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High frequency somatic embryogenesis and plant regeneration of an elite Chinese cotton variety

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Abstract. An elite Chinese cotton (*Gossypium hirsutum* L.) cultivar Simian-3 was chosen for tissue culture. Callus with a high frequency of somatic embryogenesis, somatic embryos, and regenerative plants was obtained. Callus was induced from three types of explants on MSB (MS salts with B_5 vitamins) medium supplemented with zeatin (ZT) only, but the percentage of callus induction and growth of callus varied. It appeared that it was much easier to induce callus from hypocotyl than cotyledon or root explants. The concentrations of ZT were critical to the induction and proliferation of callus. The optimum ZT concentration for callus induction was 3.0~5.0 mg/L. Two kinds of callus could be identified after 70 days of culture: embryogenic and nonembryogenic callus. Embryogenic callus developed into somatic embryos at various stages after 20 days of subculture. The capability of embryogenesis depended on the explant types. The root was the most responsive explant for production of somatic embryos, the hypocotyl was the next, and the cotyledon was the last. Moreover, a low concentration of ZT was advantageous to the induction of embryogenic callus. 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. Addition of activated charcoal or a proper combination of ZT and 3-indoleacetic acid (IAA) could promote the production, maturation and germination of somatic embryos. The best medium for the proliferation of embryogenic callus was ZH medium (Zhang et al., 1996) with 1.0 mg/L 2,4-D, 0.5 mg/L kinetin (KT) and 0.5 mg/L ZT. The best medium for the differentiation and germination of somatic embryos was MSB with 0.1 mg/L ZT and 2 g/L activated charcoal. An efficient protocol for the production of high frequency somatic embryogenesis and plant regeneration of an elite cotton variety Simian-3 has been developed. Complete plants could be regenerated through somatic embryogenesis from hypocotyl, cotyledon and root explants in 3-4 months.

Keywords: *Gossypium hirsutum* L.; Plant regeneration; Simian-3; Somatic embryogenesis.

Abbreviations: **B_5,** Gamborg et al. medium (1968); **2,4-D,** 2,4-dichlorophenoxyacetic acid; **ZT,** zeatin; **KT,** kinetin; **IAA,** 3-indoleacetic acid; **MS,** Murashige and Skoog's basal medium (1962); **ZH,** Zhang et al. medium (1996).

Introduction

Cotton is one of the most important fiber crops in the world. 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.

Cotton somatic embryogenesis was first observed by Price and Smith (1979) in *Gossypium koltzchianum*, but no plantlet regeneration was reported. 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 labs using varied strategies (Shoemaker et al., 1986; Chen et al., 1987; Trolinder and Goodin, 1987; Zhang and Wang, 1989; Voo et al., 1991; Kolganova et al., 1992; Zhang, 1994a; Zhang et al., 1996, 1999). Regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root (Zhang, 1994a) 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

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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 efficiency of regeneration via somatic embryogenesis has been improved significantly in recent years, 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. Rajasekaran et al. (1996) obtained regenerative plantlets via somatic embryogenesis from T25, GSA 78 and Acala. Kumar and Pental (1998) obtained regeneration from MCU-5; GonzalezBenito et al. (1997) obtained regeneration from CNPA Precoce 2. Zhang et al. (1999) obtained regenerative plantlets from CRI 12. Most of these cultivars, except MCU-5 and CRI 12, are obsolete. Moreover, the frequency of regeneration was low in these varieties.

Here we report a protocol for high frequency somatic embryogenesis and plant regeneration from an elite cotton variety Simian-3. This variety is one of the most important varieties in China. It is planted in most of Chinese cotton areas and is the standard cotton variety to compare new lines in regional tests in the Changjiang River Valley of China.

Materials and Methods

Seed Germination and Cultivation of Sterile Seedlings

Seeds of cotton (*Gossypium hirsutum* L.) cv Simian-3 were delinted with sulphuric acid. Plump, mature seeds were chosen and sterilized by the following procedure: seeds were initially sterilized for 20 min using 70% alcohol, scorched in the flame of an alcohol burner for a moment, then dipped and kept in sterile water for 30 min to soften the seed coats and allow their complete removal. The sterilized seeds without seed coats were then sown in test tubes containing MSB [MS (Murashige and Skoog, 1962) salts with B_5 (Gamborg et al., 1968) vitamins] medium supplemented with 6 g/L agar for germination at 28 ± 2°C under 24 h photoperiod conditions with the light intensity of approximately 2000 lx.

