized with $HgCl_2$ (0.1%) for 8 - 10 min followed by rinsing with sterile distilled water for three times and kept in sterile water overnight for germination at 28 ± 2ºC. Seeds were then placed on Murashige and Skoog (MS) based medium (Murashige and Skoog, 1962) but with half strength macronutrients, 6.5 g/L agar (Japan) and 2% (w/v) sucrose and cultured at 25 ± 2 ºC for 5 - 7 days in the dark.

Induction, proliferation and development of callus

To induce callus, hypocotyl sections 5 - 7 mm in length were excised from germinated sterile seedlings as explants were placed on MSB medium (MS medium with vitamine B5) supplemented with various combinations and concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), indolebutyric acid (IBA) and kinetin (KIN) (Table 1). The media of callus subculture were adjusted by changing the concentration of hormones according to the color and texture of the calli.

Induction of embryogenic callus

After about two months of culture, the prolifically growing and loose pre-embryogenic calli with smaller cells and very dense cytoplasm were selected and then transferred onto embryogenic callus induction media consisting of MSB supplemented with different hormones (Table 1).

Differentiation of somatic embryos and plant regeneration

Two months later, embryogenic calli with high proliferation rate were chosen and transferred onto somatic embryo induction medium, MSB supplemented with 825 $mg.l^{-1}$ NH_4NO_3, 3800 $mg.l^{-1}$ KNO_3, 2.0 g/l glutamine and 0.5 g/l asparagines, for the induction and development of somatic embryos. Then somatic embryos were transferred once again onto the same medium to induce the formation of regenerated plants.

All the cultures were maintained at 28 ± 2ºC under 14 h photoperiod with a light intensity of approximately 2000 lx provided by cool white fluorescent lamps in a growth chamber. Besides the medium for the germination of sterile seedlings, all the other media were supplemented with glucose (3%) and solidified with phytagel (2.5 g/l). The pH of all the media was adjusted to 5.85 using 1 M HCl and 1 M KOH before autoclaving at 121ºC for 15 min.

Table 1. The hormone combination in the induction media of callus and embryogenic callus.

Treatments*	Hormone combinations ($mg.l^{-1}$)		
	IBA	KT	2,4-D
Hc1	0.1	0.1	0.5
Hc2	0.1	0.1	0.1
Hc3	1.0	0.1	0.5
Hc4	1.0	0.1	0.1
Hc5	1.0	0.1	0.05
He1	0.1	0.05	0.1
He2	0.1	0.05	0.01
He3	0.1	0.05	0
He4	0.3	0.05	0.1
He5	0.3	0.05	0.01
He6	0.3	0.05	0
He7	1.0	0.05	0.1
He8	1.0	0.05	0.01
He9	1.0	0.05	0

*Hc1-5 media were used for callus induction; He1-9 media were for embryogenic callus induction.

Plant recovery

Regenerated plants with 2 - 4 leaves were transplanted to green house by graft method. Soil was kept moist and plantlets were partially covered with plastic bag to allow for gradual acclimatization. After about one week, the plastic bag was gradually opened and eventually removed to allow for their normal growth.

Statistical analysis

Each Erlenmeyer flask was considered an experimental unit. Each experiment was performed three times. An analysis of variance was performed for each experiment. The least significance difference test (LSD at P=0.01) was used for multiple mean comparisons.

RESULTS

Induction and development of callus

The two ends of hypocotyls started to swell as they were cultured in MSB media (Table 1) for 10 - 15 days, and produced a mass of calli 25 - 30 days later. However, the induction frequency, color and texture of calli were diverse among the different media and cultivars (Table 2).

The induction frequency of calli ranged from 70 to 100% among different treatments. As for medium, Hc2

Table 2. Effect of various hormones on induction of callus.

Treatments and states		Cultivars				
		Shann724	**Zhong6331**	**CCRI18**	**Liaomian12**	**Jinmian14**
Induction frequency (%)*	Hc1	98AB**	95ABC	89BC	96AB	98AB
	Hc2	100A	100A	97AB	100A	100A
	Hc3	94ABC	88C	70D	93BC	95ABC
	Hc4	100A	100A	96AB	100A	100A
	Hc5	90BC	85C	80CD	90BC	93BC
Color	Hc1	Yellow	Yellow	Off-white	Fawn	Off-white
	Hc2	Light yellow	Light yellow	Yellow	Light yellow	Light yellow
	Hc3	Yellow green	Green	Cream	Kelly green	Gray
	Hc4	Light yellow	Light green	Light yellow	Light gray	Yellow
	Hc5	Off-white	Gray	Gray	Brown	Fawn
Texture	Hc1	Loose	Loose	Compact	Loose	Loose
	Hc2	Friable	Friable	Loose	Granule	Granule
	Hc3	Hard	Hard	Hard	Compact	Compact
	Hc4	Loose	Loose	Small nibble	Loose	Friable
	Hc5	Compact	Pulpy	Hard	Pulpy	Pulpy

*Induction frequency was mean of three replicates. The induction frequency of each replicate was counted by the number of hypocotyls with callus /the total number of hypocotyls in per treatments.
**Different *letters* indicate significant differences at P=0.01.

gave the highest frequency of calli induction (Table 2). The induction frequency of callus was low when hypocotyls were cultured in the medium supplemented with low concentration of 2,4-D (0.05 mg.l^{-1}). However, high concentration of 2,4-D (0.5 mg.l^{-1}) also restricted the induction and proliferation of callus.