Induction and Proliferation of Callus

Hypocotyl sections (3-5 mm length), cotyledon pieces (10~16 mm^2 surface area), and root segments (3-5 mm length) of 7-day-old sterile seedlings were placed on MSB medium supplemented with various concentrations of ZT for the induction of callus.

Selection of High Frequency Embryogenic Cell Lines

After 80 days of culture, embryogenic callus was chosen and transferred onto MSB medium supplemented with different hormones or MSB medium without any hormones for the proliferation of embryogenic callus. After 28 days of subculture, embryogenic callus with high frequency of embryogenesis was chosen for the next subculture. Then continual subcultures were carried out in the same way. Subsequently, embryogenic callus was subcultured every 28 days on MSB medium supplemented with 0.1 mg/L ZT (zeatin) and 2 g/L activated charcoal.

Differentiation of Somatic Embryos and Plant Regeneration

High frequency embryogenic cell lines were chosen and transferred onto embryo differentiation medium (MSB medium supplemented with 0.1 mg/L ZT) for the induction and development of somatic embryos. Mature embryos were chosen and transferred onto embryo germination medium [MSB or ZH (Zhang et al., 1996) medium supplemented with 0.1 mg/L ZT and 2 g/L activated charcoal] after 30 days.

All media were supplemented with 30 g/L sucrose, and were solidified with 7 g/L agar. The pH of medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 28 ± 2°C under a light intensity of approximately 2000 lx provided by cool white fluorescent lamps with 16 h photoperiod.

Results and Discussion

Induction and Proliferation of Callus

A range of ZT concentrations were tested for callus initiation. The results indicated that callus was induced on MSB medium supplemented with a range of ZT concentrations. However, differences based on ZT concentrations and nature of the explant were observed. The induction percentage of callus initially increased and then decreased with increasing ZT concentrations, with the maximum callus observed on MSB medium with 3.0-5.0 mg/L of ZT (Table 1). Although low (0.1-1.0 mg/L) and high (7.0 mg/L) concentrations of zeatin could induce hypocotyl explant to produce callus, the induced callus grew slowly or turned brown. Addition of 2,4-D promoted the formation and growth of cotton callus (Table 2).

Several types of callus were distinguishable based on the physical appearance. Callus on MSB medium containing ZT was initially grayish yellow and friable but grew into heterogeneous types (embryogenic and nonembryogenic callus) after about 1 month. Callus on MSB medium containing 2,4-D only was homogeneous, compact and green. In contrast, callus on MSB medium containing 2,4-D and ZT and IAA was homogeneous and

Table 1. Callus induction and proliferation of an elite cotton (*Gossypium hirsutum* L.) variety Simian-3 from different explants or various concentrations of zeatin (ZT).

ZT (mg/L)	Explant	Number of explants	Number of explants with callus	Rate of callus induction (%)	Growth of callus
0.01	Cotyledon	37	11	29.7	+
	Hypocotyl	40	35	87.5	+
	Root	16	0	0.0	-
0.1	Cotyledon	33	7	21.2	+
	Hypocotyl	18	18	100.0	++
	Root	25	16	64.0	+
0.5	Cotyledon	22	17	63.6	+
	Hypocotyl	27	24	100.0	++
	Root	8	2	25.0	+
1.0	Cotyledon	36	1	2.8	+
	Hypocotyl	38	38	100.0	+++
	Root	19	19	100.0	++
3.0	Cotyledon	40	11	27.5	++
	Hypocotyl	42	42	100.0	+++++
	Root	21	21	100.0	+
5.0	Cotyledon	32	7	21.9	+++
	Hypocotyl	33	33	100.0	++++
	Root	19	19	100.0	+++
7.0	Cotyledon	31	9	29.0	++
	Hypocotyl	23	23	100.0	++++
	Root	23	12	52.2	+++
10.0	Cotyledon	37	14	37.8	++
	Hypocotyl	30	27	90.0	+
	Root	16	4	25.0	+

*Cultures were evaluated at 60 days of culture. Number of + indicates the growth of induced callus; more +, more growth of induced callus; -, indicates trace growth of induced callus.

Table 2. Comparison between the effect of ZT, IAA and 2,4-D on callus induction and embryogenesis from various explants (80 days of culture).

Hormones	Number of explants	Explants	Ratio of callus induction (%)	Embryogenic ratio (%)	Callus weight (g)
0.1 mg/L ZT	33	Cotyledon	21.2	9.09	0.129
	18	Hypocotyl	100.0	5.56	0.356
	25	Root	64.0	8.00	0.135
3.0 mg/L ZT	40	Cotyledon	27.5	0.00	0.402
	42	Hypocotyl	100.0	0.00	1.291
	21	Root	100.0	0.00	0.107
0.1 mg/L 2,4-D + 0.1 mg/L ZT	48	Cotyledon	100.0	0.00	4.872
	48	Hypocotyl	100.0	0.00	5.687
	36	Root	100.0	0.00	2.999
0.1 mg/L 2,4-D + 0.1 mg/L ZT + 0.1 mg/L IAA	60	Cotyledon	100.0	0.00	5.399
	60	Hypocotyl	100.0	0.00	6.017
	60	Root	100.0	0.00	3.680
0.1 mg/L 2,4-D	50	Cotyledon	98.0	0.00	1.000
	50	Hypocotyl	100.0	0.00	1.532
	50	Root	92.0	0.00	0.788

grayish yellow or gray and friable. Hypocotyl explants were most responsive to callus induction and proliferation. Induction percentage of callus from hypocotyl explants was 100% on MSB medium supplemented with 0.1-7.0 mg/L ZT. The optimal ZT concentration for cotton callus induction from all explants was 3.0-5.0 mg/L (Table 1).

Somatic Embryogenesis

After 70 days of culture on MSB medium supplemented with various concentrations of ZT, callus could be classified into two types: nonembryogenic callus (Figure 1 A) and embryogenic callus (Figure 1 B). Embryogenic callus was fast growing, light yellow and loose. Somatic embryos

Figure 1. Somatic embryogenesis and plant regeneration of cotton (*Gossypium hirsutum* L.) variety Simian-3. A, nonembryogenic callus; B, embryogenic callus; C, somatic embryos at various developmental stages; D, germination of somatic embryos and plant regeneration; E, regenerative plant; F, regenerated plant after transferred to soil.

at various stages of development were found in embryogenic callus. Nonembryogenic callus was slow-growing, compact, light brown or light or dark green. Some of these turned brown and died.

The ZT concentration is the key factor on the direct induction of embryogenic callus. Although high concentrations of ZT promoted the induction and growth of cotton callus, it was disadvantageous to the induction of embryogenic callus. In contrast, low concentrations of ZT (0.01, 0.1 and 0.5 mg/L) could induce cotton explants to produce embryogenic callus directly from cotyledon, hypocotyl, and root explants. On MSB medium with 1 mg/L ZT or more, embryogenic callus could not be induced directly from explants. However, embryogenic callus could be induced when callus was subcultured on these media more than twice.

In previous reports, 2,4-D was an essential hormone for the induction of somatic embryogenesis in cotton and other plants (Davidonis and Hamilton, 1983; Trolider and Goodin, 1986; Chen et al., 1987; Zhang and Li, 1992; McKersie and Brown, 1996; GonzalezBenito et al, 1997; Guis et al., 1998; Kumar and Pental, 1998; Choi et al., 1999; Zheng et al., 1999; Zhang, 2000). In this study, we have found that ZT, a cytokinin, can induce cotton embryogenic callus. After we obtained embryogenic callus on MSB medium supplemented with ZT only, we compared the method described here with the previous method, which was used to induce embryogenic callus with auxin (especially 2,4-D) and cytokinin combinations. According to our and other's results (Davidonis and Hamilton, 1983; Trolider and Goodin, 1986; Chen et al., 1987; Zhang and Li, 1992; GonzalezBenito et al., 1997; Kumar and Pental, 1998), MSB medium supplemented with 0.1 mg/L 2,4-D, 0.1mg/L ZT and 0.1 mg/L IAA is the best medium for the induction of cotton embryogenic callus. However, this callus requires multiple subcultures before embryogenesis is induced. We compared the two methods for their ability to induce somatic embryogenesis. The results indicated that although the addition of 2,4-D could promote the induction and growth of cotton callus, embryogenic callus was not observed within 90 days of culture (Table 2).

All the explants viz hypocotyls, cotyledons and root segments produced embryogenic callus on MSB medium supplemented with 0.01, and 0.1 mg/L ZT only. However, the frequency was low, since only 10% of the explants produced embryogenic callus (Table 2). The frequency of embryogenic callus was also different amongst the explants, with hypocotyl callus giving the highest frequency of embryogenic callus and cotyledons the lowest.

Regeneration varied from seed to seed in this experiment. Out of the seedlings that were tested, explants derived from some seedlings easily regenerated via somatic embryogenesis, but some proved difficult or did not regenerate at all (data not shown). This result is similar to that of previous reports (Trolinder and Chen, 1989; Gawel and Robacker, 1990; Kumar and Pental, 1998).

Embryogenic callus was transferred to MSB medium supplemented with 0.1 mg/L ZT and 2 g/L activated charcoal or without any hormones. After 7 days of subculture, embryogenic callus produced visible somatic embryos. After 20 days of culture, somatic embryos in various developmental stages could be obtained.