Yellow, light yellow, green, light green, yellow green, gray and off-white colored calli with compact, loose, pulpy, hard and friable texture were observed in the different induction media. Those yellow and light yellow calli were easily to differentiate. Those with light green and gray could also differentiate but required an extended time of subculturing. As for calli texture, the loose and friable ones differentiated most easily, the compact and hard ones slowly, and the pulpy ones all failed to differentiate. Hc2 medium, MSB with IBA (0.1 mg.l^{-1}), KIN (0.1 mg.l^{-1}), 2, 4-D (0.1 mg.l^{-1}), was found to be the best for calli induction based on induction frequency, color and texture of calli. Successive culture of the induced callus for one month could produce pre-embryogenic calli in this medium.

Induction and selection of embryogenic calli

Pre-embryogenic calli from Hc2 medium (Figure 1A) were selected to subculture on the media with various combinations and concentrations of hormones to induce embryogenic calli. After one months of subculture, various states of calli differentiation were detected for the cotton cultivars. Two types of calli, embryogenic with cream-color or light yellow and loose granular and non-embryogenic with light brown or dark green and compact or pulpy, were categorized. The calli of the five cultivars, Shann724, Zhong6331, CCRI18, Liaomian12 and Jinmian14, could differentiate into embryogenic calli only in He6 medium (Figure 1B, Table 3). And the proliferation rate among different cultivars ranged from 75 to 83%.

Differentiation of somatic embryos and plant regeneration

Embryogenic calli of these five cotton cultivars were transferred onto MSB medium supplemented with ½ × NH_4NO_3 (825 mg.l^{-1}), 2 × KNO_3 (3800 mg.l^{-1}), glutamine (2.0 g/l) and asparagines (0.5 g/l). After 15 days of subculture, embryogenic calli could produce somatic embryos (Figure 1C). Regeneration of somatic embryos into plants for these five cotton cultivars was successfully induced after subculturing on the same medium for 3-4 weeks (Figure 1D, 1E). And the ratio of plant regenera-

Table 3. Effect of various hormones on induction of embryogenic calli.

States	Treatments	Shann724	Zhong6331	CCRI18	Liaomian12	Jinmian14
Color	He1	Dark green	Light brown	Dark green	Yellow	Gray
	He 2	Brown	Gray	Dark green	Dark green	Brown
	He 3	Light brown	Dark green	Gray	Gray	Yellow
	He 4	Fawn	Dark green	Light brown	Light brown	Dark green
	He 5	Gray	Brown	Gray	Light brown	Light brown
	He 6	Light yellow	Light yellow	Cream-color	Cream-color	Cream-color
	He 7	Dark green	Light brown	Fawn	Fawn	Dark green
	He 8	Light brown	Gray	Dark green	Gray	Yellow
	He 9	Gray	Fawn	Light brown	Dark green	Fawn
Texture	He1	Hard	Compact	Hard	Hard	Compact
	He 2	Pulpy	Hard	Pulpy	Compact	Compact
	He 3	Compact	Hard	Compact	Pulpy	Compact
	He 4	Hard	Compact	Hard	Compact	Hard
	He 5	Pulpy	Pulpy	Pulpy	Compact	Pulpy
	He 6	Loose granule	Loose granule	Loose granule	Loose granule	Loose granule
	He 7	Hard	Compact	Hard	Hard	Hard
	He 8	Compact	Pulpy	Compact	Pulpy	Pulpy
	He 9	Hard	Hard	Compact	Compact	Compact
Embryogenic callus induction frequency*	He1	0	0	0	0	0
	He 2	0	0	0	0	0
	He 3	0	0	0	0	0
	He 4	0	0	0	0	0
	He 5	0	0	0	0	0
	He 6	83	77	75	80	76
	He 7	0	0	0	0	0
	He 8	0	0	0	0	0
	He 9	0	0	0	0	0

* Induction frequency was calculated by following formula: Induction frequency = weight of embryogenic callus after one months of culture / original weight of callus.
For each treatment, 3 samples were recorded and averaged.