Embryogenic callus with somatic embryos at various developmental stages were chosen and transferred into MSB medium supplemented with 0.1 mg/L ZT, 30 g/L sucrose and 2 g/L activated charcoal. After 28 days of subculture, the embryogenic callus with more extensive differentiation of somatic embryos was chosen and transferred into fresh medium of the same composition. Therefore, the high frequency embryogenic lines required more subcultures. Following this procedure, high frequency embryogenic cell lines were obtained, and these cell lines could produce more than 100 somatic embryos in one culture bottle (Figure 1C).

Table 3. The effect of various media on Simian-3 embryogenic callus in terms of growth, proliferation, differentiation and germination of somatic embryos (after 28 day of culture).

Medium code	Medium	Growth ratio of embryogenic callus[a]	% Embryogenic callus with embryos[b]	Germination ratio of somatic embryos[b] (%)
SM1	MSB	4.56	68.5	34.8
SM2	MS + 0.1 mg/L 2,4-D + 0.1 mg/L KT + 0.2 mg/L IAA	10.01	0.0	5.4
SM3	ZH+1.0 mg/L 2,4-D+ 0.5 mg/L KT + 0.5 mg/L ZT	11.52	2.0	3.2
SM4	MSB+0.1 mg/L ZT	8.76	87.8	70.0
SM5	MSB+0.1 mg/L ZT+2 g/L activated charcoal	5.38	100.0	87.8
SM6	MSB+0.1 mg/L ZT+0.1 mg/L IAA	6.23	84.9	79.1

[a]Growth ratio was calculated by following formula: (Growth ratio)=(wet weight of callus after 28 days of culture)/(original weight of callus). For each treatment, 5 samples were recorded and averaged.
[b]Values represent means of three replicates.

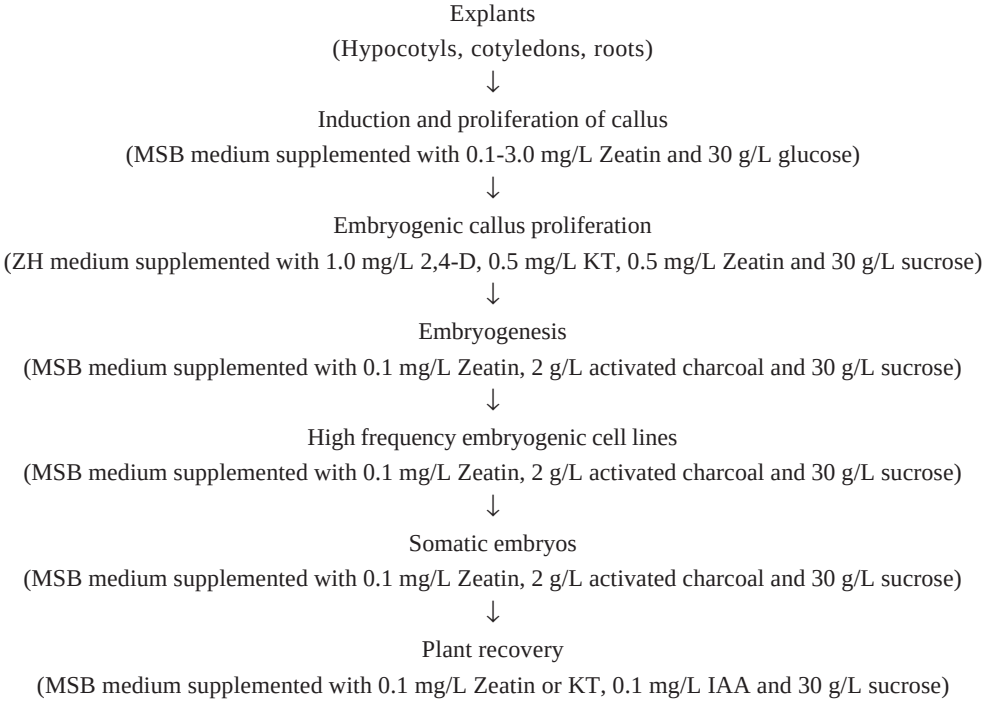

Figure 2. Protocol for somatic embryogenesis and plant regeneration of *Gossypium hirsutum* L. variety Simian-3.

Proliferation of Embryogenic Callus, Differentiation and Development of Somatic Embryos

A range of media were tested for the proliferation of embryogenic callus, differentiation and development of somatic embryos from elite cotton cultivar, Simian-3 (Table 3). Addition of 2,4-D stimulated the proliferation of embryogenic callus, but was disadvantageous to the differentiation and germination of somatic embryos. Although MSB medium without any hormones was advantageous for embryogenesis, its effects on the proliferation of embryogenic callus and the germination of somatic embryos was not adequate. The addition of activated charcoal to MSB medium or proper combination of hormones ZT and IAA promoted the differentiation, maturation, and germination of somatic embryos (Table 3).

Embryogenic callus grew and proliferated faster on Medium SM3 than on the other media. The growth ratio of embryogenic callus was 11.52 after 28 days of culture on SM3 medium, almost twice the growth ratio on the other media (Table 3). On SM5 medium, all embryogenic callus produced visible somatic embryos after 28 days of culture; but no visible somatic embryos were obtained on SM2 medium. The addition of activated charcoal, or ZT, was advantageous to the germination of somatic embryos, and the germination ratio was more than 70% on the medium supplemented with ZT only or with a combination of ZT and activated charcoal. Adding 2.4-D inhibited the germination of embryos and promoted some embryos callusing, so the germination ratio was very low on SM2 and SM3 media.

As Table 3 makes clear, the optimum medium for the proliferation of embryogenic callus was ZH medium supplemented with 1.0 mg/L 2,4-D, 0.5 mg/L KT and 0.5 mg/L ZT, and the optimum medium for the differentiation and germination of somatic embryos was MSB medium supplemented with 0.1 mg/L ZT and 2 g/L activated charcoal

Establishment of Cotton Elite Variety Simian-3 Tissue Culture and Plant Regeneration System

Development of an efficient tissue culture and plant regeneration protocol for elite cotton varieties is the first step towards the application of transgenic technology to improve cotton breeding and is, thus, the foundation of cotton biotechnology. Our initial intention was to induce adventitious buds on MSB medium with ZT, but the results were inconsistent with our intention. None of the explants formed adventitious buds, but they did produce embryogenic callus and somatic embryos. We have established the culture protocol for a new elite cotton variety, Simian-3, with high frequency embryogenesis and plant regeneration (Figure 2). With the protocol developed in this study, we have obtained hundreds of regenerated plants from Simian-3 via somatic embryogenesis (Figure 1D, E). These plants were transferred to soil and grew well (Figure 1F). This will promote the application of plant tissue culture technology in the area of selection resistance, production of artificial seeds, and genetic transformation.

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一個中國棉花優良品種高頻胚胎發生於植株再生

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一個中國棉花（*Gossypium hirsutum* L.）優良品種泗棉 3 號被選擇作組織培養的材料，選擇獲得了高頻胚胎發生，體細胞胚和再生植株的癒傷組織。在僅附加玉米素的 MSB（MS 無機鹽和 B_5 維生素）培養基上，三類外植體均誘導獲得了癒傷組織，但癒傷組織的誘導率和誘導量不同。下胚軸切斷比子葉片和根切斷更易誘導獲得癒傷組織。ZT 濃度影響棉花癒傷組織的誘導和增值。用於棉花癒傷組織誘導的 ZT 最佳濃度為3.0～5.0 mg/L。接種後連續培養 70 天可見到胚性和非胚性兩類癒傷組織。當胚性癒傷組織繼代培養到胚分化培養基上培養 20 天後，可見到不同發育時期的體細胞胚。棉花品種泗棉 3 號體細胞胚胎發生的能力依賴於外植體的種類和 ZT 濃度，根最容易誘導獲得胚胎發生，下胚軸次之，子葉最差。低濃度的 ZT 有利於棉花胚性癒傷組織的誘導。2,4-D 的添加雖然有利於癒傷組織的誘導和增殖，但卻不利於體細胞胚的分化、成熟和萌發。活性炭的添加或細胞分裂素 ZT 和生長素 IAA 的合理配合使用有利於體細胞胚的產生、成熟和萌發。在該實驗中，用於胚性癒傷組織增殖的最佳培養基為附加 1.0 mg/L 2,4-D, 0.5 mg/L KT 和 0.5 mg/L ZT 的 ZH 培養基，用於體細胞胚分化和萌發的最佳培養基為附加 0.1 mg/L ZT 和 2 g/L 活性炭的 MSB 培養基。本文建立了一種適用於棉花優良品種泗棉 3 號的高頻胚胎發生於植株再生的有效方法。利用該方法進行棉花泗棉 3號的組織培養，僅需要 3~4 個月就可以從下胚軸，子葉和根外植體通過胚胎發生途徑誘導獲得完整的再生植株。

關鍵詞：陸地棉；泗棉 3號；體細胞胚胎發生；植株再生。