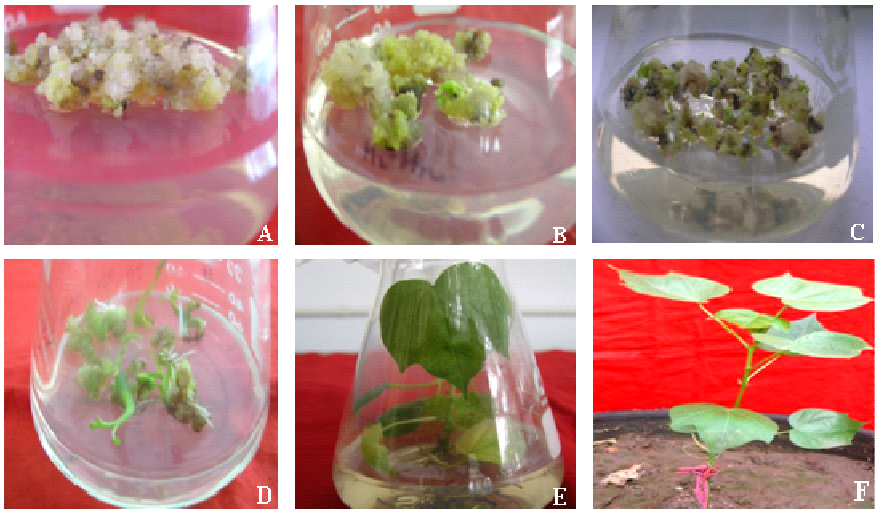

Figure 1. Somatic embryogenesis and plant regeneration of recalcitrant cottons. (A) Pre-embryogenic callus; (B) embryogenic callus; (C) somatic embryos at various developmental stages; (D) germination of somatic embryos and plant regeneration; (E) regenerated plant; and (F) grafted plant.

Table 4. The percentage of regeneration plantlets and somaclonal variation in recalcitrant cottons.

Percentage (%)	Shann724	Zhong6331	CCRI18	Liaomian12	Jinmian14
Regeneration plantlets*	30.0	22.2	19.6	20.0	18.5
Abnormal plantlets**	5.0	4.4	7.2	6.0	7.7

*Percentage of regeneration plantlets was calculated by the total number of plantlets on SEIM medium/the number of somatic embryos inoculated (100).
**Percentage of abnormal plantlets was calculated by the number of abnormal plantlets on SEIM medium/the number of somatic embryos inoculated (100).

tion ranged from 18.5 to 30.0% among different cultivars (Table 4). Besides the normal plants, some somaclonal variations were detected among the regenerated plants such as plantlets with abnormal leaves, cluster axillary shoots and main axillary shoots, and their frequency was from 4.4 to 7.7% (Table 4).

Plant recovery

Plantlets with 2 - 4 leaves survived and grew well after they were transplanted to green house by graft method (Figure 1F). Full plants were recovered under green house conditions for all recalcitrant varieties. The whole procedure, which started from callus initiation to plant establishment in a green house, took about 4 to 5 months.

DISCUSSION

In this study, the somatic embryogenesis and plant regeneration of five elite cotton cultivars (Shann724, Zhong6331, CCRI18, Liaomian12 and Jinmian14) were investigated. Plants have been regenerated from these five recalcitrant cotton cultivars for first time through a suitable tissue culture protocol described in this paper. Regeneration time is also shortened to 4 - 5 months using the combinations of IBA, KIN, 2,4-D and the regulation of nitrogen sources and amino acid at different stages of culture.

The cotton cultivars applied were resistant to *Fusarium* and *Verticillium* wilts. Up to now, the media used in some studies were still far from ideal since embryogenesis could not be initiated in many genotypes especially to recalcitrant genotypes with the protocols available (Trolinder and Xhixian, 1989; Zhang et al., 1994; Wu et al., 2004). Therefore, it is essential to establish a genotype-independent regeneration system for such cotton genotypes. Comprehensive *in vitro* regulation by use of hormones, chemicals, and amino acids at different stages may be the key factors for obtaining regenerated plants from recalcitrant genotypes (Wang et al., 2006). Here, we firstly reported successful plant regeneration from all five genotypes with *Fusarium* and *Verticillium* wilts resistance. The new protocol could shorten the time of cotton regeneration from 6 - 8 months as previously reported to 4 - 5 months. We employed this protocol to model cotton cv. '*Coker312*', and the plant regeneration was attained within three months (data not shown). Also, successful regeneration of these elite cultivars enhanced genetic diversity of regenerative cottons. The regenerative genotypes promised to in-crease efficiency of developing transgenic cotton plants, and to decrease costs of molecular breeding programs as well.

Hormone regimes have been found to be the most important factor affecting somatic embryogenesis in many species (Lin et al., 2000; Vasic et al., 2001). Different combinations of hormones have been employed by some researchers such as BR+2,4-D, 2,4-D+KIN, IBA+KIN (Firoozabady and Deboer, 1993; Xie et al., 2007). In this study, Combination of IBA, KIN and 2,4-D was employed to study somatic embryogenesis. It was found that gradual reduction in the concentration of 2,4-D and KIN was advantageous to somatic embryogenesis of five recalcitrant genotypes. This may be related to concentration of exogenous hormones accumulated during callus development (Wang et al., 2006).

As previously reported (Chia and Saunders, 1999; Witjaksono and Litz, 1999; Wu et al., 2004), we also observed that amino acids have been shown to be efficient for somatic embryogenesis and embryo development. When the embryogenic calli were transferred onto the medium supplemented with glutamine and asparagine, the number of embryos was significantly increased together with enhanced germination and embryos differentiation. This might be attributed to the transformation pathway of ammonia in somatic embryo germination of cotton. In the transformation pathway of ammonia, glutamine is transformed into stored protein through asparagines and arginine, and decomposition of stored protein provides the similar condition for somatic embryos germination (Price and Smith, 1979).

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